An investigation of the mechanisms underlying the developmental origins of health and disease and their impact on telomeres

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ABSTRACT

Telomeres are specialised nucleoprotein complexes that act as a protective "cap" for chromosomal DNA and are found at the ends of linear eukaryotic chromosomes. Telomere length (TL) decreases with age and is linked to a number of age-related disorders and disease risk factors. The metabolic syndrome, a condition with increasing global incidence and symptoms such as obesity and dyslipidaemia, is linked to accelerated telomere shortening. It is proposed that metabolic disease is "programmed" in offspring as an adaptation to unfavourable environments during development, and may be the result of gene-environment interactions. Not only telomeres, but also mitochondria, whose dysfunction is linked to an increased risk of metabolic syndrome, may play a role in this process.

To investigate the effects of unfavourable intrauterine conditions on TL, studies were performed in two dietary pregnancy models using C57BL/6J mice, and in a retrospective human cohort, namely 1335 participants in The Western Australian Pregnancy Cohort (Raine) Study, who were aged 17. Participants in the cohort were followed from 18 weeks' gestation until early adulthood as part of a longitudinal study investigating the early origins of adult disease.

In the first rodent study, pregnant dams were dietary restricted (DR) by being given 30% caloric deficit over 12 gestational days (E5.5 to 17.5); in the second study, dams received a 60% hypercaloric high fat diet (HF group)). In both animal studies, fetal hepatic TL was significantly shortened compared to controls

(CON). In DR livers there was a trend towards increased cytoplasmic localisation of shelterin protein TIN2, which is involved in both mitochondria and telomere mechanisms. Reduced TL and increased mitochondrial DNA (mtDNA) copies were found in blood cells of DR dams. Linear regression analysis showed that daily weight gain by DR dams could reliably predict maternal and fetal TL. Increased hepatic mtDNA copy number and expression of genes associated with fatty acid uptake and metabolism was observed in HF fetuses.

Peripheral blood mononuclear cell TL analysis in the human cohort study revealed that the individuals with the shortest telomeres at age 17 had mothers who were hypertensive during pregnancy. Longer breastfeeding periods were associated with longer telomeres, which mitigated the effects of hypertension on telomere length. These data highlight the sensitivity of telomeres to intrauterine and postnatal conditions and suggests their importance as signposts of future programming of disease. Moreover, combining TL and mtDNA copy number as measures may yield a more significant indicator of cellular distress and future programming of disease.

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11β-HSD1	11 beta-hydroxysteroid dehydrogenase 1
11β-HSD2	11 beta-hydroxysteroid dehydrogenase 2
36B4	acidic ribosomal phosphoprotein P0
AA	arachidonic acid
ACTH	adrenocorticotropic hormone
AFP	alpha-fetoprotein
AGM	aorta-gonad-mesonephros
ALT	alternative lengthening of telomeres
aTL	absolute telomere length
ATP	adenosine triphosphate
B6	C57BL/6J
BMI	body mass index
BMP	bone morphogenic protein
BSA	bovine serum albumin
CHD	coronary heart disease
ccf-DNA	circulating cell-free mitochondrial DNA
CNS	central nervous system
CON	control
CpG	cytosine-phosphate-guanine
Cpt1a	carnitine palmitolytransferase 1a
CR	caloric restriction
CRH	corticotropin-releasing hormone
CS	Carnegie stages
DAB	3,3′-diaminobezidine
DHA	docohexanoic acid
DHE	dihydroethidium
DNMT	DNA-methyltransferases

DNPH	2,4 dinitrophenylhydrazine
DOHaD	developmental origins of health and disease
DR	dietary restricted
E	gestational day
ELISA	enzyme-linked immunosorbent assay
FATP2	Fatty acid transport protein-2
FFA	free fatty acids
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridisation
FTO	obesity gene
GC	glucocorticoid
GR	glucocorticoid receptors
GDM	gestational diabetes mellitus
H	hours
HBCS	Helsinki Birth Cohort Study
HDL-C	High-density lipoprotein cholesterol
HF	high-fat
HP	hydroperoxides
HPA	hypothalamic-pituitary-adrenal
HSC	haematopoietic stem cells
IGFBP-1	insulin-like growth factor binding protein-1
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
IGF-IIR	insulin-like growth factor II receptor
IL-6	interleukin-6
IQR	Interquartile range
IRSAD	Index of Relative Socioeconomic Advantage and Disadvantage
IUGR	Intrauterine growth restriction

Kcal	kilocalories
KEMH	King Edward Memorial Hospital
LBW	low birth weight
LC-PUFA	long chain polyunsaturated fatty acids
LDL-C	low-density lipoprotein cholesterol
LP	low protein
LPL	lipoprotein lipase
Minutes	min
MR	mineralcorticoid receptors
mtDNA	mitochondrial DNA
mTert	mouse telomerase reverse transcriptase
mt-CytB	mitochondrially encoded cytochrome b
mt-ND1	mitochondrially encoded NADH dehydrogenase 1
mTOR	mammalian target of rapamycin
mTR	mouse telomerase RNA
NAFLD	non-alcoholic fatty liver disease
nDNA	nuclear DNA
NGFIA	nerve growth factor-inducible protein-A
NRF-1	nuclear respiratory factor 1
NRF-2	nuclear respiratory factor 2
O_2^-	superoxide
ω-3 PUFA	omega-3 polyunsaturated fatty acids
ω-6 PUFA	omega-6 polyunsaturated fatty acids
OXPHOS	oxidative phosphorylation
PAR	predictive adaptive response
PBS-T	PBS-tween
PMBC	Peripheral blood mononuclear cell

Pgc1-α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PK	proteinase K
PPAR-γ	peroxisome proliferator-activated receptor- gamma
PUFA	polyunsaturated fatty acids
PVDF	polyvinylidene fluoride
PVN	paraventricular nucleus
qPCR	quantitative real-time PCR
ROS	reactive oxygen species
S	seconds
SAM	S-adenosylmethionine
Sdha	succinate dehydrogenase complex, subunit A
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SSC	saline sodium citrate
T2D	type-2 diabetes
TBE	Tris-Borate-EDTA
Tbp	TATA-box binding protein
TBS-T	Tris-buffered saline with Tween 20
TERT	telomerase reverse transcriptase
Tfam	Mitochondrial transcription factor A
TG	Triglycerides
TAFs	telomere-associated DDR foci
TIFs	telomere-induced DNA damage foci
TL	telomere length
TR	telomerase RNA

TS	Theiler stages
WHO	World Health Organisation
WHR	Waist-to-hip ratio
WWII	Second World War
Ywhaz	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein, zeta polypeptide

CHAPTER 1

INTRODUCTION

1.1 The Developmental Origins of Health and Disease Hypothesis

1.1.1 General

Globally, overweight and obesity have reached epidemic proportions, with up to 39% of adults overweight, and over half a billion categorized as obese (1). This poses a major healthcare challenge because the occurrence of overweight and obesity also increases the probability of comorbidities including type 2 diabetes (T2D), hypertension, and hyperlipidaemia (2), which can lead to further disease or even mortality. As the incidence of overweight and obesity increases, so does the co-occurrence of the abovementioned disorders, which are major components of the metabolic syndrome (2). While initially deemed a "Western" condition, metabolic disease is now increasingly prevalent in low and middle income countries of the developing world (3). This trend is driven by several factors, including social and economic transformation, technological advances, and urbanisation (3,4). These factors are found to have a profound effect on food preference resulting in a nutritional transition to Western diets of increasing caloric density with low micronutrient quota, as foods of convenience and sedentariness become more prevalent (4-6).

Policy initiatives to counter these unfavourable health trends have tended to be lifestyle focused with greater investment on modification of behaviour (7,8). These schemes can also be self-limiting due to lifestyle drift, a shift from confronting wider upstream macrosocial health factors, to a downstream focus

on just individual health factors, principally in disadvantaged groups (8). Thus, these strategies have proven largely ineffective in undermining the global epidemic of disease.

However, a group of studies in the 1980s led by David Barker and colleagues showing a link between disordered growth and the development of comorbidities associated with metabolic syndrome (9-11) led to a paradigm shift in the understanding of metabolic syndrome pathogenesis. This early work, and studies that have followed since, have been able to more clearly establish a link between metabolic disease and exposure to suboptimal antenatal/early postnatal environments. These studies led to the generation of a hypothesis which states that challenges in prenatal and early postnatal environments are associated with increased risk of developing metabolic disease in adulthood (12). This premise is called the developmental origins of health and disease (DOHaD) hypothesis.

The DOHaD hypothesis was initially proposed by David Barker and colleagues in the 1980s, stating that events *in utero*, and in particular undernutrition, reduced fetal growth and increased the risk of developing heart disease and diabetes in later life (13,14). Prior to the development of this hypothesis, earlier epidemiological work by Forsdahl (15) in 1977 demonstrated a relationship between exposure to suboptimal early life environments and the development of coronary heart disease (CHD) later in life in a middle-aged Norwegian population (15). Forsdahl proposed that due to poverty in early life, and hence

poor nutrition as a result, there was an increased susceptibility to CHD in adulthood if living standards improved in later life (15).

Following this work, in the late 1980s David Barker and colleagues in the United Kingdom then observed that specific regions of England and Wales with high infant mortality in the early 1920s, had correspondingly high incidences of CHD between 1968-1978 (9). Consequently, it was concluded that the association of heart disease with neonatal mortality was related to poor nutrition during prenatal and early postnatal life (9). Furthermore, their finding concurred with the earlier work done by Forsdahl.

Barker and colleagues further defined the link between early life adversity and CHD by studying a cohort of men born between 1911 and 1930 in Hertfordshire, England (11). Using comprehensive birth records containing information on birth weight and infant growth, an inverse relationship was observed between weight at birth and risk of mortality from CHD (11). Death rates from CHD were lower in individuals with the highest weights at birth and at age one (11). From this it was hypothesised that the same early environmental influences that impair growth and development were also associated with increased risk of CHD in adulthood (11).

Expanding on this work, it was later demonstrated that CHD was not the only disease outcome associated with impaired fetal growth. Once again using birth

weight as a proxy indicator of early environmental exposure, studies in the Hertfordshire cohort demonstrated that intrauterine growth restriction (IUGR) had a similar association with T2D as with CHD (16). This relationship also extended to other disorders hypertension and hyperlipidaemia, which exist as comorbidities associated with the development of the metabolic syndrome (17). Later studies in Hertfordshire and other cohorts reported further associations between IUGR and other conditions including sarcopenia (18), osteoporosis (19), schizophrenia (20) and impaired cognitive ability (21). This relationship between suboptimal early life environments and risk of metabolic disease is also replicated in later cohort studies in other countries (22-25).

1.1.2 Catch-up growth

The early work by Barker formed the basis of the DOHaD hypothesis, which focused principally on intrauterine factors that affected fetal growth and development and predisposed individuals to disease in later life. However, it was later established that an interaction between prenatal and postnatal environments might elevate disease risk (26,27). Epidemiological studies were able to establish that neonates that were small at birth, but experienced the most rapid gain in size (both in weight and height) over the first year of life, or "catch-up growth" were at greatest risk of metabolic disease (28,29). The significance of growth trajectories and size during infancy in later disease is emphasised in epidemiological data from the Helsinki Birth Cohort Study (HBCS). The HBCS comprises two birth cohorts, one born earlier than the other, at the Helsinki University Central Hospital in Helsinki, Finland (30). Available data from the older cohort (born 1924-1933) includes birth characteristics and growth data

between 7 and 15 years of age. The "newer" cohort (born 1934-1944) consists of child growth data from birth until 12 years of age (26,31).

Findings in the "newer" group (born 1934-1944) showed an association between CHD risk and the occurrence of both thinness and IUGR (26). The men at greatest risk of CHD were thin at birth, with the thinness persisting through infancy until a rapid weight gain and BMI increase after 1 year (32). The highest risk was found in those who had experienced rapid weight gain at 2 years old after remaining thin until that age (33).

Compared to the men, the women who eventually developed CHD were short, not thin at birth, and became thin with compensatory height gains in the first year of life, followed by rapid BMI increases in later childhood (34). This suggests a reduced female vulnerability to intrauterine undernutrition and an increased capability for sustaining postnatal growth in suboptimal environments (34). These data from the HBCS show the significance of the early postnatal developmental window, in determining future disease risk. Hypotheses have been put forward to explain how the interaction between pre- and postnatal environments is associated with disease risk and will be discussed below.

1.1.3 The Thrifty Phenotype Hypothesis and "mismatch "

In acknowledgment of the interaction between prenatal and postnatal environments the DOHaD hypothesis was therefore modified and led to the

generation of the "thrifty phenotype hypothesis" by Barker and Hales (16,35), derived from the thrifty genotype hypothesis by Neel (36). The thrifty genotype hypothesis suggested that during evolution when food was scarce 'thrifty' genes, or genes associated with economising energy use, were selected which resulted in a "fast insulin trigger". This "trigger" enhanced the ability to store fat which increased susceptibility to diabetes (36). Alternatively, the thrifty phenotype hypothesis (35) suggested that development of the fetus is sensitive to the environment *in utero*. In a suboptimal environment, an adaptive response is instigated, which results in the fetus making adjustments for a resource-deficient future environment, based on the nutritional cues *in utero* (35). As a result the fetus would safeguard the development of specific organs such as the brain at the expense of others, including the pancreas and liver, leading to disease (35).

This notion of intrauterine adaptation in response to suboptimal environments is what was originally referred to as "programming" by Lucas (37). The central concept of programming is that early life events induce permanent or long term changes to organ structure and function during sensitive or critical periods of development (37). It has been proposed by some that the term programming is not suitable and that it be replaced by the term developmental plasticity (38,39), defined as the ability of a genotype to produce different phenotypes in response to environmental conditions (38).

The term 'Predictive Adaptive Response' (PAR) hypothesis has been applied in place of the thrifty phenotype hypothesis (40,41), which posits that the fetus uses mechanisms of developmental plasticity to establish a phenotype in postnatal life that it predicts will provide the best chance of survival (40). Therefore a "mismatch" between the predicted environment and the actual postnatal environment may elevate disease risk.

This mismatch phenomenon has been observed in two epidemiological studies of famine, The Dutch Hunger Winter of 1944-45 (42) and the German siege of Leningrad (43). During the Dutch famine period, the population in the West Netherlands region were exposed to a severe reduction in daily rations during the Second World War (WWII) (44). Daily rations of food fell from 1800 kilocalories (kcal) in late 1943 to as low as between 4-800 kcal at the height of the famine in December 1944. The availability of calories finally increased to 2000 kcal in May 1945 when the region was liberated from the Nazis (45). Data obtained from these studies were able to show associations between the period of undernutrition and adverse metabolic outcomes, but also emphasise the significance of timing and critical windows of development.

Specific phenotypes in adulthood appeared to be affected by the timing of the exposure to famine. Overall, children that were still *in utero* during the Dutch famine exhibited lower birth weights and by age 50 were glucose intolerant (46). The severity of glucose intolerance was greater when exposure to the famine

occurred during mid- or late gestation (46). Exposure to famine in early gestation was linked to greater body mass index (BMI) as well as impaired liver function, revealed by elevated fibrinogen and lower concentrations of Factor VII in middle age (47). Furthermore, early gestational exposure was also associated with a pro-atherogenic lipid profile (48) and increased CHD risk (45). Mental conditions such as schizophrenia were also reported if children were conceived during the famine (49).

This is in contrast to health outcomes resulting from the famine that took place in Leningrad, Russia, during a 900 day siege by the Germans in the WWII between 1941-44 (43). The population in Leningrad endured a period of poor nutrition that lasted 28 months, without any improvement to nutrition throughout the period (50). Despite the presence of LBW in children from both the Dutch and Leningrad famines (45,50), the rates of insulin resistance, dyslipidaemia and CHD were not different across offspring exposed to the Leningrad famine (50).

A comparison of both famines shows that the circumstances around each hunger period were different. The people in Leningrad did not only endure a more prolonged famine (>2 years as opposed to ~6 months), but the living conditions that preceded the Russian famine were also very poor (50,51). Further, while the Dutch post-liberation conditions managed to improve, data indicated that the quicker infant nutritional transition to adequacy in postnatal life elevated disease

risk much later (45). Another example of this mismatch effect as a result of PAR includes studies in Indian children who were small at birth but were exposed to abundant nutrition in postnatal life (52). Children were shown to be significantly heavier at 8 years of age, and demonstrated several cardiovascular risk factors including insulin resistance and hypertension in childhood (52). This is believed to occur as a result of the discrepancy between undernourishment in early life and a system of overnutrition in later life.

From an evolutionary standpoint, the induction of specific PARs should be advantageous for offspring, preparing them for life in a postnatal environment. However, as shown in the above studies, there may be a high cost if these predictions prove to be inaccurate (53). These studies not only highlight the importance of delivering a better "matched" early postnatal nutrition to compromised offspring, but also more importantly, signify a potential window for therapeutic intervention.

1.2 Factors that affect the development of systems involved in DOHaD

Suboptimal early environments can trigger changes during periods of developmental plasticity to generate dysfunction and hence disease in later life. There have been extensive studies that have determined the exposures that generate these long-term health outcomes in offspring. Two early life factors that have most widely been investigated are nutrition, which has been briefly introduced, and exposure to glucocorticoids.

1.2.1 Nutrition

As previously established in the above studies, sufficient nutrition throughout gestation is important for fetal development. Over time, studies have expanded from the prenatal to reflect postnatal nutritional effects as well. Thus, the interactions between the fetal adaptations from cues *in utero* and final postnatal nutrition can impact disease risk (48). The prenatal period encapsulates maternal nutrition during both periconceptional and gestational periods.

1.2.1.1 Maternal undernutrition

1.2.1.1.1 Beneficial caloric restriction vs pregnancy caloric restriction

Restricting caloric intake while maintaining optimal nutrition has long been linked to increased longevity and slowed ageing phenotypes (54). As a result, it is possible to reduce energy intake without becoming malnourished (55). In fact, caloric restriction (CR) has been associated with improvements in both humans and animals regarding insulin resistance, oxidative damage (56) as well reduced morbidity (57). However, these findings are confined to adult organisms. When applied to the fetus, maternal caloric restriction constitutes a nutritional insult in that it imposes a deficit of specific nutrients or calories required for the development of offspring (58).

Caloric restriction has been linked to the deactivation of the nutrient-sensing mammalian target of rapamycin (mTOR) signalling, which is also a factor in aging, in both adult and fetal cases. (59,60). In the case of adult CR without malnutrition, the removal of nutrients inhibits insulin signalling which, in turn

represses mTOR, decelerating aging (60). Additionally demonstrated to repress mTOR is pregnancy CR. However, this stimulates increased insulin-like growth factor binding protein-1 (IGFBP-1) expression and phosphorylation, which reduces the bioavailability of insulin-like growth factor-1 (IGF-1) (59). The primary distinction between the two is that, while mTOR acts on IGF-1 in pregnancy CR, it responds to IGF-1 signalling and insulin responsive substrates in non-malnutritive CR (59,60). mTOR plays a significant role in nutrient sensing, which has significant effects in both environments, even though other signalling pathways may be involved.

1.2.1.1.2 Maternal undernutrition and programming

Throughout the course of pregnancy an altered metabolic environment can have significant consequences for the developing fetus. Some of the earliest epidemiological evidence linking maternal undernutrition to programming of disease can be observed in the previously-discussed studies by Barker in Hertfordshire (9,11,13). While these studies set the stage for the development of the DOHaD hypothesis, they did not directly show the relationship between maternal undernutrition and the development of metabolic syndrome phenotypes in later life. They tended to focus on the use of birth weight as a crude surrogate measure for determining later disease.

Where the relationship between nutrition and disease was better clarified is in studies of adults that were still *in utero* during the Dutch Hunger Winter of 1944-45 (42). Studies of the Dutch cohort provided a unique opportunity to study a

direct link between restricted maternal nutrition and the implications for offspring health in the long term. However, the physiological mechanisms that are involved in the programming of later health outcomes still remain undefined. Therefore, the strongest evidence of a causative link between undernutrition and disease risk has largely been derived from animal models. Indeed, findings have shown that inadequate nutrition in both humans and animals compromises growth of both fetus and placenta (61,62).

Approaches for the study of nutrient restriction in animal models have included global restriction of maternal dietary intake and restriction of specific macro- and micronutrients (63). What is evident, however, is that poor maternal dietary intake is not the single limiting factor contributing to fetal undernutrition. Maternal and fetal nutrition are not identical. Other factors including reduced maternal stores, body composition and physiology, as well as constrained uteroplacental function are factors that minimise nutrient transfer to the fetus (63-65).

The programming effects of global restriction of maternal dietary intake are of particular interest in this thesis. Studies of global restriction of maternal dietary intake have been undertaken using both rodent models and sheep. The effects of decreasing maternal dietary intake by 30% (66), 50% (67-69) and even more severely, 70% (70,71) have been studied in rodent models. Maternal dietary restricted offspring tend to have low birth weights (66-68). In both mice and rats,

exposure of dams to 50% dietary restriction in the final week of gestation perturbs pancreatic development resulting in a reduced β–cell mass, which contributes to impaired insulin secretion and glucose intolerance in adulthood (67,68). Adult hypertension (66,71) and obesity (72) have also been observed in some models. While these outcomes are not present in all, catch-up growth is still present in some (66,72).

As with the human studies described above, duration and timing of nutrient exposure is a significant factor. While in rats, 50% dietary restriction in late gestation leads to impaired insulin secretion (67,68), there is no effect on the action or secretion of insulin in adulthood when the exposure occurs during the initial two thirds of gestation (69). Similarly in sheep, a 50% restriction in the first 30 days of gestation (term ≈147 days) alters cardiovascular function in young adulthood without changing birth weight (73), whereas the same dietary restriction in late gestation leads to a glucose intolerance phenotype (74).

Similar disease outcomes have also been present when there was a deficiency of specific nutrients during gestation. In rats, protein restriction has been shown to reduce nephron number in the developing kidney, which may contribute to the development of hypertension in adulthood (75,76). Hypertensive phenotypes have also been observed when there is a deficiency in the micronutrients iron and zinc during gestation (77,78).

Animal studies also show that global dietary restriction plays a significant role in the development of metabolic disease through programming of the brain. Notably, appetite and energy expenditure mechanisms are significantly affected in calorie-restricted offspring as a result of a significant decrease in concentration of an adipocyte-derived peptide hormone called leptin (79,80). Two isoforms of leptin exist, with the long isoforms highly expressed in the hypothalamus and the short isoforms in the tissue periphery (81,82). Across various species, leptin plays a prominent role in satiety by promoting energy expenditure and reducing intake of food, thus stabilising adiposity (83). While caloric restriction suppresses offspring leptin (79,80), adult plasma leptin levels are high in obese individuals, and this is also accompanied by leptin resistance (84).

An observed consequence of this is behavioural modification; adult offspring of severely dietary restricted (DR) rat dams displayed changes in feeding habits, such as hyperphagia (over-eating), augmented by a hypercaloric post-weaning diet (70), and a tendency toward sedentariness (85). Changes in cognition have also been noted. Offspring of DR dams have a reduced learning capacity (86). One other significant programming target that involves the brain, which is impacted by maternal dietary restriction during pregnancy, is the hypothalamic-pituitary-adrenal axis, which is discussed in more detail below (2.2.2).

Epidemiological studies of the mismatch between pre- and postnatal life are supported by experimental work in animals. In a model of maternal undernutrition using sheep, a normal feeding regimen was introduced in postnatal life. Sheep demonstrated altered growth patterns with less weight gain in the first 31 days of gestation but greater postnatal growth rates prior to weaning (87). The mismatch in nutrition led to cardiovascular dysfunction and altered renal function in adulthood (87). Using a system of maternal gestational malnutrition in rat dams with low protein (LP) diets, followed by a period of overfeeding, resulted in offspring with IUGR that exhibited rapid catch-up growth (88,89). Further, in adulthood, rodents showed various symptoms associated with metabolic disease including hyperinsulinemia, glucose intolerance, insulin resistance (88), as well as hypertension and renal dysfunction (89).

Although it be might expected that matching the inadequate diets of the intrauterine environment to the postnatal environment might produce a positive outcome, studies in rats have shown otherwise. Exposing rat dams to LP diets throughout gestation and lactation periods resulted in offspring with increased lipid accumulation in the liver pre-weaning, and compromised hepatic structure and function in adulthood, all precursors to disease (90,91). Given the example of the Leningrad siege where no disease symptoms were apparent in offspring in adulthood despite pre- and postnatal nutritional adversity (43), these findings may suggest that there are other interacting variables which contribute to the

final phenotype. Thus, there are limitations to the animal model, and a better understanding of the interaction between environments might provide a significant window for future interventions.

1.2.1.2 Maternal overnutrition

Although there is strong evidence that nutritional insufficiency during gestation can program susceptibility towards metabolic disease, it has been proposed that overnutrition *in utero* can also contribute to the development of obesity and its related disorders (92,93). Interest in fetal overnutrition began following the development of a hypothesis by Jørgen Pedersen in the 1950s, which suggested that in diabetic pregnancies, maternal hyperglycaemia caused excess glucose transfer to the fetus (94). Consequently, the fetal hyperglycaemia that develops elevates fetal insulin production and secretion which promotes fetal overgrowth leading to adverse outcomes in later life (94).

Norbert Freinkel later modified Pedersen's hypothesis, retaining the principle of increased blood glucose, and broadening it to include maternal plasma free fatty acids (FFAs) and amino acids as other nutrients that could bring about similar outcomes when in excess (92). This is known as the fuel-mediated teratogenesis concept which postulates that in diabetic pregnancies, the fetus is exposed to excess "mixed fuels" *in utero* which may lead to, in addition to higher birth weights, congenital malformations and greater risk of developing T2D and obesity (92). More recently, it has been proposed that fetal overnutrition also occurs in obese non-diabetic pregnancies, resulting in long-term changes, including alterations to appetite, neuroendocrine function and metabolism (93).

This collection of understanding is now referred to as the developmental overnutrition hypothesis.

1.2.1.2.1 The influence of maternal body composition

The worldwide prevalence of obesity in adulthood has almost doubled over the past three decades (95), and of particular concern is the parallel global rise in the rates of overweight/obesity in pregnant women and in children (96). In the United States alone, a third of women are obese, with two thirds maintaining an overweight status (97). It was previously hypothesised that exposure to maternal diabetes during pregnancy might adversely affect intrauterine conditions leading to an intergenerational "vicious cycle" of obesity and T2D (98). More recently, it has been proposed that this intergenerational effect is more likely due to exposure to maternal obesity whilst *in utero* (99).

Maternal obesity has been linked to fetal growth abnormalities. A particularly concerning trend is the presence of macrosomia (birth weight >4,000g) rather than IUGR, following exposure to both maternal diabetes and obesity during gestation, which in turn, also puts offspring at risk of metabolic disease (100,101). This was initially illustrated by studies of the Pima Indians in Arizona, a cohort where women had pre-existent diabetes and gestational diabetes mellitus (GDM) (101). Exposure to diabetic and GDM pregnancies resulted in large-for-gestational-age offspring, which also tended to be heavier in childhood after the age of 5 (102). Data from this cohort and other later studies of diabetic

pregnancies served to expand the outcomes of the overnutrition hypothesis to include fetal obesity/adiposity (103-105), a significant risk factor for childhood obesity and impaired metabolism (106).

Similar to diabetic pregnancies, higher fetal adiposity is also independently associated with maternal obesity, as determined by BMI (107). It has been determined that women with BMIs above 40 are at a high risk of passing on their obesity to their offspring (108). Studies have shown a dose-dependent link between maternal obesity and macrosomia (109). Despite the fact that obesity in pregnancy is strongly associated with GDM (110), fetal exposure to solely maternal obesity can double the risk of developing metabolic syndrome (107). Indeed, results from various studies suggest that fetal overgrowth has an even stronger association with maternal pre-pregnancy BMI, and maternal plasma triglycerides (TG) than with GDM (111-113). This trend in growth can occur in non-hyperglycaemic mothers (114), suggesting that fuels other than glucose may be involved in the processes governing fetal overgrowth.

It was previously hypothesised by Szabo that pregnancy obesity, excessive weight gain and diabetes caused increased adiposity in neonates as a result of increased placental transfer of maternal FFA to the fetus (115). Subsequent studies support this notion, denoting a positive association between both higher birth weights and neonatal adiposity with not only maternal FFA, but also TG in non-hyperglycaemic GDM pregnancies (101,111). Furthermore, children born to

obese mothers have been shown to develop insulin resistance phenotypes (116). Evidence suggests that maternal lipid metabolism in obese mothers is altered and this may contribute to fetal overnutrition by maternal lipids (117).

Over the course of pregnancy, maternal metabolism and endocrine function is altered to accommodate the nutritional needs of the developing fetus. Analogous to changes in protein and carbohydrate metabolism, there are two phases of lipid metabolism in pregnancy (118). The first phase is anabolic and lasts through the first two trimesters of gestation. The anabolic phase is marked by an increase in maternal insulin sensitivity, which in conjunction with elevated estrogen and progesterone, promotes lipogenesis and suppresses lipolysis, accumulating deposits of long chain polyunsaturated fatty acids (LC-PUFA) in adipose tissue (118-120). In addition to enhanced lipogenesis, maternal body fat also accumulates as a result of hyperphagia (118) and increased activity of the enzyme lipoprotein lipase (LPL) in adipose tissue which hydrolyses circulating TG for tissue uptake (121).

The second phase is catabolic and occurs in the final third (late second into the third trimester) of pregnancy (118). This phase is characterised by enhanced lipolysis and mobilisation of fats (119). As the maternal metabolic state transitions from anabolic to catabolic there is an accompanying increase in maternal insulin resistance and a suppression of LPL activity (118). This increased lipolytic activity in the presence of maternal insulin resistance elevates

the concentration of circulating FFA and glycerol (118). The elevated maternal lipids are now available for transfer to the fetus, and are thought contribute to the fetal fat deposition that occurs towards the end of pregnancy (111). The availability of fatty acids, particularly LC-PUFAs is essential for the developing nervous system (122).

This process of lipid metabolism has been shown to differ between obese and non-obese women in pregnancy. The amount of fat that accumulates in early pregnancy is greater in lean women than in obese women, likely due to greater insulin sensitivity in the former (117). Further, indications from previous work by Catalano *et al.* (123) demonstrated that the pattern of lipogenesis and lipolysis during pregnancy differs between lean and obese women. Lean women demonstrated greater pre-pregnancy lipogenesis, which was also maintained in early pregnancy, and increased lipolysis in later pregnancy (123). In obese women however, while lipogenesis occurs pre-conception, in early pregnancy it occurs to a lesser degree than in lean women, and the transition from anabolic to catabolic state occurs much earlier (123). Notably, higher circulating levels of circulating lipids are present in obese pregnancies (111-113,124) and this may indeed affect lipid transfer to the fetus. However, more work is still required to determine the mechanisms through which this may occur.

1.2.1.2.2 The influence of maternal dietary intake

Compared to fetal undernutrition, there is still a dearth of information regarding the contribution of maternal diet to fetal overnutrition in humans. Hyperglycaemic diets during pregnancy have been associated with increased fetal adiposity and higher birth weights (125), which can be compounded by maternal overweight and obesity (126). **The interest in this thesis is specifically on the effects of a high fat (HF) diet exposure.** Through the use of animal models, the effects of maternal feeding of HF and diet-induced maternal obesity during gestation have been examined, in addition to programming outcomes of such exposures.

In animals, diets high in fat have been used extensively to model obesity, and other metabolic risk factors particularly with rodents. Evidence from animal studies has paralleled many of the findings observed in epidemiological studies of maternal overnutrition. Exposure to a HF diet during gestation has been shown to have significant effects on adult offspring. Male offspring of pregnant rats fed a HF diet during gestation have higher body weights and glucose intolerance (127). If maternal HF feeding is extended from gestation into the lactation period, rat offspring demonstrate cardiovascular dysfunction, altered liver lipid metabolism, hypertension, elevated leptin levels, and impaired pancreatic β-cell development causing hyperglycaemia (128-132). While these studies have demonstrated the long-term programming effects in adult offspring there are few studies that have reported effects in younger offspring pre-

weaning. One such study reported higher levels of circulating triglycerides in neonatal offspring following exposure to a HF diet during gestation (133).

Similar to DR animal studies, HF diets have also been shown to play a role in programming of offspring behaviour. Elevated anxiety has been demonstrated in offspring of both rats (134) and non-human primates (135) in response to prenatal HF exposure. Excessive intake of food by the mother during either pre- or postnatal periods has been shown to program postnatal fetal appetite (136-138). Postpubertal offspring of HF-fed rat dams displayed changes in food preference and feeding habits, including hyperphagia and an increased preference for fat (136). This has been attributed to HF-induced increased proliferation of neurons in hypothalamus that secrete peptides responsible for control of food intake and body weight (136). Similar to dietary restriction, HF exposure has also been shown to influence the HPA axis; to be discussed below (Section 2.2.2).

Several animal studies suggest that maternal HF diets during pregnancy might induce metabolic syndrome-type phenotypes in offspring. In spite of this, the phenotypic outcomes in offspring may be dependent on the type of fatty acids they are exposed to. A large number of animal studies have employed the use of saturated fats (63), which are associated with many of the adverse outcomes described above. In contrast, unsaturated fatty acids are associated with positive cardiovascular health (139).

In rats a deficiency in omega (ω)-3 polyunsaturated fatty acids (PUFAs) in the perinatal period elevated blood pressure in later life (140). The most abundant fatty acids in the brain and the retina are arachidonic acid (AA), which is an ω-6, and docohexanoic acid (DHA) which is an ω-3 PUFA (139). In humans a prenatal deficiency of the ω-3 DHA is associated with impaired retinal function and learning ability (122). The ω-6 acid AA is believed to play significant role in promoting growth in early life and is a critical component of membrane lipids including phospholipids in cells (141).

In addition, exposure to PUFAs has been suggested to counter the effects of suboptimal prenatal environments. In a postnatal environment, human milk provides a source of PUFAs (139). A longer duration of breastfeeding is associated with protection against the development of overweight and obesity both in adults (142,143) and children (144,145). In rats, postnatal feeding with ω-3 fatty acids has been shown to counter programmed hyperleptinemia and hypertension in offspring exposed to excessive glucocorticoid exposure *in utero* (146).

In concert with these nutritional effects, over the last ten years interest has grown in the role of the microbiome. This has brought to light a new hypothesis based on the microflora makeup of the gut (147), which postulates that early life exposures alter the development of the human gut microbial makeup. Not only is microbiome of the mother associated with maternal metabolism but it can also be vertically transmitted to offspring which means it may pose significant changes in microbiota of the neonate (148,149). While not the focus of this thesis

and well-reviewed (148,149), in the future, it may prove significant in examining log-term alterations to microbiota that are inherited in offspring of undernourished and hypercaloric pregnancies.

1.2.2 The Hypothalamic-pituitary-adrenal axis

Upon exposure to stressors, an array of responses, collectively called the stress response, is activated in order to maintain the homeostatic state of the cell (150,151). This homeostatic response may involve the combined action of nervous, endocrine and immune systems, triggering responses at the molecular, cellular, physiological and behavioural levels (150,151). This response to stress is managed through activation of the hypothalamic-pituitary-adrenal (HPA) axis and activation of the sympathetic nervous system (152). The activation of the HPA axis results in a more sustained stress response through the release of corticosteroids, steroid hormones secreted by the adrenal glands and are the end point of the axis (152). The adrenal corticosteroids are comprised of two types: GCs and mineralocorticoids.

1.2.2.1 Anatomical organisation of the stress response

The HPA axis is comprised of elements that are located in both the central nervous system (CNS) and in peripheral tissue (153). The CNS component comprises the hypothalamus containing neurons of the paraventricular nucleus (PVN), and the anterior lobe of the pituitary gland, which lies below. The peripheral tissue component is the adrenal gland (153). Through a coordinated

series of actions hormonal signals are released from these structures to mediate the stress response.

Under the influence of stress, circadian rhythm or other stimuli, the hypothalamic PVN synthesises and secretes corticotropin-releasing hormone (CRH), which is the main regulator of the HPA axis, (and another hormone called vasopressin) (154). Once CRH is secreted it stimulates the corticotrope cells of the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into the bloodstream, targeting the cortex of the adrenal gland. ACTH then stimulates the adrenal cortex to synthesise and secrete GCs (Figure 1.5) (155,156). In humans the principal GC is cortisol, whereas in rodents it is corticosterone. GCs traverse the blood-brain barrier to the brain where they bind to two distinct receptors through which they exert their effects, mineralcorticoid (MR) and glucocorticoid receptors (GR) (157,158). MR are activated by low basal levels of cortisol under normal physiological conditions, whereas during stress, higher cortisol levels activate the GR (157). GR mediate the activity of GCs in the cell (159). Once bound to a corticosteroid, activated GR dimerise and translocate into the nucleus from the cytoplasm where they induce or repress target gene transcription, and behave as ligand-dependent transcription factors (160).

Activity of the HPA axis can be inhibited by negative feedback from elevated levels of circulating GCs (156) (Figure 1.1). Increased levels of GCs can also affect one other structure in the brain, the hippocampus, which is abundant in both MR

and GR and plays a significant role in the GC negative feedback response (Figure 1.1) (161). In the absence of stimuli, the hippocampus normally prevents HPA axis activation by blocking the activity of CRH-containing cells. However, periods of protracted stress or cortisol exposure can be damaging to the hippocampus, diminishing hippocampal neurogenesis and reducing hippocampal volume (162,163), which may impede its ability to adequately regulate the HPA axis.

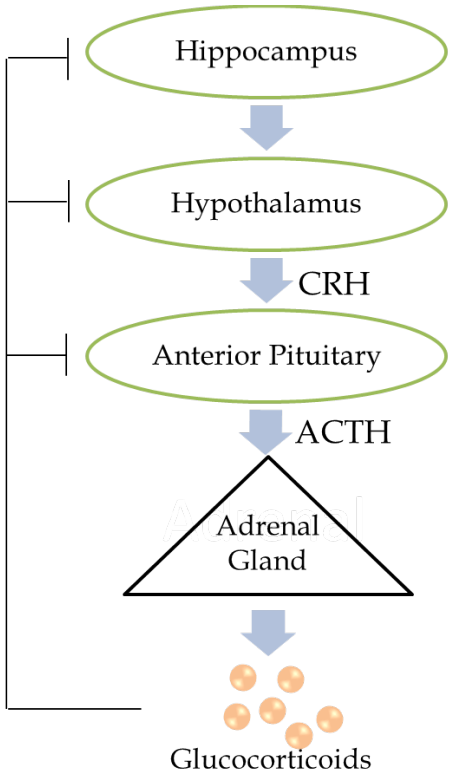

Figure 1.1 Glucocorticoid regulation in the HPA axis.
Adapted from Vedder 2008 (164). Under the influence of stress, corticotrophin releasing hormone (CRH) produced by the hypothalamic paraventricular nucleus (PVN) stimulates the anterior pituitary gland to synthesise and secrete ACTH, which, in turn stimulates glucocorticoid release from the adrenal gland

1.2.2.2 Glucocorticoids and pregnancy

GCs play an important role in embryogenesis (165) and are crucial for the growth and maturation of various fetal tissues including the lung, liver, kidneys, and gut

(166). Throughout the course of pregnancy the maternal HPA axis undergoes significant changes as levels of cortisol gradually increase, and normal basal responses to GCs are tempered (167).

In late gestation, mammalian fetal GCs experience a final surge for organ maturation in preparation for neonatal life (168). Both in humans and mice this late-stage increase comes about as the result of two sources. The first source is fetal and arises from glucocorticoid synthesis (*de novo*) by the fetal adrenal gland (169,170). This increase in *de novo* glucocorticoid synthesis is also accompanied by a rise in fetal production of GR near term, which plays a significant role in the preparation of the liver for carbohydrate and lipid metabolism in early postnatal life (171,172). The second source is the maternal plasma (173,174).

Despite these increases, over the course of a normal pregnancy, fetal glucocorticoid concentration is maintained at lower levels than the mother (175). Given the significantly higher concentrations of active GCs in maternal circulation, it is therefore necessary to manage fetal exposure so that it is not in excess. This role is carried out by the placenta, which, in addition to its role in nutrient exchange, acts as a glucocorticoid barrier between maternal and fetal circulations (176). Notably, the bioavailability of GCs is regulated by two isoforms, 1 and 2, of the enzyme 11 beta-hydroxysteroid dehydrogenase (11β-HSD), which interconverts the active cortisol (in humans)/corticosterone (in rodents) and their inactive forms, cortisone/11-dehydrocorticosterone (177).

The 11β-HSD2 isoform is present in the placenta, and catalyses the inactivation of maternal GCs, thus reducing fetal exposure (178,179). This provides a barrier for the exclusion of up to 90% of active maternal GCs from the fetus (180). However, in late gestation, 11β-HSD2 levels decrease to allow more maternal cortisol/corticosterone to be available to the fetus. Acting in concert with 11β-HSD2 is a second protective placental transporter called P-glycoprotein, which mediates the removal of GCs (181). Unlike 11β-HSD2 whose expression increases steadily throughout gestation, dropping near term (173,174), P-glycoprotein elevates mid-gestation and steadily decreases as pregnancy progresses (182).

As a result of these effects, synthetic pharmacological GCs have been used to treat women at risk of preterm birth to promote precocious lung maturation and, less frequently, congenital adrenal hyperplasia to improve neonatal viability (183,184). The therapeutic effectiveness of synthetic GCs is due to the inability of 11β-HSD2 to efficiently metabolise them and therefore they are able cross the maternal-fetal barrier (185,186). In spite of the benefits of such treatments, studies have indicated that several acute and chronic effects occur in situations when the fetus is exposed to higher levels of GCs.

1.2.2.3 Glucocorticoids and programming

Fetal overexposure to GCs can result when synthetic steroids are administered to mothers as discussed above (183,187,188), during high maternal stress

(189,190)., or when corticosteroids cannot be properly metabolised (191,192). A notable consequence of increased glucocorticoid presence is impaired growth, which has already been identified as a significant indicator of adversity *in utero*, and is linked to development of metabolic disease (9,11,13). IUGR has been observed both in humans (187,188) and animals (193,194) following antenatal glucocorticoid treatments. Notably, lower birth weights in humans have also been accompanied by elevated umbilical cord CRH and cortisol when prenatal corticosteroids were given (195,196). When clinically equivalent doses of betamethasone were given to pregnant ewes, femur lengths, as well as brain, liver and kidney sizes are reduced (197).

Similarly, intrauterine growth is impaired when endogenous maternal GCs are elevated by factors such as maternal disease stress (195), hypoxia (198,199) and malnutrition (200-202). The ability to metabolise GCs ties into the effectiveness of 11β-HSD2 in managing the maternal-fetal barrier in the placenta. Under normal circumstances 11β-HSD2 levels increase throughout pregnancy, but decrease closer to term (173,174). However, in humans, some growth-restricted fetuses have been observed with drastically reduced expression of placental 11β-HSD2 (191,192). In rare instances, there are individuals that possess a deleterious mutation that causes an 11β-HSD2 deficiency, who also report severe growth deficits at birth (203).

Of the listed factors that elevate endogenous GCs**, the effects of nutrition are of particular interest to this thesis.** Maternal protein or global dietary restriction in rats (202), mice (204) and sheep (200,205,206) suppress 11β-HSD2 in late gestation. Using a mouse model Knight and colleagues in our research group previously imposed a 30% maternal dietary restriction on two strains of laboratory mice: the A/J strain and the C57BL/6J (B6) strain from E6.5 to E17.5 (201). They observed fetal growth restriction in conjunction with larger placentae, indicating placental inefficiency, as well as elevated maternal plasma corticosterone in the dietary restricted group, suggesting increased GC passage into the fetal compartment (207).

The later life consequences of HPA axis programming in humans and animals are well documented. Elevated GCs have a demonstrated risk of hyperglycaemia (193,208) and hypertension (208-210) when they are exogenously administered. Once again dysfunctional metabolism is linked to stimuli such as nutrition which also increase early life endogenous GCs. Using this mouse model, previous data were able to demonstrate adult metabolic syndrome phenotypes including hyperlipidaemia and glucose intolerance in response to undernourishment during gestation (211). The development of these phenotypes coincided with elevated endogenous maternal corticosterone and suppressed 11β-HSD2 in the placenta at E17.5 (201).

In addition to metabolic disease outcomes, in excess, GCs have been linked to profound programming effects in the neuroendocrine system (194,212). The

perinatal period is a critical window for brain development. In sheep, fetal exposure to synthetic GCs resulted in hyperactivation and hyper-responsiveness of the fetal HPA axis leading to elevated ACTH and cortisol in early life (213,214). In humans this inappropriate pattern of HPA axis hyper-stimulation is also shown across infancy and into adolescence following maternal prenatal psychological stress (215-217).

The long-term implications for mental disorders are substantial in offspring that might have been exposed to maternal stress. Schizophrenia in adults has been linked to undernutrition, as reported in offspring of the Dutch Hunger Winter (218), as well as overnutrition following maternal obesity (219,220). Further, maternal obesity (221,222) as well as anxiety/depression (223) are also identified as risk factors for the development of attention deficit hyperactivity disorder (ADHD). Failure of the placental glucocorticoid barrier is also associated with behavioural programming. In rats, experimentally blocking the activity of placental 11β-HSD2 programs anxiety behaviour in adult offspring (212).

HPA axis effects on offspring are not limited to exposure to adverse intrauterine environments. Studies have also shown that maternal behaviour in early postnatal life has implications for the offspring HPA axis in adulthood. Rat pups that were exposed to more frequent licking/grooming-arched back nursing in their first postnatal week had greater hippocampal GR expression and increased sensitivity to the GC negative feedback, suggesting a less exaggerated response to stress (224,225). Taken together, these data suggest that early life stress, and

the fundamental alterations in HPA axis function that occur, can have long-term consequences for disease. An understanding of the molecular mechanisms involved in mediating these outcomes may help clarify understanding of disease progression and its underlying pathophysiological processes.

1.2.3 Sex differences

Indeed, while the abovementioned factors, nutrition and glucocorticoids, have significance for programming, offspring sex also appears to both dictate response to both factors, as well as future severity and incidence of disease phenotypes. Various studies employing the use of animal models such as rats (226-228), mice (211,229,230) and sheep (231,232) have demonstrated sexually dimorphic responses to early life stressors. This is not limited to models but is also clearly shown in humans (26,233,234).

The reasons for this remain unclear. However, one often explored hypothesis is differences in male and female growth rates *in utero* (235). Speed of growth may in fact be a significant factor when it comes to assessing the effect of an insult during a specific developmental time point. *In vitro* evidence in mice shows that male cultured preimplantation embryos grow at a higher rate than females (236). Moreover, in humans, intrauterine post-morphogenesis growth is also shown to be more rapid in males, accounting for the generally higher birth weight (237). This developmental difference in males across various species may suggest why they are more sensitive to insufficient nutrition *in utero* (66,202,232), and why female fetuses may have a greater susceptibility to overnutrition (131).

Not limited to nutrition, sex-specific responses are influenced by timing of glucocorticoid exposure. Female offspring of rat dams with elevated stress in the final week of gestation demonstrated higher ACTH and corticosterone when restrained, whereas there was no observed effect in males (189). When triggered by undernutrition, elevated endogenous glucocorticoids not only program a hypertensive phenotype in both male and female sheep offspring, but substantially increased resting cortisol in the first year of life (238).

The highlighted sex differences in the literature associated with DOHaD emphasise the importance of examining males and females separately. The effects dependent on sex are wide-ranging and will require a significant amount of research, before determining stimuli and mechanisms for specific health outcomes.

1.3 Impacted organs: The liver

As initially discussed, exposure to a less than adequate early life environment may impact the development of several tissues and organs involved in insulin-mediated regulation of metabolism, such as the liver (35). Consequently, the effects of perturbations in the development of the liver have been linked to the development of metabolic disease (239). Given the relevance of this particular organ to the studies conducted in this thesis, a comparative outline of the structure and development of human and mouse livers is detailed below.

1.3.1 Liver Structure

The liver is the single largest interior organ of the body and performs many of the same functions in humans as in mice (240). In spite of this, species-specific differences in liver gross structure, relative size and position are present between humans and mice. While the organ develops similarly, variations also exist within the various stages of liver development.

The human liver constitutes roughly 2.5% of total body weight (152). It is located in the superior right-hand quadrant below the diaphragm in the cranial abdomen, and comprises four discernible lobes (Figure 1.2A, B): the left, right, caudate and quadrate. The right lobe is the largest lobe (241). The liver is suspended above the gall bladder and an extensive blood vessel network by a series of prominent ligaments (241).

Similarly, the mouse liver (Figure 1.2C, D) is also located below the diaphragm in the cranial abdomen (240). However, unlike humans, the mouse liver is spread across the entire space inferior to the diaphragm, occupying a third of the anterior abdominal cavity (242) and making up 5% of total body weight (240). The liver in the mouse also possesses four lobes: left, right, caudate, and a fourth distinct lobe, which is the largest, called the medial lobe (Figure 1.2C, D) (240). The medial lobe is partitioned into left and right portions by a deep cleft through which the gall bladder protrudes (242) (Figure 1.2D). Unlike in humans, the ligaments that suspend the mouse liver are less distinct (240).

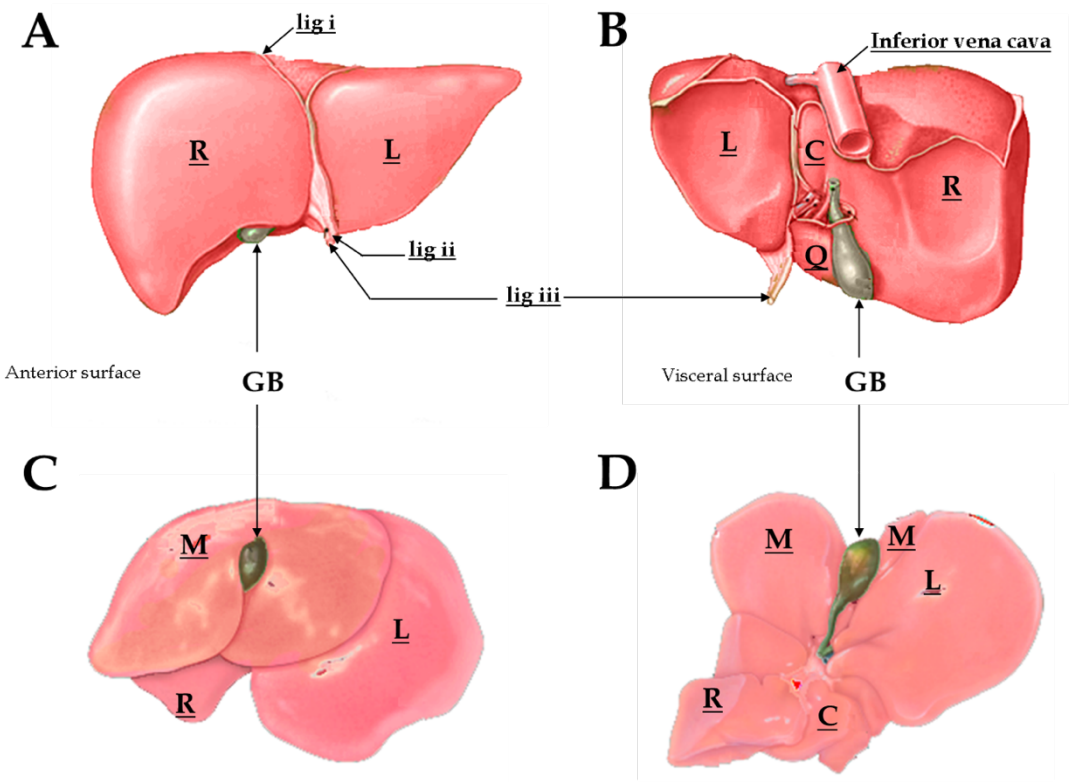

Figure 1.2. Anatomy of the human and mouse liver.
(A), (B) The human liver comprises four discernible lobes: right (R), left (L), caudate (C) and quadrate (Q) and is suspended above the gall bladder (GB) by prominent ligaments (lig i, ii, and iii). (C), (D) The mouse liver also has four lobes: right (R), left (L), medial (M) and caudate (C). Suspensory ligaments in the human liver are not present in the mouse liver. In the mouse liver, the GB does not fully lie below the liver, but protrudes through the middle of the medial lobe. Figures modified from Treuting (240).

Human and mouse livers possess various cell types which are organised very similarly between species. These include parenchymal cells called hepatocytes, and bile duct epithelia (cholangiocytes), non-parenchymal sinusoidal cells consisting of endothelial cells and hepatic macrophages called Kupffer cells, and non-parenchymal peri-sinusoidal fat storing cells called stellate (Ito) cells and hepatic natural killer cells called pit cells (242-244). The remainder of the liver is comprised of an extracellular compartment that includes sinusoids which are lined by the non-parenchymal cells, the perisinusoidal space called the space of

Disse and bile canaliculi (241,244). Hepatocytes comprise 60% (52% in mice) of the adult liver cell number (243,245). Hepatic sinusoidal endothelial cells comprise 20% (18% in mice) of total liver cells, while stellate cells, which represent 5% to 8% of total cells (8% in mice). The remaining endothelial cells make up the remaining cell number (12-15% in humans or 22% in mice) (243,245).

As in humans, the architecture of the murine liver is optimised for function due to the presence of a portal triad, comprised of branches of the hepatic artery, portal vein and hepatic ducts (245,246). The hepatic portal vein provides 40% of the liver's oxygen for metabolism and 70% of total liver blood supply, and the hepatic artery supplies 60% of the liver's oxygen and the remaining 30% of the liver's blood (246). This hepatic design helps to facilitate the exchange of materials between the blood and therefore allows the liver to more easily carry out its many functions in the body (246).

1.3.2 Liver Function

The liver has several important functions including synthesis of plasma/serum proteins (including clotting factors and transport proteins such as albumin and transferrin), and removal and degradation of compounds such as serum proteins, erythrocytes, microbes and toxic endogenous compounds (247). However, one of the most important functions of the liver is its role in nutrient metabolism (152). The liver metabolises both carbohydrates and fats, and maintains a dynamic equilibrium of catabolism and anabolism (246). The regulation of this equilibrium is dependent upon signalling from hormones secreted by organs such as the

neighbouring pancreas, adrenal glands, thyroid and adipose tissue, as well as input from the nervous system (247,248).

It is important that an adequate level of circulating glucose is maintained for the central nervous system, which uses glucose as its main metabolic fuel (246,248). When blood glucose levels are very high, the liver converts glucose into glycogen, fatty acids or amino acids for storage (152). However, when blood glucose concentrations are low, glucose can be produced by degradation of the glycogen depots in the liver or muscle cells (247).

During the course of digestion, fats are broken down to fatty acids and glycerol (247). In order to store the metabolised fats, the fatty acids and glycerol then undergo an esterification reaction to form TG (247). These triglycerides are stored in the hepatocytes cytosol as lipid droplets and used to help maintain the energy balance of the cells and hence the whole organism (243). Fatty acids are typically derived from phospholipids or TG and are stored either in the liver or in other sites such as adipose tissue (243). They may be used as a component of the cell membrane, or as a fuel source following beta- (β–) oxidation (249), to yield the highest adenosine triphosphate (ATP) production of any metabolic fuel (243). Due to the essential role the liver plays in maintaining homeostasis, it is important that the liver develops properly. The process of liver development is described below.

1.3.3 Liver Development

The process of intrauterine development is very similar between humans and mice. In humans this period of development is divided into three stages: pre-implantation, embryonic, and fetal (250). The embryonic stage is the major period of organogenesis (organ formation) (251). The length of time an organism spends *in utero* and thus, within a given developmental stage, differs between species. The duration of normal human gestation is roughly 38 weeks, whereas in mice it is between 19-20 days (252). Furthermore, the timing of birth is set very differently between these species.

The human embryonic period covers the first 8 weeks (~60 days) of development, during which organogenesis occurs (251). At the end of the 8-week period, the conceptus is no longer called an embryo and the term "fetus" is applied. This new fetal period lasts from week 9 to birth, during which the fetus remains *in utero*, allowing organ maturation and body growth to occur (251). However, in mice, once organs are fully formed, the mouse is born almost straight away (253). Because the mouse has a much shorter gestation period, the term embryo is applied throughout all developmental stages post fertilisation until birth (254). Therefore the progression of mouse development *in utero* is indicated by the number of days post conception (255). The timing of the first day of pregnancy is determined by the discovery of a copulatory plug and is designated embryonic day (or E) 1 or 0.5 (255). For the purpose of this thesis E0.5 will be used to denote embryonic day 1, and E18.5 for final gestational day 19.

Stages of development in the human embryo are characterised using criteria established by the Carnegie Institution of Washington, and are called Carnegie stages (256). Carnegie stages comprise a total of 23 steps and only cover the embryonic phase of development (Figure 1.3). Once the fetal phase begins, Carnegie staging no longer applies (256). Carnegie stages were originally determined exclusively by examining the development of external characteristics (256). However, staging criteria has expanded to include age in days (post-fertilisation), internal morphology and embryonic (crown to heel) length (257,258). Conversely stages of embryo development in the mouse are characterised using Theiler stages (TS) (Figure 1.3), which are comprised of 26 prenatal (22 embryonic followed by 4 fetal) and 2 postnatal periods covering the first 20 days post conception (259,260). However, in order to compare changes in appearance that occur during the embryonic period across different species only Carnegie staging is used (Figure 1.3).

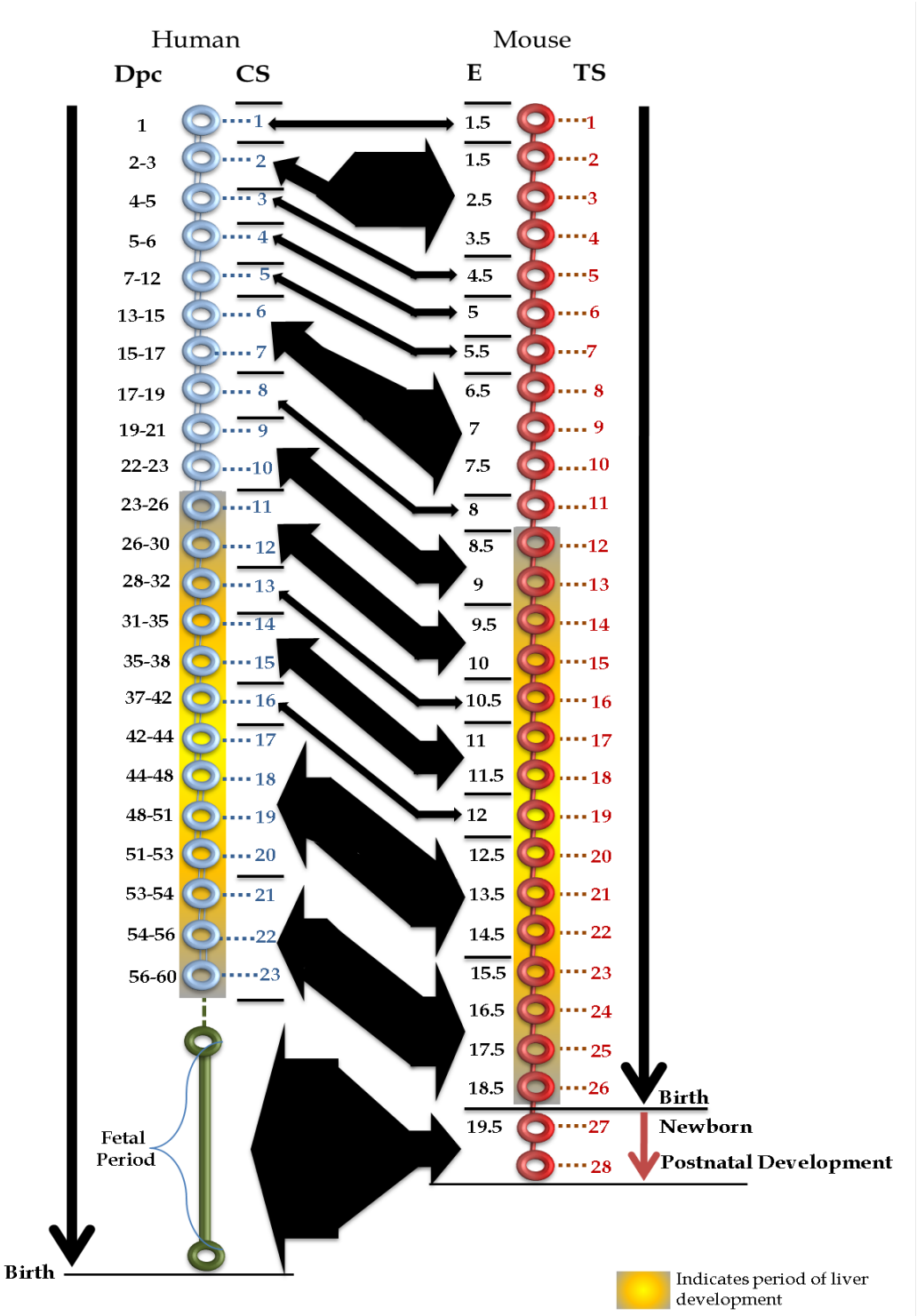

Figure 1.3. Comparison of developmental stages of human and mouse embryos, including periods of liver development.
Carnegie stages (CS) of human development are indicated in blue. The equivalent Theiler stages (TS) of mouse development are represented in red. Gestational timeline is expressed using days post conception (Dpc) in humans and gestational day (E) in mice. Period of liver development is highlighted as between CS 11-23 in humans and TS 12-26 in mice. (Adapted from Xue *et al*. (253) and Theiler (260)).

Many similarities exist in the mechanisms that govern liver development in humans and mice. In both species liver development commences during embryogenesis and proceeds as a multi-stage process, which is regulated by various genes and pathways (251,261). Embryonic development of the human liver begins in the third to fourth week of gestation, corresponding to Carnegie stage (CS) 11, which is equivalent to E8.5 or Theiler stage (TS) 13 in the mouse (252,262). Following fertilisation and eventual blastula formation, the blastula reorganises into the gastrula with three germ layers ectoderm, mesoderm and endoderm, the latter from which the liver derives (263).

During this period the endodermal epithelia lie in close proximity to mesenchymal cells in the septum transversum of the developing heart (246). The cardiac mesoderm and developing cardiac organ then release two classes of proteins, fibroblast growth factor (FGF) and bone morphogenic protein (BMP), which stimulate the underlying endoderm to commit to differentiate into primitive hepatocyte precursors such as hepatoblasts, Type I hepatocytes, and prehepatocytes (264,265). Further FGF and BMP secretion stimulates the formation of a hepatic diverticulum at CS 11/TS 13 ((246,252). This diverticulum, which consists of primitive hepatocytes, then grows out from posterior foregut of the endodermal epithelium (246,252).

These primitive hepatocytes also begin to aggregate into small cords (262). This process of "fate-mapping" and outgrowth of cells is marked by increased

hepatoblast expression of albumin (Alb) (264). By the end of the first month of gestation in humans (CS 12) there is an increase in the expression of Alb and other hepatocellular proteins alpha-fetoprotein (AFP), and transferrin indicating the ability of the primitive cells to synthesise and secrete proteins (246). In mice, once liver primordial outgrowth is initiated, AFP expression is increased by E8.5 (TS 11-12) and decreases over the course of the pregnancy, with a corresponding increase in albumin expression within 2 days of AFP (254,265).

At CS 13/TS 16, the cords of cells at the liver diverticulum begin to proliferate to significantly increase hepatic cellular mass (262). As development enters CS 14/TS 17, the hepatic glands enlarge and become more vascular (262). While organogenesis proceeds at CS 12/TS15 in the endodermal layer, the visceral mesodermal layer begins to evolve into a unique axial structure consisting of the dorsal aorta, genital ridges and mesonephron (266). This structure is called the aorta-gonad-mesonephros (AGM) region (251,267). By the end of CS 14/TS 17, the AGM is fully formed and begins to produce stem cells which have the ability to differentiate into the various cell and tissue types in the blood (268). This production of cells and tissue components of the blood is known as haematopoiesis (247).

These hematopoietic stem cells (HSCs) invade the fetal liver from the AGM region through the established circulation and initiate liver hematopoietic function. As a result, the HSCs colonize the liver and are able to expand their

mass and diversify their lineage (262). As the liver matures and becomes more metabolically active, the HSCs then relocate to the bone marrow or spleen, which then assume the primary haematopoietic role at the end of TS 21 (262).

Continuous proliferation of epithelia occurs in CS 18/TS 21. The bile ducts also reorganise, establishing continuity between the liver and the gut, but only in humans (262). Notably, this remodelling happens in mice only in the postnatal period (254). Between CS 18-23/TS 21-26 biliary ductules develop and organise near the connective tissue portal. By CS 23/TS 26 (57 days in humans/E 17.5 in mice), more than 90% of the hepatic structures present in the adult liver are now present in the embryo (256).

Once the liver is formed during the embryonic phase, the organ continues to further enlarge in the fetal phase. In humans, initially liver growth occurs as a result of hepatocyte proliferation (246). However, as the second trimester begins, mitosis is regulated by growth factors insulin-like growth factor I and II (IGF-I/II) (269,270). By the third trimester, mitosis decreases and hepatic organ mass increases due to enlargement of individual hepatocytes (271). When the human fetus is born, there is on-going mitosis to increase liver organ size since the neonatal liver only contains 20% of the hepatocytes present in the adult liver. This growth persists until the end of the second decade of life (271). At birth, hepatocytes are specialised so that they now have a dual-surface functionality (272). One surface, which is sinusoidal, is responsible for nutrient and oxygen

absorption from the hepatic portal vein. The other surface, which is canalicular, transports bile and metabolic end products (272). The liver is now able to carry out various metabolic functions including glycolysis, gluconeogenesis, detoxification and hydrolysis of cholesterol esters (272).

Alternatively, once mice are born, liver organ size is augmented by significant increases in hepatocyte proliferation (273), with a more than 3-fold increase in liver mass by postnatal day 9 (274). By four weeks of age, there is a marked decrease in proliferation as the liver-to-body weight ratio approaches adult levels (275). At this point the liver histological architecture resembles that of 3-month-old mice (276). The developmental stages of the liver are summarised in Figure 1.4.

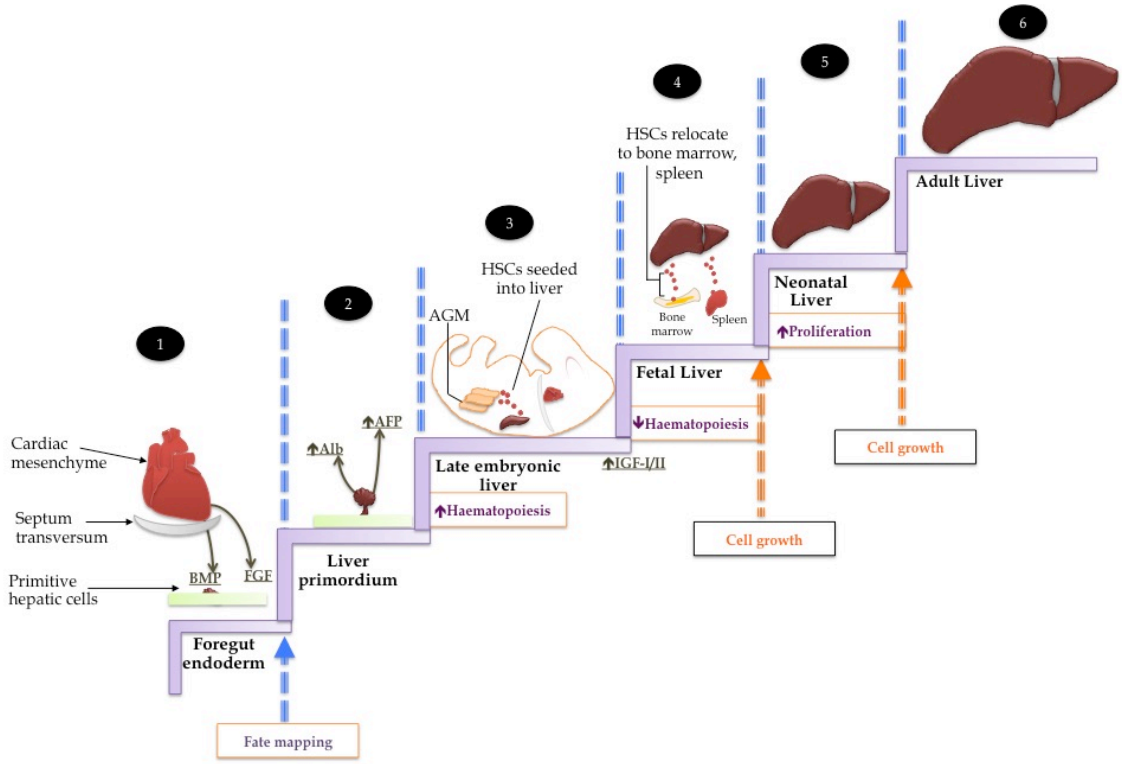

Figure 1.4. Summary of liver development.
1) Hepatic cells arise from foregut endodermal cells in response to Bone morphogenic protein (BMP) and Fibroblast growth factor (FGF) signals from cardiac mesenchyme and septum transversum. 2) Maturation of the liver primordium is accompanied by increased expression of Albumin (Alb) and alpha-fetoprotein (AFP). 3) In late embryo phase the aorta-gonadal-mesohephros (AGM) develops and AGM HSCs begin to invade the liver converting the liver into a haematipoietic organ. 4) As the liver matures and metabolic activity increases leading up to HSCs relocate to the bone marrow or spleen reducing liver haematopeitic function. 5) Hepatocytes continue to proliferate at birth and into neonatal life. 6) Hepatocytes further mature postnatally as cell proliferation is terminated. All events in boxes with **Purple** text (fate mapping, haematopoiesis, proliferation) occur at equivalent time points in humans and mice. Events that are boxes with **Orange** text (cell growth) are unique to humans at those specific time points i.e. birth and at the end of the neonatal phase. Proteins expressed IGF-I/II (Insulin-like growth factor I/II) Adapted from Kinoshita and Miyajima (264).

1.4 Molecular Mechanisms

Overall, the underlying molecular mechanisms of DOHaD still remain unclear. The abundance of data, from both epidemiology and animals, suggests that developmental and early life environments can affect specific genotypes generating phenotypic outcomes for susceptibility to metabolic disease. While tracking of the molecular mechanisms from compromised early life to the disease pathogenesis is incomplete, possible mechanisms have been proposed involving genetic and epigenetic processes.

1.4.1 Genetics/Epigenetics

1.4.1.1 Genome

It has been clearly demonstrated that ante- and postnatal environments contribute to metabolic dysfunction. However, differences in disease prevalence rates between individuals suggest that genotype also defines health outcomes in later life (277). Furthermore, it has been proposed that the aberrations that lead to these conditions may derive from interactions between an individual's genes and the environment (278-280). A significant body of work implicates single nucleotide polymorphisms (SNPs), variations in the DNA sequence of a gene that occur when a single nucleotide is changed (281), as being key determinants of risk (282).

Several SNPs associated with metabolic disease have been identified. Examples of such polymorphisms include those that occur in the multi-isoform gene peroxisome proliferator-activated receptor- gamma (PPAR-γ) (283). PPAR-γ is a

hormone receptor in the nucleus that regulates target gene expression by mediating the effects of ligands, and plays a crucial role in metabolism (283). The PPAR-γ variant where the amino acid alanine is replaced with proline at codon 12 (or Pro12Ala) has previously been linked to the development of type-2 diabetes (284). Alternatively, the Pro12Pro genotype has stronger links to DOHaD, demonstrating associations with small birth size and insulin resistance in later life, as well hypertension and dyslipidaemia (285-287).

Other SNPs associated with metabolic syndrome phenotypes include those identified in the glucocorticoid receptor gene (288-290). Given the critical role of GR in HPA axis regulation, it is also of note that polymorphisms have been linked to programming of psychological stress disorders (291,292). Furthermore, notably, data from the Helsinki Birth Cohort identified the potential for haplotypes of GR to modify associations between birth length and metabolic and stress axis dysfunction in adulthood (293).

The identification of polymorphisms may not only be useful for evaluating candidate genes for developmental programming of disease, but also assessing potential effects of interventions. The potential benefits of longer periods of breastfeeding were shown by Briollais and colleagues in carriers of a high-risk allele on the obesity gene (FTO) associated with higher BMI in adolescents of the Western Australian Pregnancy (Raine) Cohort (294). Breastfeeding was able to counter the effects of the allele, particularly in girls (294). Follow-up studies using British Avon Longitudinal Study of Parents and Children (ALSPAC) cohort

revealed that at least 5 months of exclusive breastfeeding acted adversely against the FTO risk allele and was linked to a lower BMI (295). Taken together, these data suggest a highly complex interaction between genes and environment that contributes to disease outcomes, and is in agreement with the principles of the developmental plasticity hypothesis. However, genotypic alterations are not the only outcomes of exposure to suboptimal early life.

1.4.1.2 Epigenome

Gene-environment interactions can lead to a grouping of several mechanisms that alter gene expression and chromatin structure without making changes to the overall DNA nucleotide sequence (296). This collection of mechanisms is heritable and are called epigenetic modifications or marks (296). They can be inherited through mitosis between cell generations or via meiosis between generations of a species. Epigenetic modifications include DNA methylations, histone modifications, and the imprinting of non-coding RNAs (297-302).

1.4.1.2.1 DNA Methylation

The best characterised of all epigenetic modifications is DNA methylation. Methylation of mammalian DNA occurs when a methyl group is added to the 5′ position of a cytosine ring structure at a site where a cytosine nucleotide occurs next to a guanine nucleotide, creating a cytosine-phosphate-guanine (or CpG) dinucleotide sequence (299). CpG structures are not evenly distributed, occurring once every 80 dinucleotides throughout most of the genome (303). About 2% of

the genomic structure comprises sections containing a greater density of CpG dinucleotides called CpG islands (303,304).

Methyl group transfer to cytosine during methylation is catalysed by a specialised group of enzymes called DNA-methyltransferases (DNMTs), of which there are 3 classes DNMT1, DNMT3a and DNMT3b (305,306). DNA methylation plays a significant role in normal embryogenesis and all 3 DNMTs are all essential during the developmental process (307). Notably, methylation patterns are determined during embryogenesis and into early postnatal life (308,309). Once the variations in cellular methylation patterns are established, they are accurately maintained by DNMTs so that they can be inherited by subsequent generations of daughter cells (308,309). The methyl groups that are added to cytosine are derived from methionine, a non-polar amino acid which is used to synthesise the universal methyl donor S-adenosylmethionine (SAM) (310). SAM is the principal source of methyl groups for DNMT mammalian methylation reactions (310,311).

Differences in DNA methylation patterns can lead to genome wide effects or, more locally, affect specific genes. Whole genome alterations to DNA methylation patterns have been linked to diseases such as cancer (312). The most clearly defined effect of methylation is on a smaller scale, in the modulation of gene promoter regions associated with active transcription (313). CpG islands generally contain promoters whose activity is linked to their unmethylated state

(304,313). Once these islands are methylated their associated genes can be transcriptionally repressed (314). This transcriptional silencing is present in genes on the inactive X-chromosome in normal females (315), and in instances of an epigenetic phenomenon known as genomic imprinting (316).

Genomic imprinting occurs when two alleles at a specific locus are present, but only one is expressed, while the other remains in an inactive state, based on parental origin, without DNA sequence alterations (316,317). This process involves methylation of specific alleles in the CpG islands (318). Given the diploid genetic constitution of mammals, both maternal and paternal copies of a gene are necessary and have the ability to be active within a cell (319,320). However, imprinting limits the expression of the inherited gene to one of the parent's chromosomes. Therefore, the genes derived from one parent "override" the expression of the genes derived from the other in a "parent-of-origin" dependent fashion (38,320). This results in the expression of specific alleles from each parent where both genomes are equally represented, resulting in optimal fetal growth and development (321).

A good example of the contribution of genomic imprinting to balanced fetal growth is with the genes IGF-II/Igf-II) and IGF-II receptor (IGF-IIR/Igf-IIr), in both humans and mice, which exhibit distinct methylation patterns depending on which parent they originated from (319,322). IGF-II is paternally expressed and plays a significant role as a potent growth factor, and in regulating fetal

nutrient supply in the placenta (323,324). IGF-IIR in contrast is a maternally expressed gene and is responsible for degrading placental IGF-II (325). Thus, the placenta is exposed to two contrasting selective pressures; "pro fetus" which ensures nutrient transfer so that developing offspring are able to survive to pass on the genome, and "pro mother" which preserves maternal resources so that they are not depleted to the point of maternal deterioration during either pregnancy or lactation (326). This balance of maternal and paternal gene expression is important for optimal fetal development.

1.4.1.2.2 Histone modifications and other epigenetic mechanisms

The other most commonly discussed epigenetic marks are histone posttranslational modifications, which alter the structure of chromatin. Structurally, histones are made up of amino-(N)-terminal tails, which extend outwards to contact the nucleosomes that lie adjacent (327). There are various ways by which histones are modified including acetylation, phosphorylation, methylation and sumoylation, which all affect transcription (297,300-302). Other mechanisms that have emerged more recently and have been shown to play roles in epigenetic processes are non-coding RNAs, which have been well reviewed (298,328).

1.4.1.2.3 Epigenetics and DOHaD

Epigenetics has received considerable attention and has emerged as a possible molecular mechanism for explaining the DOHaD paradigm. Several pathological

conditions have been linked to the dysregulation of epigenetic processes, such as early stage atherogenesis associated with CHD, and in T2D (329,330). While the epigenetic modifications described above are recognised as playing an important role in pathogenesis of disease, presently the specifics of their involvement in programming are still not fully understood. Moreover, despite their identification, the challenge of contextualising the gene signalling and regulatory paths related to such changes to the epigenome still remains. This especially remains a problem in human studies and therefore animal studies have mainly been conducted in this area.

Several studies have examined the effect of early nutrition, both pre- and postnatal, which has been shown to affect offspring susceptibility to disease in later life (13,14,37). Moreover, various data have established a link between specific nutritional components in maternal diet and the development of these disorders (331). It is evident that changes in DNA methylation occur in response to less-than-optimal environments *in utero*. The influence of diet on methylation is shown through a process called one-carbon metabolism, which increases the bioavailability of SAM, the principal methyl group donor (332,333). The one-carbon metabolism process relies on micronutrients such as B vitamins folic acid, B2, B6 and B12, as well as glycine and serine, in order to generate methionine (332,333).

Wolff *et al.* (334) conducted the initial study that demonstrated that these micronutrients in maternal diet could modulate both gene expression and methylation, using the *agouti* viable yellow mouse (A^{vy}). The A^{vy} mice express the *agouti* gene as a result of a viral intracisternal A particle which generates a yellow coat and transcriptional silence in offspring during myelination, but an *agouti* coat and gene expression if unmyelinated (334). By exaggerating the concentrations of folic acid, B12, zinc, methionine, betaine and choline in maternal diet Wolff and colleagues were able to observe significant methylation changes in the intracisternal A particle (334).

Building on the success of Wolff *et al*. (334), a later study was able to link Vitamin B and methionine concentrations to developmental programming of disease (335). Decreasing sheep periconceptional B12, folate and methionine within the normal physiological range led to extensive alterations to DNA methylation pertinent to health outcomes in adults (335). These changes in methylation status of the CpG sites were associated with the development of insulin resistance, increased adiposity and elevated blood pressure in adult offspring, principally manifest in the males (335). Hence, the effects of epigenetic changes on gene expression may elevate risk of disease in later life (336).

Diet-induced epigenetic alterations are also affected by deficiencies in macronutrients. In rats, maternal protein deficiency alters methylation patterns of specific metabolic disease-related hepatic genes, PPAR-α and GR, in offspring.

Notably these effects can be reversed via folic acid supplementation (337). Further, in another rat model, brains of offspring from uterine artery-ligated rat dams show decreased methylation along with decreased Dnmt1, as well as increased histone acetylation, which accompanies their IUGR phenotype (338).

As previously discussed, (Section 2.2.2), postnatal life also predicts improved stress axis responses in adult offspring, as shown in rats who were exposed to more frequent licking/grooming-arched back nursing in the first week of life (224,225). These effects on offspring HPA axis have been linked to epigenetic modifications of the exon1 promoter of the GR gene (339). Several transcription factors interact with the GR promoter, notably nerve growth factor-inducible protein-A (NGFI-A), which is expressed in the brain and is a potent transactivator (340,341). The exon 1_7 NGFI-A binding site of the GR promoter is hypomethylated in offspring who were nurtured more and increasingly binds to NGFI-A (339). This coincides with increased hippocampal GR expression and thus enhanced HPA feedback regulation in the better-nurtured offspring (339). However, in spite of the ability of low licking/grooming-arched back nursing to program certain adverse stress responses via epigenetic modification, this programming may be reversed via methionine supplementation (342).

In humans, the relationship between epigenetic dysregulation and the development of disease is highlighted through findings in cohort studies. Such findings include data on offspring from the Dutch Hunger Winter, who

demonstrated a hypomethylation of the IGF-II gene in adulthood (343,344). Interestingly, IGF-II hypomethylation has also been observed in children of obese parents, particularly where the fathers were obese (345). Complementing these and findings from animal studies, neonates who were exposed to high B12 during pregnancy had reduced methylation of IGF binding protein-3 levels (346). In addition, the placentae of IUGR infants have been shown to have significantly altered methylation patterns in numerous loci (347). This outcome may be attributed to maternal and paternal imprinted genes in the placenta, given their proposed role in regulation of fetal nutrition and growth (323-325).

1.4.1.2.4 Transgenerational transmission

Data from animals and epidemiology have also proposed that transgenerational transmission of epigenetic changes can occur. In guinea pigs, maternal prenatal exposure to synthetic GCs produced an altered DNA methylation pattern in offspring, which persisted into adulthood. This altered methylation pattern was also passed on to the next generation (348).

In humans, it has been suggested that inherited epigenetic changes that promote vulnerability to diabetes are influenced by previous parents' and grandparents' nutrition (349,350). The influence of inherited epigenetic changes has also been suggested in offspring from the Dutch Hunger Winter. Female offspring that were born to undernourished mothers during pregnancy had lower birth weights and increased risk for insulin resistance (42). In addition, the daughters

of those females also had low birth weights (42). Taken together these data strongly suggest a significant impact of early life stressors to influence several generations.

A more detailed discussion of genetic/epigenetic involvement in the programming of disease lies beyond the scope of this thesis, but has been well reviewed (351,352). **The focus of the work presented in this thesis is the molecular mechanisms associated with ageing and senescence, and the potential for their involvement in programming of metabolic disease.**

1.4.2 Telomeres

Cellular aging or "senescence" is a stress response that results in a cell's permanent withdrawal from the cell cycle, and changes cellular morphology and function resulting in impaired homeostasis (353). It is important to note that the occurrence of cell cycle arrest alone is not senescence. Senescence occurs when during cell cycle arrest, cells are continually stimulated to grow and increase their mass (354). This clash between blocked cell cycle progression and pro-growth stimulation leads cells to incorrectly enter S-phase and thus lose their proliferative potential (354).

This definition of cellular senescence was initially adopted as a result of early studies by Hayflick, which outlined that the number of divisions that normal non-transformed cells undergo *in vitro* is finite (355). This limitation on the number of cell divisions is called the Hayflick limit. Senescence can be initiated by a variety of stimuli. One such stimulus is DNA damage or loss at the ends of

the chromosomes from specialised structures called telomeres. It is suggested that a potential mechanism involving telomeres may link adverse early life exposures to the development of age-related disease.

Telomeres were originally defined as regions of DNA located on the ends of linear chromosomes which are required for replication and stability of those chromosomes (356). This functional definition of telomeres was originally put forward by Blackburn and Szostak in 1984 (356). However, as the molecular structure of telomeres has been further elucidated, this definition has since been revised. Telomeres are presently defined as specialised nucleoprotein complexes located at the ends of linear eukaryotic chromosomes that maintain their stability and integrity (357). In addition to revisions to their definition, the term "cap" is now applied to describe a protective function of telomeres. Telomeres provide a protective "cap" for chromosomal DNA by preventing the ends from fusing together (recombination), and in so doing; help to maintain genome stability (358). This ability to cap chromosomes is facilitated by telomere-associated proteins (359); discussed below.

1.4.2.1 Telomere Structure

Telomeres are made up of tandemly repeated nucleotide sequences. The telomeric repeats have been identified as (TTAGGG)n in vertebrates (360). Telomeric DNA is comprised of G-rich chromosomal DNA double-strands oriented 5′ to 3′ towards chromosome ends so that each G-rich strand possesses

an overhang at the 3′ end (361) (Figure 1.5). The G-strand overhang is present because of what is known as the "end replication problem"(362). The "problem" arises due the lagging strand that is created during semi-conservative DNA replication(363). Short RNA primers are synthesised by the enzyme RNA primase, and DNA polymerase extends them further to create Okazaki fragments. After the original RNA primers are removed, it becomes a challenge to synthesise the lagging strand sequence that complements the chromosome ends and this creates an overhang. Once DNA replication is complete, telomeres are synthesised by either the leading strand or the lagging strand, and overhangs are generated on either end of the DNA, depending on which strand was used (364).

Structurally the telomere is a duplex loop, which is created by the telomere looping back on itself (365). This loop invades an interior segment of the duplex telomere DNA to form a structure called a telomere or t-loop (365) (Figure 1.5). The presence of the t-loop is believed to protect the DNA from damage, and in particular from double stranded breaks (DSBs) in the structure, thus inhibiting aging (366,367). DSBs are repaired using two main pathways: homologous recombination, which requires a DNA template to accurately repair the break (368), and non-homologous end joining (NHEJ), which repairs the broken strands, and disregards homology altogether, thereby causing deletions and insertions which can lead to tumour cell formation (369). The presence of the t-loop structure is believed to stop recombination via the latter pathway (366).

A portion of the telomere architecture includes specialised telomere-repeat binding proteins which also help to protect the chromosomal ends (370) (Figure 1.5). This collection of proteins was named shelterin by de Lange (359). Shelterin is composed of six subunits (Figure 1.5) and includes telomeric repeat binding factor 1 and 2 (TRF1, TRF2), the transcriptional repressor/activator protein Rap1, TRF1-interacting protein 2 (TIN2), protection of telomeres 1 (POT1 or Pot1 in rodents) and TIN2-organising protein TPP1 (359). The presence of shelterin, and specifically the activity of TRF2 ,is crucial for the formation of the t-loop (371,372). TIN2 has a significant role in telomere stabilisation, acting as a linchpin molecule for the maintenance of shelterin complex molecules TRF1, TRF2, and TPP1 (373,374).

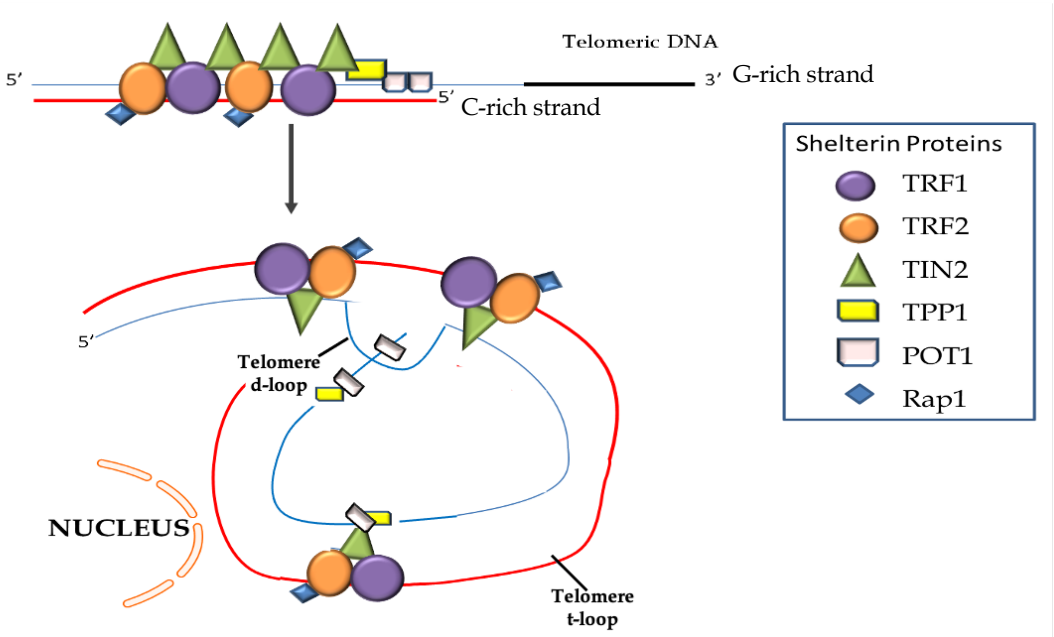

Figure 1.5. Telomere structure and associated proteins.
Telomere DNA is doubled stranded chromosomal DNA comprised of a G-rich strand oriented 5′ to 3′ and a C-rich strand which is the lagging strand. The duplex telomere loop is generated by the telomere looping back on itself and invading an interior segment to form a structure called a telomere or t-loop. Telomere repeats associate with shelterin, which consists of telomere proteins telomeric repeat-binding factor 1 (TRF1) and TRF2, TRF2-interacting factor repressor/activator protein 1 (RAP1), TRF1-interacting nuclear protein 2 (TIN2); protection of telomeres 1 (POT1), and POT1- and TIN2-interacting protein (TPP1). (Adapted from O'Sullivan and Karlsleder (375)).

1.4.2.2 Telomere shortening and aging

While several hypotheses have been proposed to explain the process of aging, including hypomethylation of DNA (376,377), the telomere length hypothesis has emerged as a significant frontrunner. During DNA replication in S-phase, the progressive loss of telomeric repeats results in telomere "shortening" both *in vitro* (378) and *in vivo* (379). When telomeres are severely shortened, the DNA damage response (DDR) is activated, which results in cell cycle arrest and likely forces the cells into senescence, a major factor in disease pathogenesis (380,381) (Figure

1.6). Both telomere-associated DDR foci (TAFs) and telomere-induced DNA damage foci (TIFs), which act as senescence markers, can form when the DDR is activated at telomeres (381,382). While senescence can lead to programmed cell death by apoptosis in situations such as embryonic development (383), the former does not always precede the latter. During particular processes or stress responses, senescence and apoptosis pathways are both active, and which one actually occurs first depends on the type of cell and level of stress (384). The significant impact of short telomeres on cell replication and viability led to the hypothesis that the presence of critically short telomeres is the main event that triggers cell senescence (378). However, regardless of telomere length, senescence can be characterised by an increase in the number of TIFs and the persistence of TAFs (385,386). Telomere length (TL) has emerged as a widely used biomarker of aging (387). . Measurement of TIFs and TAFs, which is more specific than telomere length, may provide additional information that tracks the severity of the ageing phenotype (385,386).

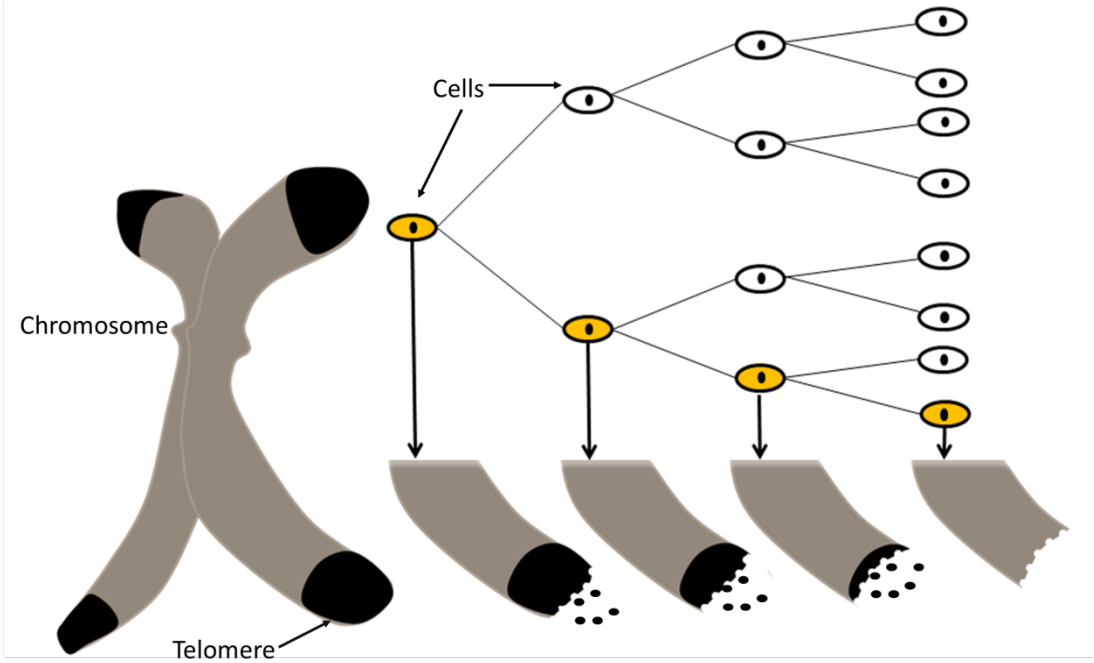

Figure 1.6. Telomere shortening.
Telomeres shorten with every cell division. As telomere repeats are lost, the cells can go into replicative senescence.

1.4.2.3 Telomere length variability

Telomere length (TL) varies between mammals, but is also heterogeneous between individual tissues (388-390). Human TL is between 10-14 kilobases (kb) (391), whereas in laboratory mice (Mus musculus) such as C57Bl/6J strains, TL is hyper-variable and lies between 30 and at least 150 kb (392). However, in other wild-derived strains of mice such as Mus caroli and Mus spretus, TL ranges from 20-30 kb and 5-20 kb respectively (393).

TL decreases with increasing age of organisms. Age-related decreases in TL have been observed in different human tissues, including the liver (394) and

lymphocytes (395). The rate of human telomere shortening is estimated at approximately 75 base pairs per population doubling of cells (379). Similarly, aging mice have also displayed telomere shortening in the brain and spleen (389). Despite having longer telomeres, the rate at which mouse telomeres shorten is 100 times faster than in humans (396). Due to significantly longer TL the shortening of telomeres in mice does not serve the same role showing the replicative age of the cells (397), and therefore can present challenges for assessing longevity.

1.4.2.4 Telomeres and senescence markers

Senescence is regulated via two separate but interconnected pathways that are sensitive to pro-senescence signalling. One pathway is the p53 senescence pathway and the second involves another major tumour suppressor called pRB (Figure 1.7) (398). It has been demonstrated that telomere shortening may signal senescence in the cell by up-regulating p53 activity (399). This is due to the ability of p53 to sense the loss of telomeric DNA on chromosome ends (400). This sensing takes place indirectly as telomere loss triggers several DNA damage response factors, including γH2AX and 53BP1 (382), which in turn contribute to p53 activation. The p53 interacts with a specialised family of protein kinases called cyclin-dependent kinases (CDKs) which regulate cell cycle progression (400). The loss of proliferative potential experienced by senescent cells is associated with elevated expression of cyclin-dependent kinase inhibitors (CDKIs) as well as p53 and pRB (398).

Two classes of CDKIs have been identified in senescent mammalian cells which are products of two loci, INK4 and the CDK inhibitory protein/kinase inhibitor proteins (CIP/KIP) (401,402). From INK4, the CDKIs commonly expressed during cellular senescence are p16 (also called p16INK4a, or cyclin-dependent kinase inhibitor 2a (CDKN2a)) and ARF (403). The CDKI expressed from CIP/KIP is p21 (also called P21CIP1 or CDKN1a) (404).

The p53-p21 pathway functions by inhibiting ARF and Mdm2 which in turn stabilises p53 allowing the CDKI p21 to be induced, leading to growth arrest and senescence (Figure 1.7) (398). In the latter pRB-p16 pathway, pro-senescence signals induce p16 expression directly, suppressing CDK activity. This suppression of CDKs blocks the inactivation of pRB by phosphorylation, allowing pRB to then induce senescence (Figure 1.7) (398). There is also an interaction between both pathways in that p53-induced p21 can suppress CDK activity (Figure 1.7). In so doing pRB is then activated and the senescence pathway proceeds. Thus, the elevated expression of both CDKIs and the tumour suppressors makes them prominent markers of cellular and tissue senescence.

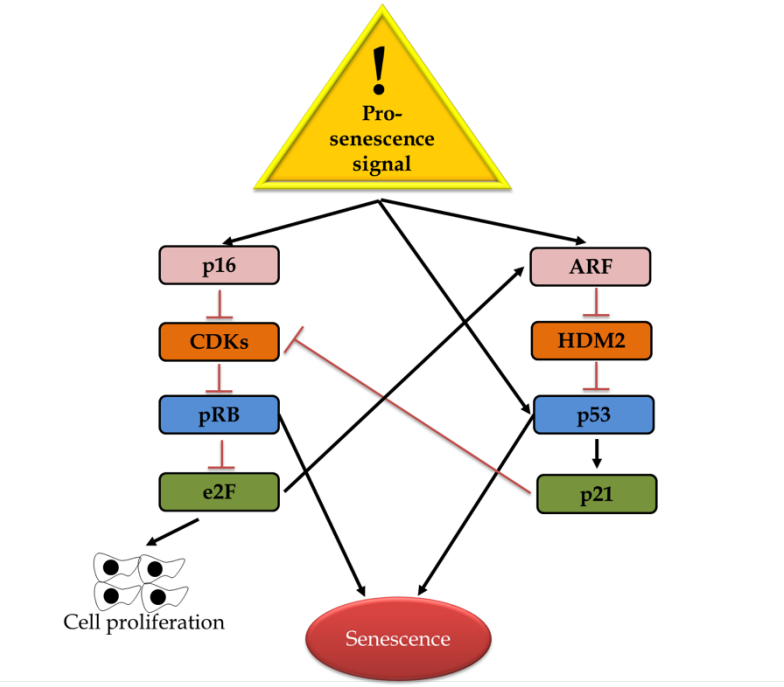

Figure 1.7 Markers of senescence.
Stress signals trigger pro-senescent mechanisms by engaging p53 or the p16 tumour suppressor pathways. Once activated, p53 then upregulates the CDKI p21 which stops the inactivation of pRB, bringing about cellular senescence. Alternatively if the CDKI p16 is activated, proliferation is halted by inhibiting E2F activity. E2F can also limit cell proliferation by suppressing ARF. Adapted from Campisi (398).

1.4.2.5 Telomere maintenance

1.4.2.5.1 The telomerase enzyme

Telomeres are maintained by the enzyme telomerase, a specialised DNA polymerase that synthesises telomeric repeats onto the ends of chromosomes (405). Telomerase is a ribonucleoprotein enzyme complex that is comprised of a reverse transcriptase catalytic subunit (TERT) and an 11-nucleotide spanning region of long noncoding RNA called TR or TERC which acts as a template for the synthesis of telomere repeats (406-408). A third subunit is also present called telomerase associated protein 1 (TP1 or TEP1) (409). However, TEP1 does not play a role in telomerase activity or maintenance of telomeres (410), and

experiments *in vitro* have shown that TERT and TR alone are sufficient for enzyme catalytic activity be initialised (411,412).

In humans the subunits are referred to as hTERT and hTR, whereas in mice the subunits are *mTERT* and *mTR* (413,414). TERT protein and nucleotide sequences, as well as TR RNA sequences, are relatively conserved between humans and mice, in addition to function (413). Despite similarities in structure and function human telomerase has a higher rate of activity and processivity than the mouse (397). Although telomerase activity and increased TERT expression are shown to be correlated in studies (415,416), the two are not always synonymous (417). TR expression may remain unchanged despite changes in telomerase activity (418,419). TERT can be a rate-limiting factor for telomerase activity in a variety of tissues(48) and cancers (420) where it is widely expressed. However, in tissues such as the brain, TERT expression postpartum is not the same as telomerase activity (414,421,422).. After birth, brain telomerase activity decreases while TERT is retained and performs non-canonical functions unrelated to telomere maintenance (414,421,422). This will be discussed below.

Evidence has shown that telomerase works by preferentially elongating the shortest telomeres (423,424). More than the concentration of telomerase, the shortest telomeres are the chief determinants of cell and tissue viability (396,425). This has been demonstrated in mice where the shortest telomeres are the first to be elongated in the offspring from a cross of long and short telomere mice (423).

Furthermore, the recruitment of telomerase to the telomeres is a slightly different arrangement in humans and mice. Elongation of telomeres in humans is initiated when telomerase subunits hTERT and hTR are trafficked to foci within the nuclei called Cajal bodies, where telomerase is assembled and then activated before being dispatched to the telomeres (426). Mouse telomerase subunits are trafficked to other foci in the nucleus and the process does not involve the Cajal bodies (427). However, once the cell enters S-phase and DNA replication is ongoing, telomerase localises to the telomeres to synthesise new repeat sequences (426,427). Telomerase is recruited for telomere extension by the shelterin protein TIN2, which has been identified as a negative TL regulator through its interaction with TPP1 (428,429).

1.4.2.5.2 Detection of telomerase in tissues

Telomerase activity has been largely associated with immortalised cells and various cancers (430). However, under normal physiological conditions, human telomerase activity is detectable in proliferative cell types such as stem cells, lymphocytes, germline cells, but not in most somatic tissues (421,431). Mouse telomerase activity, on the other hand, can be identified in several somatic tissues including the liver, testes, spleen, thymus and lymph nodes (389,432).

During gestation, telomerase activity has been identified in both human and mouse embryonic tissues (421,432,433). The enzyme's activity is progressively

down-regulated soon after birth (414,421). An example of a tissue where this occurs is the brain in the mouse where telomerase activity can be detected in the developing mouse cerebral cortex at E15, but activity gradually decreases by postnatal day 16 (432) and is maintained at low levels in the brain up to late fetal development (433). In humans fetal expression of telomerase in the adrenal glands, lungs, skin and liver is lost by 2 months of age in neonatal life (421).

Despite the absence of telomerase activity in postnatal life, the expression of the subunit TERT is continually found in several tissues. Telomerase activity cannot be inferred directly from TERT because RNA template TR and reverse transcriptase TERT must be assembled in the holoenzyme structure before telomerase activity can be initiated for telomere elongation (417,430). However, it has been noted that in mice *mTert* is continually expressed in tissues that are both telomerase positive (testis and liver) and negative (brain) in adulthood (433). Conversely in humans the expression of hTERT without full activity of the telomerase enzyme is shown in cancer cells (434). TERT also serves a number of functions unrelated to telomere maintenance, so telomerase activity cannot be inferred directly from TERT.

1.4.2.5.3 Alternate roles for TERT

Several lines of evidence have indicated alternative roles for TERT that are independent of its role in telomere maintenance. Alternative roles for telomerase

are attributed to activity of only the catalytic TERT subunit in both mice and humans. One of the more significant non-telomeric roles attributed to telomerase is its role in promoting cell survival. In mice, elevated *mTert* expression has been shown to counteract neuron ischemia in the brain (435). In humans, increased hTERT expression, without full activity of the telomerase enzyme, has been shown to counteract p53-induced apoptosis in cancer cells, inhibit caspase activation via the mitochondrial apoptosis pathway, and to increase resistance to apoptosis following exposure to genotoxicity (436-438).

Another identified role of telomerase is in the management of oxidative stress. Telomere DNA is particularly vulnerable to damage from oxidative stress. This hypersensitivity to oxidative damage has recently been named TelOxidation (439). Although telomere DDR can expedite telomere shortening in response to oxidative stress, shortening is not always the result (440).

1.4.2.5.4 Alternative lengthening of Telomeres

An alternative method of telomere elongation, which is independent of telomerase, has been observed principally in tumour cells, cell lines, oocytes and pre-implantation embryos (441-443). This alternative lengthening of telomeres (ALT) is also characterised by extreme heterogeneity of TL (444), high rates of telomere exchange (445), and the presence of ALT-associated promyelocytic leukaemia bodies (446). The replication of telomere DNA by ALT is mediated through a homologous recombination mechanism (447). Only 15% of tumours

have been shown to use this method for telomere maintenance, whilst the remaining 85% use still telomerase (444). More recently, it has been suggested that ALT plays a significant post-fertilisation role in embryos prior to implantation by promoting telomere lengthening until just before the blastocyst stage, at which point telomerase would continue to maintain telomeres (442). The mechanistic basis for using ALT over telomerase for the maintenance of telomeres in these instances is still yet to be determined.

1.4.3 Oxidative Stress

It is well documented that early life stressors such as impaired nutrition, exposure to GCs and catch-up growth following LBW can program metabolic disease (448). The exact mechanism by which this occurs is unknown. One hypothesis is that oxidative stress is involved in the mechanism of cellular events that links poor early life exposures and increases risks of the metabolic and degenerative disorders (449). Hence, understanding the role of oxidative stress in health may provide an alternative therapeutic target for treatment or overall prevention of these disorders.

Oxidative stress occurs when there is an overproduction of reactive oxygen species (ROS), and an inability of the cell to effectively detoxify them, resulting in damage to tissue, thereby altering metabolic homeostasis (450). ROS are generated primarily by mitochondria, specialised organelles, which play a significant role in metabolism, de novo compound biosynthesis and energy production in the cell. ROS are produced as metabolic by-products of oxidative

phosphorylation (OXPHOS), or as a result of interactions with xenobiotic compounds (451,452). Examples of ROS include superoxide (O_2^-), hydroxyl (OH^-) and peroxynitrite ($ONOO^{\bullet-}$) (453).

During periods of cellular stress, ROS levels increase dramatically and potentially cause alterations in cellular structure and function. To counter increasing concentrations of ROS, cells possess a defence system comprised of antioxidant enzymes (454). The primary antioxidant enzymes that are essential in aerobic cells include superoxide dismutase (SOD) catalase and glutathione peroxidase (455). SOD catalyses the conversion of O_2^- anions to hydrogen peroxide which is further converted to water and oxygen by catalase and glutathione peroxidase, (455).

The effects of ROS can be either physiological or pathophysiological in cells. Under normal conditions relatively low concentrations of ROS are produced which play a significant role in cellular signalling, acting as second messengers in signal transduction pathways (456). However, in excess, ROS are damaging to macromolecules in the cell including DNA, proteins, and lipids (450). The continued oxidative damage to cellular macromolecules is believed to be a significant factor in tissue functional decline associated with aging (457) and aging-related disorders such as diabetes, obesity and Parkinson's disease (458-460).

The accumulation of ROS-induced damage and the role of such damage in the development of aging and aging related disorders forms the basis of another hypothesis called the free radical theory of aging originally proposed by Harman (461). Due to their role in ROS production during aerobic respiration, the hypothesis was then expanded to include mitochondria, which were found to be especially vulnerable to ROS (462). Excess ROS has an adverse impact on mitochondria directly causing them to release high levels of ROS into the cytoplasm, and in a knock-on effect, trigger the release of ROS from other mitochondria, which further damage cellular components (463). Thus, mitochondria play a significant role in ROS-mediated aging mechanisms.

1.4.3.1 Mitochondria, ROS and aging

A number of metabolic conditions such as metabolic syndrome and obesity have been shown to accelerate aging and have been associated with telomere shortening (464). Further, as these conditions can be programmed during fetal development (13,26), it has been postulated that exposure to adverse conditions *in utero* may also induce premature aging in humans (465). In addition to the increased prevalence of cellular ROS, the aging process is also characterised by a progressive decline in tissue function (466).

While TL has been highlighted extensively as a marker of aging (467,468), an alternative hypothesis has been proposed, which suggests a substantial role for mitochondria. The mitochondrial aging hypothesis posits that dysfunctional mitochondrial respiratory complexes accumulate with age, causing a decline in

ATP synthesis, and therefore deterioration in the bioenergetic capability of cells (469,470). This decline in bioenergetic capacity may be accompanied by increasing oxidative stress and oxidative damage (471). Elevated ROS are especially damaging to mitochondrial DNA (mtDNA), which encodes subunits of OXPHOS complexes, which are required for respiration and ATP synthesis (472). Damage to mtDNA induces alterations such as single and double strand breaks, point mutations, and large deletions of mtDNA which can result in the loss of more than half of the mitochondrial genome (473,474). Importantly, the incidence of mtDNA deletions correlates with lower respiratory activity, and has been observed in rat and primate livers (475,476) as well as human skeletal and cardiac muscle (477,478). Furthermore, mtDNA mutations are associated with the generation of various degenerative phenotypes including myopathies, neuropathies, diabetes, in addition to premature aging and compromised lifespan (479,480).

1.4.3.2 Mitochondrial biogenesis

It is believed that a greater number of mitochondria or increased mitochondrial mass confers greater protection to the cell against metabolic failure and cell death (481). Thus, mitochondrial biogenesis, the growth and division of pre-existing mitochondria, is critical for cellular defence (482). The process of mitochondrial biogenesis is stimulated by environmental factors such as physical activity, caloric intake, cell division and oxidative stress (482). A significant factor involved in mtDNA replication and maintenance is mitochondrial transcription factor A (TFAM or Mtfa), a high-mobility group protein characterised initially

for its role in mitochondrial transcription (483). Tfam is actively involved in maintaining the mitochondrial genome by transcribing and replicating mtDNA (482) and is associated with sensing and repairing damaged mtDNA by ROS (484).

Tfam is a significant component in a mitochondrial biogenesis signalling cascade, and lies downstream of main regulator peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α), and two nuclear transcription factors: nuclear respiratory factor 1 (NRF1) and 2 (NRF2) (482). In response to environmental stimuli, PGC-1α activates the nuclear transcription factors NRF1 or NRF2, causing Tfam to be transcribed and then translocate to the mitochondria where mtDNA replication occurs (Figure 1.8) (482). Once replication and transcription are complete the mitochondria may then divide synchronously with cell division (485). Following the formation of new daughter cells, mitochondria can then fuse with other dysfunctional mitochondria in order to reduce toxic stress effects (486). This mitochondrial biogenesis process is important for the maintenance of functional mitochondria, but also ensures that mitochondria are passed on to new cells once mitosis has occurred.

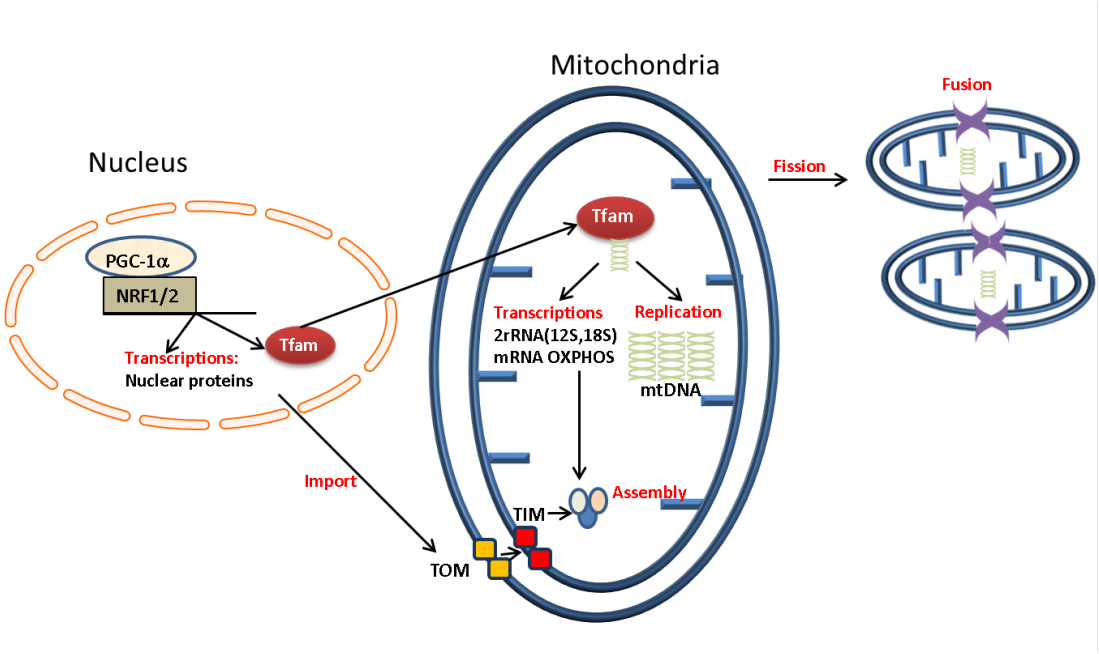

Figure 1.8. Summary of mitochondrial biogenesis.
PGC-1α activates nuclear transcription factors Nuclear respiratory factor 1 and 2 (NRF1/NRF2) leading to transcription of nuclear- encoded proteins and mitochondrial transcription factor Tfam. Tfam activates transcription and replication of the mitochondrial genome. Nuclear-encoded proteins are imported into mitochondria through the outer- (TOM) or inner (TIM) membrane transport machinery. Nuclear- and mitochondria-encoded subunits of the respiratory chain are then assembled, before the mitochondrion undergoes fission and then fusion with other mitochondria. Adapted from Ventura-Clapier *et al.* (482).

Impaired mitochondrial biogenesis has been implicated in degenerative diseases such as Alzheimer's where hippocampal PGC-1α, NRF1, NRF2 and TFAM are all downregulated in conjunction with reduced mtDNA copy number (487). Conversely, increased TFAM expression has been associated with a reduction in mtDNA content in aging (488). It has been suggested that the inverse relationship between TFAM and mtDNA may be attributed to enhanced TFAM expression or to a slower degradation of TFAM (489).

1.4.3.3 Diet and mitochondrial biogenesis

Diet appears to play a significant role in the regulation of mitochondrial biogenesis. Recent data has shown that extended periods of high fat feeding in rodents results in elevated PGC-1α and TFAM, increases mtDNA in skeletal muscle (490), inducing rapid changes in mitochondrial morphology (491). While it would be expected that prolonged HF diet feeding might create an environment that impairs metabolism by suppressing the PGC-1α-Tfam pathway, it has been proposed that the observed increase in PGC-1α expression might be an adaptation to enhance tissue oxidative capacity (490). The increase in mtDNA, and thus mitochondrial content, may be an adaptation of the tissue to increasing amounts of free fatty acids (492). Increased mitochondrial biogenesis may be a possible mechanism to prevent impairment of mitochondrial function, as a result of either increasing oxidative stress or the accumulation of toxic lipids (lipotoxicity) (493).

Interestingly, PGC-1α and TFAM can also be upregulated by caloric restriction (489). Caloric restriction without malnutrition prevents the onset of aging by increasing the expression of genes involved in mitochondrial metabolism, improving insulin sensitivity and increasing levels of circulating adiponectin, a key hormone that regulates glucose concentrations and fatty acid oxidation (494-496). This complementary increase in PGC-1α expression is also accompanied by increases in nutrient-sensitive regulators such as mammalian target of rapamycin (mTOR) (497). These effects reveal that diet has the ability to metabolically program mitochondria and thus alter their metabolic state. The impact of diet on

mitochondria during early prenatal development has not been fully investigated. Because of the sensitivity of mitochondria to the external environment and their contribution to aging and aging-related disease, new efforts aim to expand the developmental origins hypothesis to include a role for mitochondria in the development of metabolic disease (498).

1.4.4 Telomere and Mitochondrial involvement in DOHaD

As described previously, there is an established association between exposure to suboptimal prenatal conditions and adverse long-term health outcomes. More recently, evidence has come to light, highlighting a role for telomeres in mechanisms linking stress *in utero* and disease risk (499). However, evidence of telomere involvement is still limited. Epidemiological studies have shown links between shorter telomeres and higher risk of disorders such as hypertension, cardiovascular disease, T2D, obesity, as well as mortality (500-503). Shorter telomeres in adults and children have been associated with risk factors for metabolic disease (501,504,505). Upon examination of animal models, mice born with drastically short telomeres have later life impaired insulin secretion and glucose intolerance (506).

Studies have also begun to link TL with activity of the HPA axis. Chronic life stress as well as prenatal exposure to stress is linked to telomere shortening and reduced telomerase activity, and an inverse relationship between cortisol/corticosterone and TL and telomerase activity has been demonstrated (507-509). The role of cortisol in telomere shortening is further supported by *in*

vitro studies in which T lymphocytes treated with synthetic cortisol displayed significantly reduced telomerase activity (510). Further, in mice, reduced telomerase expression in the hippocampus has been linked to depression-related behaviour (511). These data highlight the potential detrimental effects of high levels of cortisol on TL, adding to a growing body of literature on the relationship between excessive stress and disease (448).

The relationship between TL and nutrition is still not fully realised. Despite the identification of specific diets such as vegetarian, Mediterranean, calorie-reduced, omega-3, vitamin or polyphenol-high systems as being telomere-preserving (512-517), studies also point to diet not having a clear effect (518). While the links continue to be further strengthened, there is indeed still a dearth of information with respect to direct effects of early life nutrition. Studies in rats have demonstrated that maternal undernutrition, in particular protein restriction, is associated with telomere shortening in tissues of the aorta and in pancreatic islets in later life (519,520). Protein restriction also shortens kidney telomeres in rats following postnatal catch-up growth (521,522).

However, data that reflects the effects of global dietary restriction and maternal HF diet exposure on telomeres during gestation is still scant. Many of the abovementioned studies have focused on postnatal outcomes of adverse early life exposures. Thus, no studies have examined the mechanistic effects of these two dietary interventions on telomeres and telomerase dynamics during fetal

life. Furthermore, there are no studies that have assessed the relationship between early life adversity, growth trajectories, and indices of metabolic disease and TL in humans, particularly in adolescents, before onset of disease.

Mechanisms have been revealed involving mitochondria crosstalk, by which dysfunctional telomeres may contribute to impaired metabolism (523,524). This has been complemented by data demonstrating telomere shortening, significantly lower mtDNA and elevated reactive oxygen species in T2D patients (525-527). Previous studies have confirmed a telomere-independent effect in which TIN2 is found in mitochondria and regulates mitochondrial function, ATP synthesis, and oxygen consumption. (528). These findings highlight a common mechanism and possible interaction between telomeres and mitochondria in the pathogenesis of disease. Given that mitochondria play a significant role in meeting the energy demands of the cell in energy metabolism (529), it remains unsurprising that obesity-related pathologies may be associated with dysfunctional mitochondria; mechanisms associated with telomere shortening and accelerated aging.

The potential role of mitochondria in the programming of disease has been studied using animal models. Low protein, global food restriction, uterine artery ligation, and HF diets have all been shown to program impaired insulin secretion and mitochondrial dysfunction in islet cells of adult rats (132,530-532). Undernutrition increases the number of mtDNA mutations and lowers

mitochondrial activity in β–cells of the pancreas in rats, which is associated with diabetes pathogenesis (532). In humans, lower mtDNA content has been observed in both growth-restricted and macrosomic neonates (533).

As with telomeres, a link has also been made between mitochondrial dysfunction and HPA axis function. However, information on this is still sparse. In rats, prenatal exposure to the glucocorticoid DEX during gestation is associated with suppression of fetal mitochondrial function and increased susceptibility to oxidative stress in neurons (534). Furthermore, specific mitochondrial genome polymorphisms have been identified which are associated with insulin resistance and diabetes (535). In humans, epigenetic modifications have been shown to affect mitochondrial function. Methylation of the gene mitochondrial NADH dehydrogenase 6 (MT-ND6) correlates with the degree of fatty acid deposition (steatosis) in the livers of patients with non-alcoholic fatty liver disease (NAFLD) (536).

However, as with telomeres, while these studies in mitochondria have demonstrated links between mitochondrial dysfunction and disease in later postnatal life, no studies have clarified the effects of undernutrition and overnutrition on mitochondria during fetal life. Further still, **no studies have looked at the effects of exposure of such drastic dietary interventions on the functional interaction between telomeres and mitochondria in early life, which is a significant focus of this thesis.**

1.4 Summary of the exposures and possible programming mechanisms during critical developmental windows

The above-discussed studies have indicated the importance of early life exposures to developmental outcomes. In addition to the type and severity of early life exposure, one other factor involved in programming of metabolic outcomes is the timing of exposure. As explained above, the adverse exposure may occur in a vulnerable or critical window of development, and affect specific tissues, increasing susceptibility to disease in later life. Presented below is a diagrammatic summary of various critical windows of development and the various exposures and proposed mechanisms associated with disease outcomes (Figure 1.9).

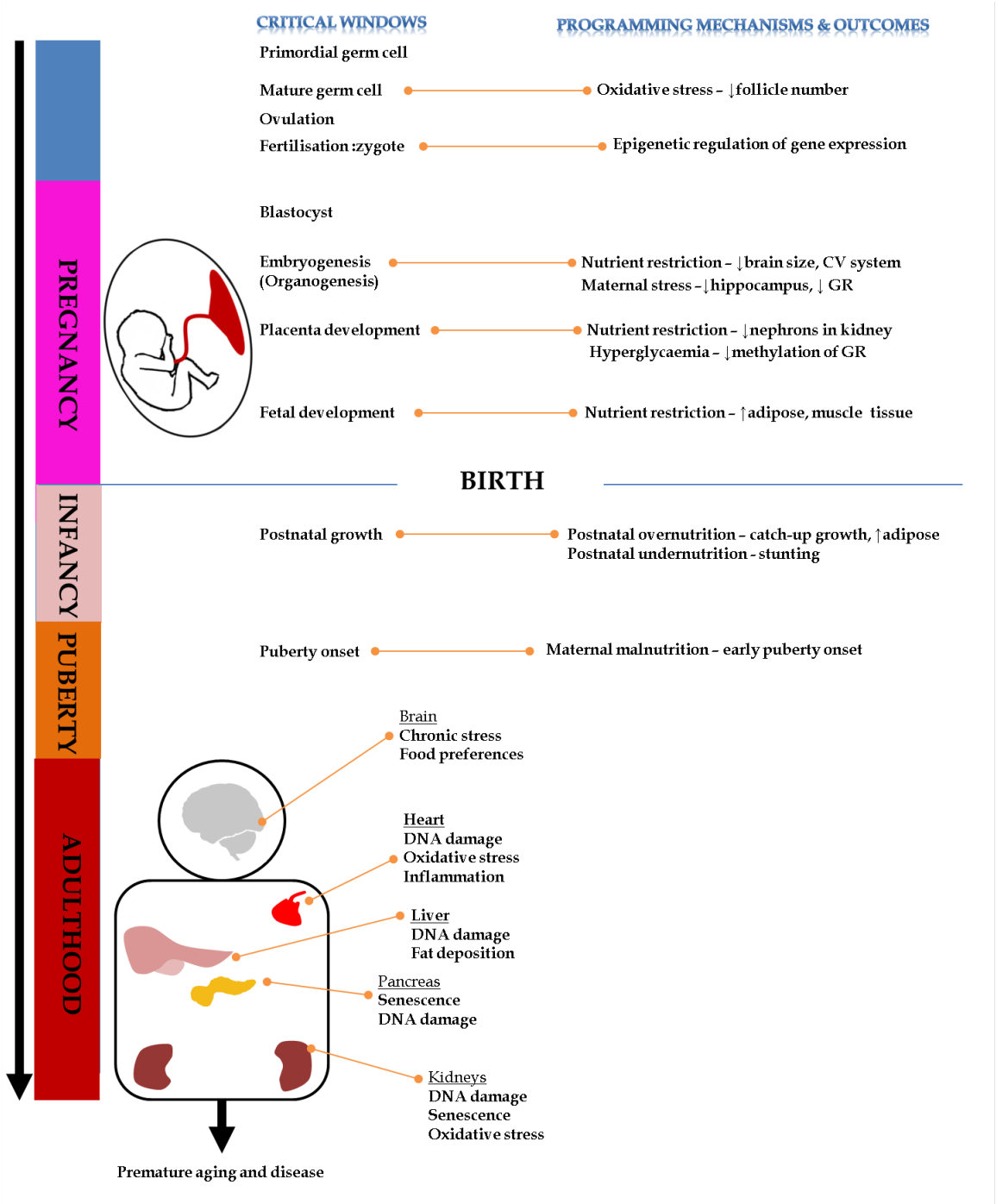

Figure 1.9 Summary of the critical windows of development and initiating mechanisms that underlie disease programming
(adapted from Hallows *et al.* (465) and McMillen & Robinson (12)).

CHAPTER 2

EXPERIMENTAL AIMS AND OBJECTIVES

The aim of the studies in this thesis was to compare the effects of intrauterine undernutrition and nutritional excess on fetal telomeres, and to determine whether child telomere length may indicate future disease risk in advance of a full metabolic syndrome phenotype. In light of the various findings in the literature, it has become clear that the genome plays a significant role in the programming of disease. TL has been investigated in several studies in the context of psychological stress, metabolic disease and mortality. However, the relationship between TL and telomerase activity in DOHaD requires further clarification.

Links between shorter telomeres, obesity and metabolic syndrome-related variables have been shown in humans (537). Conversely, the relationship between TL, metabolic disease risk, and growth trajectory in humans has not been fully investigated. The work in this thesis presents a unique opportunity to concurrently investigate the mechanisms of DOHaD and their impact on telomeres in both animals and humans.

While undernutrition has been associated with shorter telomeres, and has formed the basis of the DOHaD hypothesis, there is still comparatively very little known about the effects of high-fat feeding during gestation. Moreover, there is

still a paucity of information concerning the effects of HF diets on TLs in fetal life and this also warrants investigation.

Therefore, the overall aim of this thesis was to test two hypotheses:

1. **An adverse environment in early life (antenatal, postnatal or a combination of both) compromises telomere length before the onset of metabolic disease.** *Using our previously established murine model of undernutrition (211) and a hypercaloric high-fat diet, this thesis examined the impact of both extreme dietary interventions on TL and telomerase dynamics in mice with a specific focus on late fetal life. This thesis also evaluated the association of human TL in late adolescence with intrauterine and postnatal exposures.*

2. **An adverse environment in early life compromises metabolism in offspring via a telomere-associated mechanism.** *Using the same murine models above, this thesis examined the effects of diet on mitochondrial function; since recent work has suggested a mechanistic link between telomere shortening and mitochondrial dysfunction which compromises metabolism (523). Also, this thesis also investigated links between TL and current metabolic status in the adolescents in a human cohort.*

Three studies were carried out, with the first two focussed on the animal model, and the latter on a prospectively recruited human cohort, namely the Raine cohort.

2.1. The Animal model

In order to more directly study the impact of early life nutrition on gene-environment interactions, our research group have used the C57Bl/6J (B6) mouse. Our lab previously established a murine model of programming by undernutrition, with two strains C57BL/6J (B6) and A/J99 (A/J) (201,211). Both strains differ physiologically as well as in their responses to changes in their environment. Distinct strain differences are present between A/J and B6 mice in response to a 30% reduction in total antenatal caloric intake. Fetal body weights were lower in the calorie-restricted groups than *ad libitum*-fed mice in both strains. The low-birth-weight pups from both strains exhibited rapid catch-up growth. However, the impact of dietary restriction was more pronounced in B6 offspring, which exhibited a more severe metabolic phenotype in adulthood. Given the susceptibility of the B6 mouse to metabolic syndrome phenotypes (538), this thesis has further investigated whether having this increased sensitivity may also affect outcomes in telomeres and mitochondria.

2.2. The West Australian Pregnancy Cohort (Raine) Study

The last decade has seen an increase in the use of human cohorts to study gene-environment interactions. In order to expand the study of DOHaD to humans the work presented in this thesis utilised a prospective cohort, the Western Australian Pregnancy Cohort (Raine) study.

The Western Australian Pregnancy Cohort (Raine) Study is one of the largest global prospective cohorts examining pregnancy, and the period of development

from childhood, adolescence through to adulthood. Whilst there are many other cohort studies, the Raine Study is one of the few where information is present on both the mother during and after pregnancy as well as child development. The Raine Study commenced in Perth, Western Australia, in May 1989 at the maternity hospital King Edward Memorial Hospital (KEMH). Via the Telethon Institute for Child Health Research, the Raine study has tracked the offspring of 2,868 live births by 2,804 women from a recruitment pool of 2900 pregnancies. In total, 96% of the initial population sample was retained since the study's inception.

The origins of the Raine study stem from a randomised controlled trial assessing whether intensive ultrasound imaging and Doppler flow studies would improve perinatal outcomes (539,540). Pregnant women were recruited at gestational ages 16 to 20 weeks in the maternity hospital King Edward Memorial Hospital (KEMH). Eligibility criteria for the study were dependent on the following: English proficiency in mothers was high enough to understand the implications of participating in the study. Mothers were also expected to give birth at KEMH and show a willingness to reside in Western Australia for the coming years to allow for follow-up studies with children (539).

Women were randomly allocated to either an intensive ultrasound or regular ultrasound group. The intensive group underwent ultrasounds and a Doppler studies at 18, 24, 28, 34 and 38 weeks, whereas the regular group attended

appointments at 18 weeks and additional scans only performed at a doctor's request (540). At 34 weeks women then provided information by questionnaire on what they were exposed to between weeks 18 and 34 (540). A detailed obstetric history of the pregnancy and labour was made at birth and also included neonatal information (326). A questionnaire was then used to record clinical information as well as dysmorphology and neurological function in the neonate. Three days postpartum, mothers completed a questionnaire on initial feeding practice and postnatal temperament. This initial study showed no evidence that intensive ultrasounds and Doppler flow studies improved outcomes for either mothers, fetuses or neonates (540). Thus, it was not necessary to increase the number of scans. However, it was noted that the number of growth-restricted fetuses was higher in the groups that had been exposed to repeated ultrasounds (540).

Both groups of women from this work and their children were retained for the duration of the Raine study in order to assess outcomes in later life. After birth progressive follow-ups occurred at the ages of 1, 2, 3, 5, 8, 10, 14, and 17, the age of interest in this thesis (Figure 2.1). Later follow-ups that have occurred have been at ages 18, 20 most recently 23, using a combination of questionnaire and clinical examination.

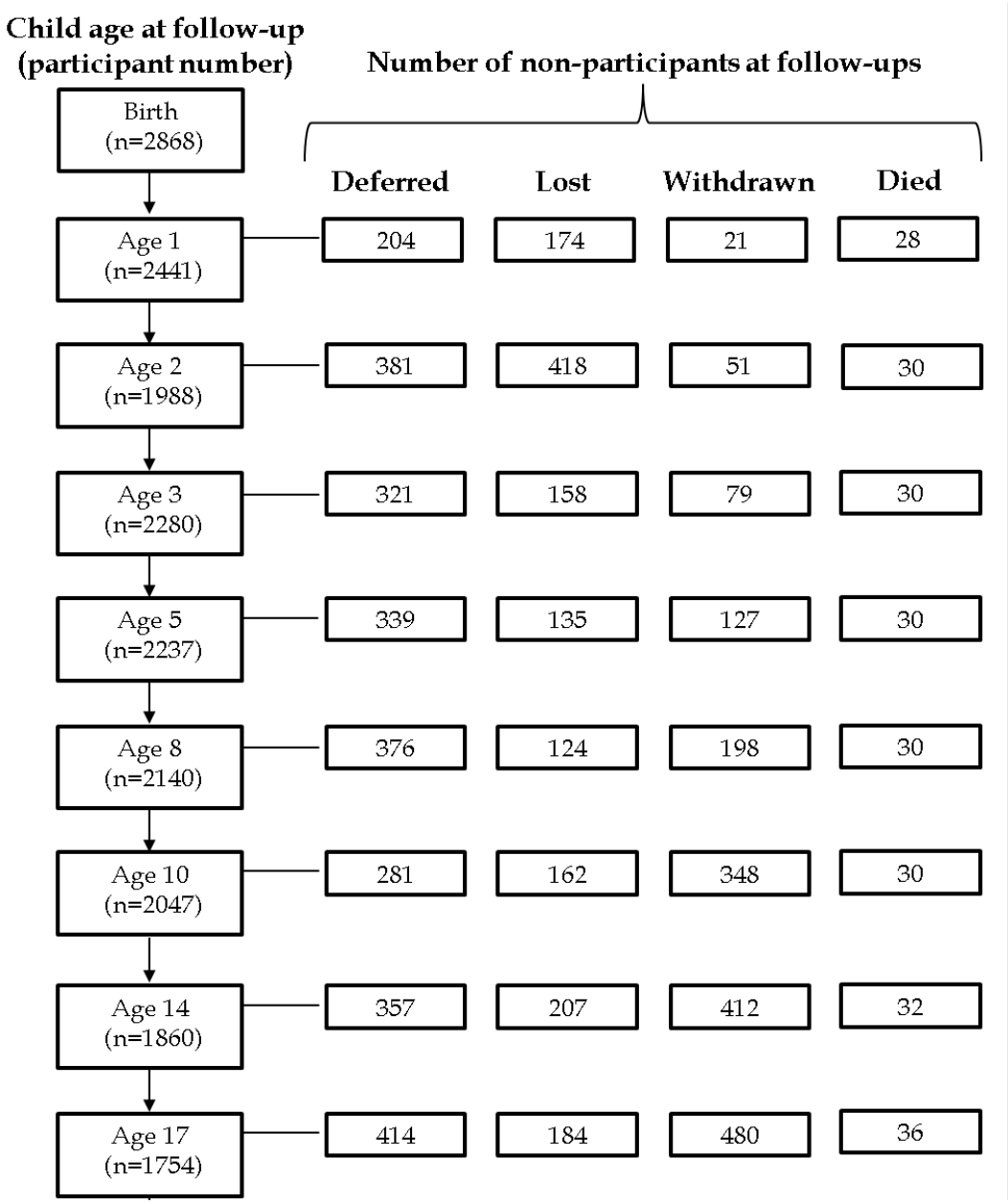

Figure 2.1. The Western Australian Pregnancy Cohort (Raine) Study participants.

Age of participants corresponds to year of follow-up, from birth up to the follow-up at 17 years old. Terms: "Deferred" refers to those who remained a part of the cohort but did not participate in the current follow-up. "Lost" refers to those whose location was unknown at each follow-up. "Withdrawn" refers to those who completely withdrew from the cohort. "Died" refers to instances when the children in the study were deceased.

CHAPTER 3

EFFECTS OF UNDERNUTRITION ON TELOMERES AND MITOCHONDRIA

3.1 INTRODUCTION

Previous studies have verified the importance of nutrition during critical periods of growth, particularly as it pertains to developmental programming of disease (39). Undernutrition during gestation leads to impaired intrauterine growth and low birth weight, and has long-term effects on offspring health, altering stress axis function, and increasing the risk of metabolic disorders (11,13,218). Despite the proven long-term metabolic outcomes of inadequate nutrition, these effects may not manifest until later in life.

Indeed, conditions in the postnatal environment may be able to modify the programming effects of prenatal stress, either favourably or by exacerbating the subsequent health outcomes that lead to these adverse effects (9,11,26,27). Although some studies have reinforced a link between undernourished pregnancy and obesity and its related pathologies, the mechanisms underlying the pathogenesis of disease are still not fully established. Numerous clinical and animal studies report on these long-term postnatal outcomes, with relatively few studies focussed on prepartum outcomes. **This study was designed to focus on the fetal outcomes, particularly telomere length (TL), following exposure to maternal undernutrition.**

Evidence suggests that telomeres may be a common thread in the mechanisms linking early life stress to disease in later life (499). Telomere shortening is associated with aging in both humans (394,395) and mice (389). As outlined in my review of the literature, human telomeres are between 10-14 kilobases (kb) in length (391), whereas in laboratory mice (*Mus musculus*) such as C57Bl/6J strains, TL is hyper-variable and lies between 30 and at least 150 kb (392). However, in other wild-derived strains of mice such as *Mus caroli* and *Mus spretus*, TL ranges from 20-30 kb and 5-20 kb, respectively (393). However, there is no definitive mouse model for DOHaD study of long-term fetal programming.

The reason for examining the impact of telomeres might be twofold: Firstly, studies show that TL is heritable (541-543). Secondly, these studies imply that there is a plasticity to telomere length that is imposed by conditions *in utero* (544). It is likely, therefore, that telomere dynamics are affected by similar parental health and intrauterine factors associated with disease outcomes. Epidemiological studies show an association between shorter telomeres and higher risk of disorders such as hypertension, cardiovascular disease, T2D, obesity, and stroke (500-503,545). While these associations have established a strong relationship between telomeres and metabolic syndrome, they are not proof of causality.

The strongest evidence linking telomere length to developmental programming of disease comes from animal models, with adult rodents displaying shorter

telomeres following intrauterine protein-restriction in addition to compromised metabolism if born with a significant telomere deficit (506,521,522).

Research has linked oxidative stress to the disease-programming effects of maternal malnutrition. (546). High levels of reactive oxygen species (ROS) have been implicated in the pathogenesis of disease (547,548). Furthermore, prenatal stress has also been shown to damage mitochondria, specifically mitochondrial DNA (mtDNA) due to excessive ROS presence in cells (549). It has previously been shown in human studies that exposure to prenatal stress shortens offspring telomeres, and this is observed in adulthood (499). Moreover, this has also been demonstrated post-weaning in animal studies. However, a significant issue that is yet to be addressed is whether, under the influence of prenatal undernutrition, a) TLs are modified in both mothers and fetuses; b) there is a global change in TL in across all tissues; and c) how the relationship between telomeres and mitochondria might be affected during fetal life.

It was previously demonstrated that exposure of C57Bl/6J mice to global nutrient restriction from E6.5 to E17.5 resulted in metabolic syndrome-like phenotypes in offspring, accompanied by greater fat mass and glucose intolerance in adulthood (201,211). In order to elucidate the mechanisms linking these detrimental long-term effects of exposure to prenatal undernutrition, and given the previous evidence from our well-characterised mouse model of undernutrition, it was hypothesised that maternal undernutrition will adversely affect telomeres and

thus reduce telomere length, suggesting a mechanism by which cellular stress leads to metabolic dysfunction.

3.2 METHODS

3.2.1 Diet

The diet used in this study was a purified control chow diet (Dustless Precision Pellets® (diet # S0173; Bio-Serv, Frenchtown, New Jersey), which was given with sterile water. These standard Dustless Precision Pellets® are a grain-based formula comprising of one-gram pellets. According to the manufacturer's specifications, the nutritional content of Dustless Precision Pellets® was 21.1% protein, 4% fat, 55% carbohydrate, 4% fibre, 6.7% ash with less than 10% moisture. The weight of a single grain pellet was 1 gram. In order to achieve the desired weight for the restriction study, pellets were trimmed in size, and a milligram balance was used to achieve the desired weight. Pellets were kept whole. If the sample fragmented, a new pellet was used to ensure only whole shaved pellets were introduced.

3.2.2 Animals and experimental treatment

All animal procedures were performed by me, with the assistance of Dr Kristin Connor, at the Lunenfeld-Tanenbaum Research Institute within the Toronto Centre for Phenogenomics (TCP) animal facility, in Toronto, Canada. All protocols concerning animal care and treatment were approved by the Lunenfeld-Tanenbaum Research Institute Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care. Further approval

to complete animal work in Canada was obtained from the Animal Ethics Committee of The University of Western Australia.

5-week-old nulliparous female C57BL/6J (B6) inbred mice were acquired from The Jackson Laboratory (Bar Harbor, Maine). The mice were kept under 12-hour light, 12-hour dark conditions in clear sterile polysulfone microisolator cages containing woodchip bedding. From date of arrival the animals were fed sterile Dustless Precision Pellets® *ad libitum* with sterile water. Female weights were recorded weekly in the pre-pregnancy period to ensure health and wellbeing. Once the females reached 10 weeks of age, they were mated with an experienced male of the same strain. Prior to mating, the estrus cycle of the females was determined through visual observation by assessing the degree of swelling, moistness, and pinkness of the vaginal opening (550). This was also done by the gentle insertion of a probe. During proestrus and into estrus, the epithelial lining of the vagina thickens. The outer layer of the epithelium becomes cornified and sloughs off into the lumen. Once removed, shed cells could be observed on the probe. This combination of assessments was used to determine the readiness of the females to mate.

Females were placed with mature males in the late afternoon (between 2.30-3 pm) and left overnight. Following overnight mating, females were examined early the next morning (by 9.30 am) for the presence of a vaginal spermatozoa plug. In the absence of a plug, if the female still appeared to be in estrus, she was left with the

male for an extra day. The morning on which the plug was present was designated gestational day (E) 0.5. Once a vaginal plug was observed, females were housed individually in fresh cages for the remainder of the experiment.

In this study *ad libitum* feeding was achieved by administering 10 dustless pellets to each cage trough, corresponding to a total of 10 g of food per dam each day. This facilitated the weighing of the food on each day of gestation. Baseline values were obtained using body weights and food intakes from ad libitum feeding to determine the weight of food to feed the pregnant DR dams so that a 30% caloric restriction was applied throughout. This entailed averaging the weights of food consumed by the females on the control diet (CON). The average daily intake of food was calculated from E0.5 to E17.5 CON females and then used to calculate total calories consumed each gestational day using the kilocalorie value of the diet. 30% of the kcal value was taken for each day and the value converted back to a weight in grams. The weights obtained from these calculations were used for all DR dams until E17.5, after which *ad libitum* feeding was applied.

Dams were weighed daily from mating until postmortem at E18.5. Mated females were randomised to two groups (Figure 3.1): 1) control females were placed on a diet of Dustless Pellets *ad libitum* throughout pregnancy (CON, n=7); 2) females calorically restricted by 30% of CON food between E5.5 to 17.5, after which dams were fed *ad libitum* (DR, n=7).

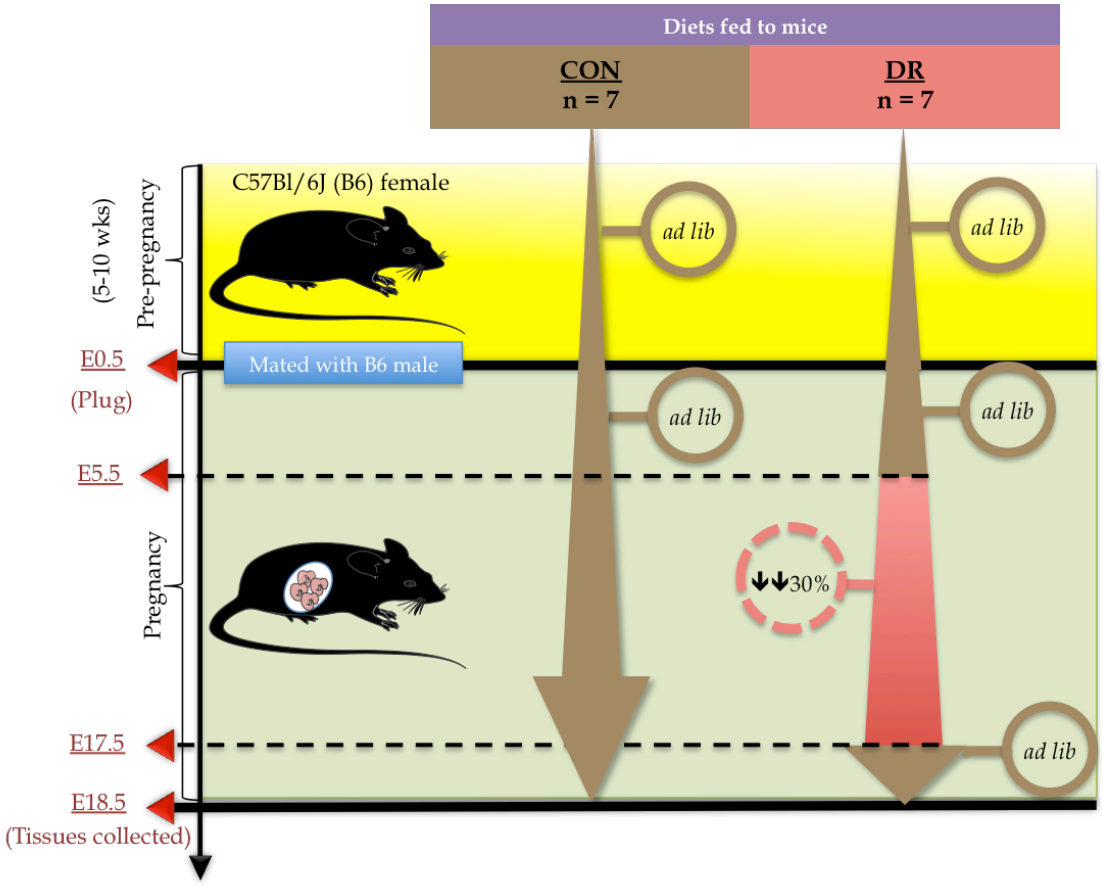

Figure 3.1. Experimental design for the diet study.
Dietary restricted females were placed on the same diet as controls. At E5.5 a 30% reduction in caloric intake was imposed until E17.5, when they were returned to normal ad libitum feeding for 24 hrs.

For the CON group the food was weighed daily from E0.5 to E18.5 every morning. The cage bedding was also checked for large pieces of food and these were incorporated into the recorded amount. Once the food was weighed, the balance of what had been consumed was then added to bring the total weight of food in the trough back to 10g. This was done for the duration of the pregnancy. Every 3 days the food was completely refreshed with 10 new pellets, and mouse cages were changed and replenished with new bedding.

3.2.3 Tissue collection

3.2.3.1 Dams

At E18.5, dams were sacrificed in a non-fasting state as fasting would have adverse implications for the fetuses. All dissections were performed between the hours of 9.00 and 11.00 am. Dam weight was recorded prior to isoflurane anaesthesia, and cervical dislocation. Dams were decapitated immediately after cervical dislocation. Trunk blood was collected in microcuvette tubes coated with lithium-heparin (Sarstedt, Nümbrecht, Germany). Uterine horns were rapidly removed, bisected longitudinally along the abdomen, and placed in a dish with saline on ice to keep moist for removal of the pups and placentae. A section of the mid portion of the maternal small intestine was collected and frozen for later analyses.

Maternal bone marrow was collected from the femurs and tibiae. Femur and tibia bones were cut at the hip joint and placed in ice cold CMF solution (Ca2+ and Mg2+-free HBSS, 10 mM HEPES-bicarbonate, and 2% volume/volume (v/v) FBS, pH 7.2) immediately following dissection. For the femur, the bone was held in place in a 10 cm dish using forceps, with the knee portion angled downwards. Scissors were placed just above the knee joint. Keeping forceps and scissors close together to prevent shattering of bone, the knee joint was cut off. For the tibia, the knee joint was rested with the knee portion facing downwards on a tissue and the knee joint was cut off. For the distal end of the tibia, the end of the portion of bone containing red marrow was identified. Scissors were used to cut the bone just below the end of the marrow region.

In order to collect bone marrow, a 23 ¾ - gauge needle was filled with CMF solution and inserted into the knee joint end of both bones. The bone marrow was the flushed into a 6-well culture plate. CMF solution was passed through until the colour of the bone changed from dark red to white, signifying the complete flushing of bone marrow. The bone was then discarded. Marrow was taken up into a syringe and then expelled 3 times to disperse it and was then divided into aliquots. The marrow was quickly centrifuged in 1.5 ml microcentrifuge tubes at 3000 rpm for 30 s at room temperature. The supernatant was discarded, and the marrow aliquoted and quickly frozen at -80 ^{o}C.

3.2.3.2 Offspring

Collection

All pups and placentae were removed from their amniotic sacs and weighed. As part of the protocol, 2 males and 2 females from each litter were selected, with a specific bias for pups in the middle away from uterine horns. Earlier studies showed that fetal weight in mice could be affected by position in the uterine horn (551,552). This uterine position effect has demonstrated that the heaviest fetuses are on the ends closest to either the ovaries or cervix as a result of exposure to a difference in blood flow and nutrient quota which can be lower in the mid horn (551,552). While such restrictions in flow and food are already present, collecting from the mid-horn allows for a more thorough study and reduces the variation that might be observed in the cervical or ovarian positions. Pups were killed by decapitation and trunk blood was collected in lithium-heparin-coated microcuvette tubes (Sarstedt, Nümbrecht, Germany). Fetal blood glucose was

measured using a One Touch Basic Glucometer (LifeScan Canada Ltd, Burnaby, B.C.).

From 1 male and 1 female, the liver, kidneys and small intestine were removed and tissue samples were snap frozen in liquid nitrogen and stored at -80 ^{o}C. For the second male and female, the livers and kidneys were fixed in 10% neutral buffered formalin (EMD chemicals, Gibbstown, NJ) overnight at 4 ^{o}C with gentle rocking on a nutating mixer (VWR International, West Chester, PA, US), followed by 3 washes with phosphate buffered saline (PBS) at room temperature for 10 min, and dehydration in 70% ethanol. Fixed tissues were stored in 70% ethanol at 4 ^{o}C for up to 1 week. The tissues were then processed and wax embedded at the Centre for Modeling Human Disease Pathology Core. All blood samples were centrifuged at 2,500 x g for 10 min at room temperature to obtain plasma. Both the remaining red blood cell pellet and plasma were stored at -80 ^{o}C for further use.

Sexing

Initially, sex was estimated visually from noting fetal anogenital distance. Tails were also collected to confirm the sex of the fetuses by PCR genotyping and agarose gel electrophoresis by identifying amplicons of X and Y chromosomes using the REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, Oakville, Ontario). All DNA isolation and sexing by PCR was performed with the help of laboratory technical staff. The methodology followed procedures outlined by the Clapcote and Roder PCR protocol, using a combination of PCR and agarose gel

electrophoresis (553). All primers used throughout this study were synthesised by Eurofins MWG Operon (Eurofins MWG Operon, Huntsville, Alabama). The sequences for the primers used in this kit are in Table 3.1.

Table 3.1 PCR primers for sexing PCR

Gene	Sequence (5′ → 3′)	Amplicon size (bp)
Jarid1	Fwd 5′-GCACAGGACCTCAGGGACCCAG-3′	
Jarid1D	Rev 5′-CAGAGGCATTCATCGATGAGG-3	331
Jarid1C	Rev 5′-TGAGTTGGTACGACGAAGCTGCAG-3	302

Extraction and Tissue Preparation solution from REDExtract-N-Amp Tissue PCR kit were pre-mixed in a ratio of 4:1 (20 µl to 5 µl per tail). Between 0.5 to 1 cm of collected mouse tail was placed into Extraction/Tissue Preparation mixture and incubated at room temperature for 10 min. The samples were then transferred to a heating block where they were further incubated for 5 min at 95 ^{o}C. 20 µl of Neutralization solution B was then added to the digested tissue, which was then vortexed.

Following the extraction, the DNA was amplified via PCR using an Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany. The Jarid primers where mixed together in nuclease-free water to achieve a final primer concentration of 25 µM. For each sample, the PCR mixture comprised of 10 µl REDExtract-N-Amp PCR Reaction Mix, 0.3 µl of primers, 7.2 µl of water, and 2.5 µl of the tissue extract DNA for a 20 µl total reaction volume. In the thermal cycler, the mixture was

initially incubated at 94°C for 3 min for initial denaturation, followed by 45 cycles of 94 °C for 15 s, 65 °C for 45 s and 42 °C for 30 s. Once the cycles completed the DNA underwent a further 10 min extension at 72 °C. The amplified PCR product was then analysed by agarose gel electrophoresis stained with *SYBR® Safe* DNA Gel stain (Invitrogen Canada Inc., Burlington, ON, Canada). Two bands on the gel represented the males, whereas a single band indicated females.

3.2.4 Nucleic acid isolation

Genomic DNA and RNA were jointly isolated using the Qiagen Allprep® DNA/RNA Micro Kit (Qiagen Canada, Qiagen Inc., Mississauga, Ontario, Canada) according to manufacturer's protocol. However, the methods of disruption and homogenisation differed between blood and the remaining tissues. Once homogenised, the remaining steps were the same for all tissues.

Frozen blood cell pellets were gently thawed on ice. A 23¾ -gauge needle on a syringe was then used to disrupt the cells. The cell pellet was taken up and then expelled from the syringe 25 times to thoroughly break up the cells. 10-15 µl of cell mixture was taken up and placed in 350 µl of RLT plus buffer containing β-mercaptoethanol and vortexed vigorously for 20-30 s.

In contrast, for the remainder of the tissues 15 mg of liver, kidneys and small intestine were homogenised in 350 µl of RLT plus buffer containing β-mercaptoethanol using the Qiagen Tissuelyser II (Qiagen Canada, Qiagen Inc.,

Mississauga, Ontario, Canada). Tissue disruption was carried out for 3 min at high speed (30 Hz) at room temperature in 2 ml microcentrifuge tubes, each containing a stainless-steel bead. Tissue disruption was only completed once to prevent shearing of DNA. The resulting homogenate was then transferred to Allprep® DNA spin columns (Qiagen Canada, Qiagen Inc., Mississauga, Ontario, Canada) and centrifuged for 30 s at 8000 x g. The flowthrough was then put aside, and the DNA columns were placed in new spin baskets and stored at 4 ^{o}C for later isolation steps.

3.2.4.1 Total RNA purification

Flow through from the DNA spin columns was used for RNA purification. 350 µl of 70% ethanol (50% ethanol for liver samples) was added to the flowthrough and pipet-mixed. The flowthrough was added to RNEasy MinElute spin columns and centrifuged for 15 s at 8000 x g. Flowthrough from the spin basket was then discarded. The RNEasy MinElute spin column (Qiagen Canada, Qiagen Inc., Mississauga, Ontario, Canada) was sequentially washed with 700 µl of Buffer RW1 (700) and 500 µl of Buffer RPE. Each wash was followed by centrifugation for 15 s at 8000 x g and a discarding of the flowthrough. 500 µl of 80% ethanol was added directly to the spin column followed by centrifugation for 2 min at 8000 x g. The spin basket was replaced with a new one and the column centrifuged at full speed to remove any remaining ethanol. The column was then transferred to a 1.5 ml microcentrifuge tube. 16 µl of RNAse-free water was then added to the centre of the spin column membrane followed by 10 min incubation

at room temperature. The column and tube were centrifuged for 1 minute at full speed to elute the RNA.

3.2.4.2 Genomic DNA purification

The DNA spin column was removed from 4 °C washed with 500 µl of Buffer AW1 and centrifuged for 15 s at 8000 x g. 500 µl of Buffer AW2 was then added and the column centrifuged for 2 min at full speed. The column was then transferred to a 1.5 microcentrifuge tube. 50 µl of warm Buffer EB (pre-warmed to 70 °C) was added to the spin column; the column was then incubated for 10 min at room temperature and centrifuged for 1 minute at 8000 x g to elute the DNA.

3.2.4.3 Nucleic acid quantification

All DNA and RNA were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA). The spectrophotometer was blanked against EB buffer for DNA samples and RNase-free water for RNA. 2 µl of sample was used when quantifying nucleic acids. All RNA used in cDNA synthesis had A260/A280 and A260/A230 ratios of 1.80 or above. The A260/A230 ratio is particularly relevant for RNA in that low A260/A230 ratios are indicative of contamination by residual guanidine and other organic contaminants. RNA samples were stored at -80 °C.

Only DNA with 260/280 nm and 260/230 nm ratios of 1.8 were used for analyses (554). DNA integrity was further confirmed by loading on a 1% (w/v) agarose

gel. Samples were stained with SYBR® Safe DNA Gel stain (Invitrogen Canada Inc., Burlington, ON, Canada) and imaged using Image Lab software GelRed (Biotium, Hayward, CA 94545). Only single-band, non-fragmented DNA was used for subsequent downstream analysis. Genomic DNA samples were stored at 4 ^{o}C for short-term storage, and transferred to -80 ^{o}C for longer-term storage.

3.2.5 mRNA relative quantification

3.2.5.1 cDNA synthesis

RNA was kept on ice throughout the making of the reaction mix. All RNA was normalised to 10 ng/µl in RNAse free water. Single-stranded cDNA was synthesised using the iScript Reverse transcription Supermix reaction system (Bio-Rad Canada, Mississauga, Ontario, Canada). A reaction mastermix was made containing a mixture of 4 µl of 5x iScript RT Supermix and 6 µl of RNase-free water per reaction. 10 µl of total RNA from each sample was seeded into the reaction for a total RNA concentration of 100 ng in a 20 µl reaction volume (final concentration 5 ng/µl). The reaction mix was then transferred to an Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany) and incubated for 5 min at 25^{o}C for priming, 30 min at 42^{o}C for reverse transcription and 5 min to inactivate the enzyme. The resultant cDNA was stored at -20 ^{o}C.

3.2.5.2 Primer design and real-time quantitative PCR

Primers were either designed using the NCBI Primer-BLAST software or adopted from the literature. Primer sequence specificity was tested using the UCSC *in*

silico PCR from https://genome.ucsc.edu/index.html, which is able to use primer pairs to search the entire genome in both humans and mice. Primer sequences are listed in Table 3.2.

Primers were validated by quantitative real-time PCR (qPCR) using serial dilutions of stock cDNA of between 1:3 and 1:10 in RNase-free water. Standards were generated from 3-fold serial dilutions of pooled cDNA from reverse-transcribed liver samples in RNase-free water (3^1 to 3^6-fold dilutions). A standard curve was run for each qPCR plate to estimate the amplification efficiency of the PCR reaction. qPCR of standards and samples was performed using the Bio-Rad CFX 384 system (Bio-Rad Canada, Mississauga, Ontario, Canada). All qPCR reactions were performed in triplicate in a 5 µl reaction volume for all samples. Each PCR reaction contained 1x LuminoCt® SYBR® Green qPCR ReadyMix™, 300 nM of forward and reverse primers, and RNase-free water. Concentration of primers and reagents used are summarised in Table 3.2. Cycling conditions were: 95 ^{o}C for 20 s, followed by 35 cycles of 95 ^{o}C for 5 s and 60^{o}C for 20 s. For all primers a melt curve from 65 ^{o}C to 95 ^{o}C showed a single PCR product (Figure 3.2).

All genes of interest were normalised to the geometric mean of three reference genes succinate dehydrogenase complex, subunit A (SDHA), TATA-box binding protein (TBP), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-

activation protein, zeta polypeptide (YWHAZ). Stability of the reference genes was checked by one-way analysis of variance (ANOVA) to confirm minimal variation across diet groups. Relative expression values were calculated and adjusted for PCR reaction efficiencies using Pfaffl's relative ratio, which is built into the Bio-Rad CFX software.

The relative expression calculation by Pfaffl (555,556) takes into account the amplification efficiencies of the reaction for both gene of interest and reference genes. In order to determine reaction efficiency every run with samples had a standard curve. Reaction efficiency was calculated by the following equation (555):

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Cq,\ \text{target}\ (\text{control - sample})}}{(E_{\text{reference}}))^{\Delta Cq,\ \text{ref}\ (\text{control - sample})}}$$

E_{target} is the efficiency of the target gene. E_{ref} is the efficiency of the reference gene. Given that multiple reference genes were used for each PCR cycle, a geometric mean was used to calculate a normalisation factor for both control and treatment groups. $\Delta Cq,_{\text{target}}$ (control - sample) is the difference between the Cq (or C_T) value of a chosen control against which every sample is normalised, and the test sample being analysed for the target gene. The $\Delta Cq,_{\text{reference}}$ (control - sample) is the same principle, substituting the values for the gene of interest for the geometric mean of the reference genes.

Table 3.2 PCR primers for gene expression analysis

	Sequence (5′ → 3′)	Amplicon Size (bp)	Accession Number/reference
mTert	Fwd 5′-TGGGTCTCCCCTGTACCAAA-3′ Rev 5′-GCCTGTAACTAGCGGACACAGA-3	68	NM_009354.1
c-myc	Fwd 5′-GTTGGAAACCCCGCAGACAG-3′ Rev 5′-CGGAGTCGTAGTCGAGGTCA-3′	83	NM_001177352.1
Sdha	Fwd 5′-CTTGAATGAGGCTGACTGTG-3′ Rev 5′-ATCACATAAGCTGGTCCTGT-3′	87	RT Primer DB
Tbp	Fwd 5′-CGGACAACTGCGTTGATTTTC-3′ Rev 5′-AGCCCAACTTCTGCACAACTC-3′	115	NM_013684
Ywhaz	Fwd 5′-GCAACGATGTACTGTCTCTTTTGG-3′ Rev 5′-GTCCACAATTCCTTTCTTGTCATC-3′	149	NM_011740

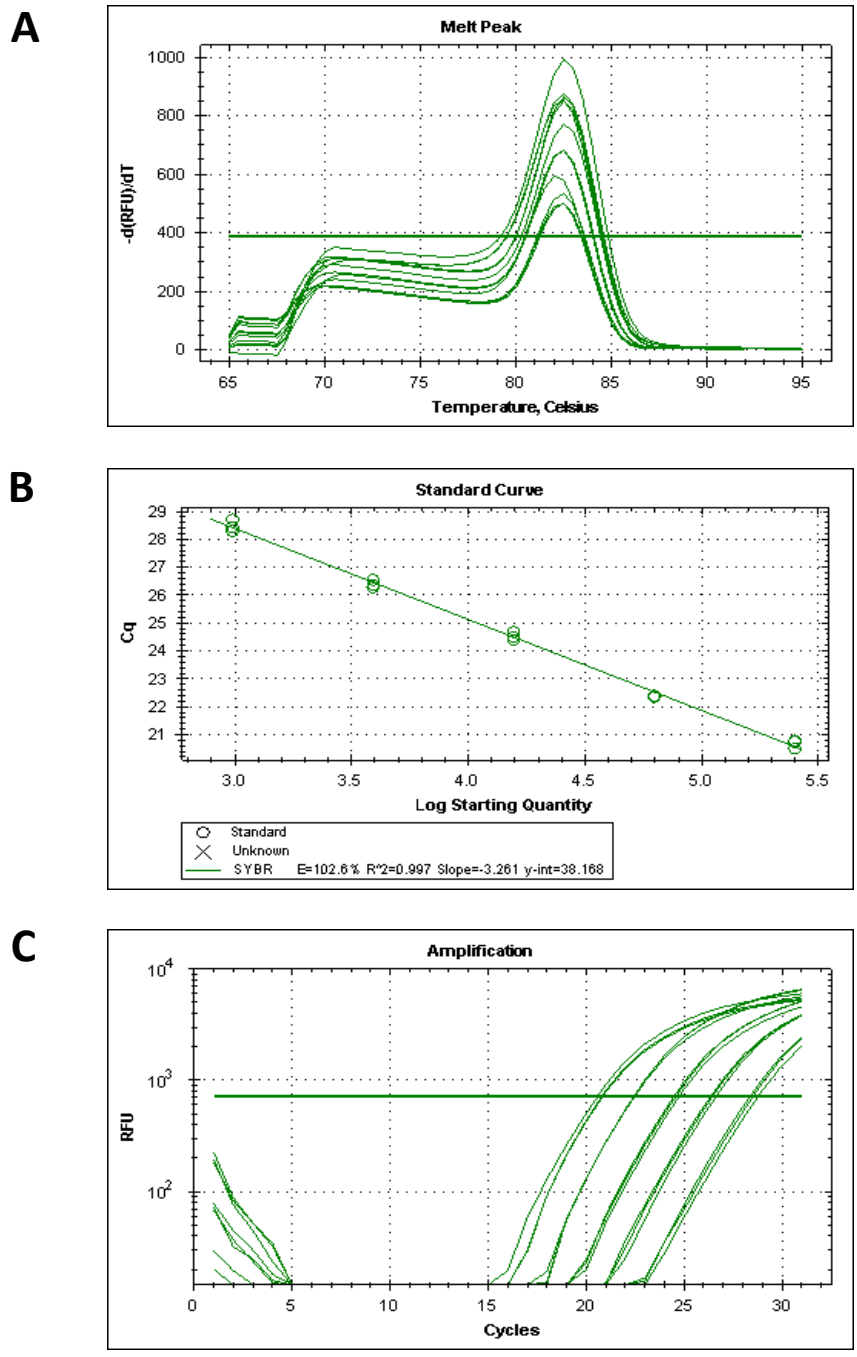

Figure 3.2. Example plots of (A) Sample melt curve. (B)Sample standard curve, (C) Standard amplification curve.

A single melt peak across all samples indicated amplification of the same product. The standard curves are generated from the threshold cycle (Ct) values of serially diluted pool of cDNA, plotted on a Log_{10} graph for the quantitation of relative expression. The standard amplification curve shows where the measurement of fluorescence intensity is above background, as indicated by the horizontal line.

3.2.6 Mitochondrial DNA Copy Number Quantitation

The assay for quantitation of mitochondrial (mtDNA) and nuclear DNA (nDNA) was based on qPCR of genomic DNA (557). This was performed on maternal and fetal DNA. The nDNA was detected using primers for β1-globin, and for the detection of mtDNA primers for mitochondrially encoded NADH dehydrogenase 1 (mt-ND1) and mitochondrially encoded cytochrome b (mt-CytB) were chosen (Table 3.3). qPCR was carried out as described in Section 3.2.3.2. The amount of mtDNA was expressed as a ratio of mtDNA/nDNA.

Table 3.3 PCR primers for mtDNA quantification

`	**Sequence (5′ → 3′)**	**Amplicon Size (bp)**	**Reference**
mt-ND1	Fwd 5′-AATCGCCATAGCCTTCCTAACAT-3′	115	(558)
	Rev 5′-GGCGTCTGCAAATGGTTGTAA-3′		
mt-CytB	Fwd 5′-GCTTTCCACTTCATCTTACCATTTA-3	90	(559)
	Rev 5′-TGTTGGGTTGTTTGATCCTG-3		
β1-globin	Fwd 5′-GCACCTGACTGATGCTGAGAA-3	64	(523)
	Rev 5′-TTCATCGGCGTTCACCTTTCC-3		

3.2.7 Telomere length measurement

Absolute telomere length (aTL) was measured in extracted DNA from maternal and fetal tissues by qPCR assay as described by O'Callaghan and Fenech (560) . This is a modification of the qPCR protocol devised by Cawthon (561), which generates a relative expression TL value. The relative TL is a ratiometric value calculated by normalising a telomere repeat copy number to a single copy gene (T/S ratio) in experimental samples as compared with a reference DNA sample

(562). The aTL O'Callaghan quantification method utilises oligomer standards of known size, which are used to generate aTL values.

(a) Oligomers

Telomere primer sequences were obtained from Cawthon (562) (Table 3.4). The reference gene used was murine acidic ribosomal phosphoprotein P0 (*36b4*). All Telomere and *36b4* standard sequences were confirmed using *in silico* PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) on whole genome mounts. Primers were validated before use in telomere qPCR.

Table 3.4. Oligomers used for aTL PCR

Type	Name	Sequence (5′→3′)	Amplicon Size (bp)	Accession number/ reference
Primers	Tel	Fwd 5′-CGGTTTGTTTGGGTTTGGGTTT GGGTTTGGGTTTGGGTT-3′ Rev 5′-GGCTTGCCTTACCCTTACCCTT ACCCTTACCCTTACCCT-3′	76	(562)
	36b4	Fwd 5′-ACTGGTCTAGGACCCGAGAAG-3′ Rev 5′-TCAATGGTGCCTCTGGAGATT-3′	78	(560)
Stds	Tel	TTAGGGTTAGGGTTAGGGTTAGGGTTA GGGTTAGGGTTAGGGTTAGGGTTAGGG TTAGGGTTAGGGTTAGGGTTAGGG	84	(562)
	36b4	ACTGGTCTAGGACCCGAGAAGACCTCC TTCTTCCAGGCTTTGGGCATCACCACGA AAATCTCCAGAGGCACCATTGA	77	NM_007475

(b) Procedure

Quantified genomic DNA was normalised to 10 ng/µl in sample normalisation tubes using nuclease-free water. A PCR master mix solution was prepared for a total reaction volume of 5 µl. Each master mix solution contained 1x LuminoCt® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich, Oakville, Ontario), 0.6 µM of both forward and reverse primers, and sterile water. Cycling conditions were: 95 ^{o}C for 10 min, followed by 30 cycles of 95 ^{o}C for 10 s, 56^{o}C for 15 s and 72 ^{o}C for 5 s.

The JANUS automated liquid handling workstation (Perkin Elmer, Massachusetts, USA) was used to aliquot DNA template and master mix into 384 well PCR reaction plates. qPCR was performed in a Bio-Rad CFX 384 cycler. All reactions were carried out in triplicate with a non-template control and a reference DNA sample included in each run.

A standard curve was generated by performing qPCR on serial dilutions of Telomere and 36B4 standards (diluted 10^{-1} to10^{-8}). The standard curves were used to measure the amount of telomeric sequence per sample in kb and the diploid genome copies of 36B4 in each sample. The amount of oligomer in the most concentrated standards for both Telomere and 36B4 was calculated using the method by O'Callaghan and Fenech (560) (Figure 3.3). Standard curve and sample values were determined using Bio-Rad CFX Manager Version 2.1

software from the starting quantity values determined from the standard curve quantities of telomere and 36B4. Absolute values were calculated by dividing the telomere kb per reaction value by diploid genome copy number to give a total telomeric length in kb per diploid genome.

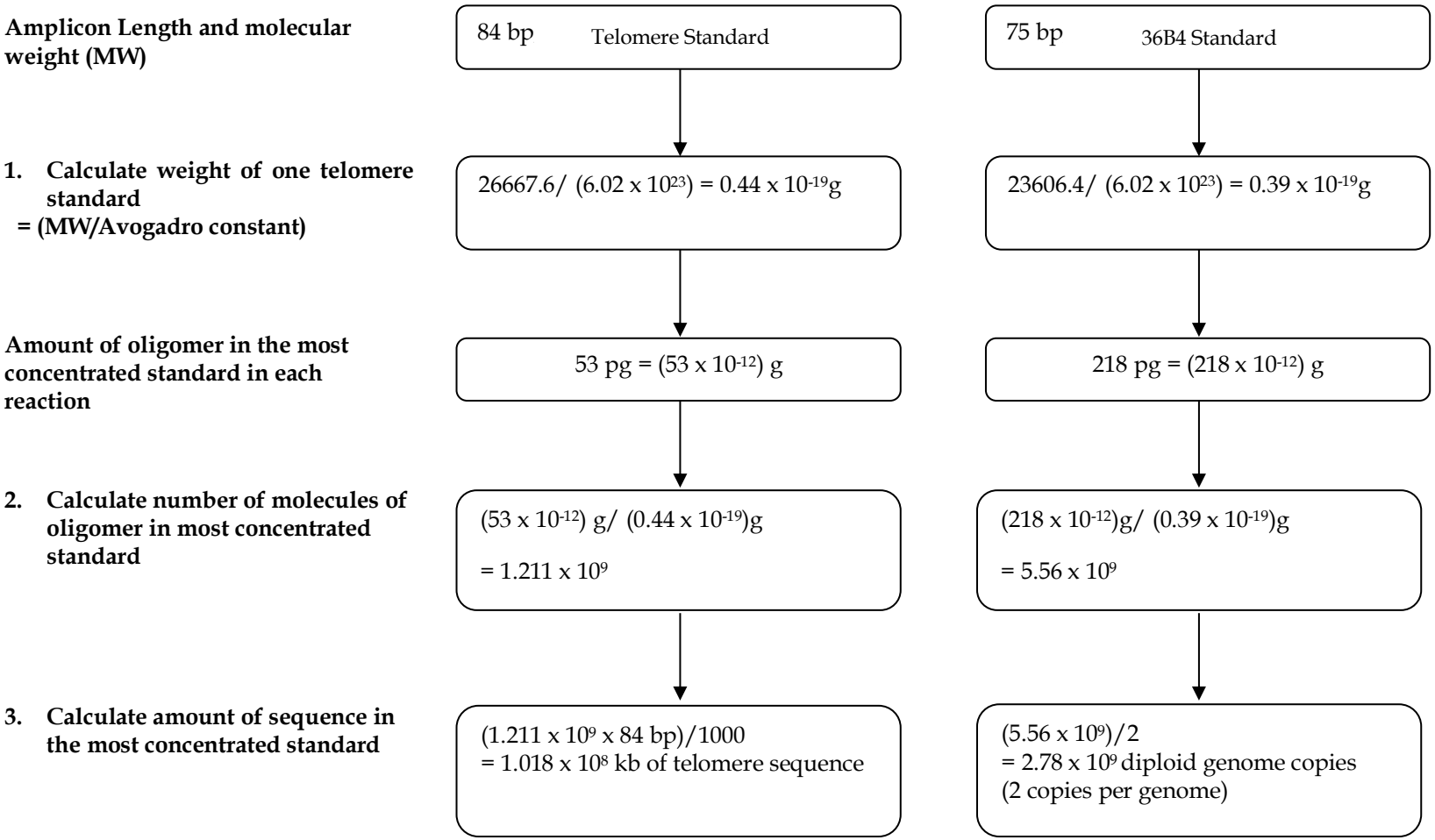

Figure 3.3. Calculation of absolute quantities of standards for standard curves
(adapted from O'Callaghan (560))

3.2.8 Sectioning and staining

Paraffin wax-embedded blocks of whole fetal liver were sectioned at 5 μm thickness using a Leica RM 2255 microtome (Leica Microsystems Inc., Buffalo Grove, IL, USA).

3.2.8.1 Immunohistochemistry

To assess the level of cellular proliferation within fetal livers, paraffin-embedded sections of whole fetal liver were stained with primary antibodies for KI67 (mouse monoclonal; Leica Novocastra, Newcastle Upon Tyne, UK)) Sections were deparaffinised in xylene (for 10 min once, 5 min two times) and rehydrated in a descending ethanol series (three changes of 100% ethanol, 5 min each, followed by one change of 95, 90, 80, 70% and 50% ethanol, 5 min each). Sections were then washed twice in double distilled water (ddH_2O) for 5 min. To quench endogenous peroxidase activity, the sections were incubated in a solution of 0.3% (KI67 in 100% methanol) v/v hydrogen peroxide for 30 min at room temperature.

Antigen unmasking and retrieval were then performed using a microwave oven with ten power level settings. Antigen unmasking was performed by heating sections in 1X Dako antigen unmasking solution in ddH_2O (Dako, Mississauga, Ontario, Canada) for 5 min on full power. This was repeated at 5 min intervals for a total of 25 min, and then followed by cooling on ice for 10 min. Antigen retrieval was then performed by immersing the sections in 0.01 M sodium citrate buffer (citric acid and sodium citrate, pH 6.0) and heating for 5 min and then 3

min at power level 6, with ice cooling for 10 min at the end of each heating period. Sections were then washed twice for 5 min in 1X PBS and then blocked with Dako protein blocking solution (1-2 drops per section; (Dako, Mississauga, Ontario, Canada) for 1 hour (h) at room temperature in a humidified chamber. The concentration of primary antibody used for KI67 was 1:200. Antibodies were diluted in Dako antibody diluent (Dako, Mississauga, Ontario, Canada) overnight in a humidified chamber at 4 ^{o}C.

Following overnight incubation with the primary antibody, sections were washed for 5 min in 1X PBS-tween (PBS-T) (0.1% v/v), followed by two more 10 min PBS-T washes and a single wash in regular 1X PBS for 5 min. Sections were then incubated with a secondary biotinylated anti-rabbit IgG (1:200; company, location; diluted in Dako antibody diluent solution) for 1 h at room temperature in a humidified chamber. Following incubation with secondary antibody, sections were washed for 5 min in 1X PBS-T, followed by two more 10 min PBS-T washes and a single wash in regular 1X PBS for 5 min. 1-2 drops of Dako solution (Dako, Mississauga, Ontario, Canada) or Invitrogen streptavidin-HRP (Invitrogen Canada Inc., Burlington, ON, Canada) were added to the sections and then incubated for 1 h at room temperature in a humidified chamber. Sections were then washed once in 1X PBS-T followed by a single wash in 1X PBS.

The chromogen 3,3'-diaminobezidine (DAB) was used to visualise the antibody-antigen reactions, via the production of a brown precipitate on the antigen site.

The DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA) was used and DAB was prepared with 2.5 ml of ddH_2O, with kit components: 1 drop of reaction buffer, 1 drop of H_2O_2 and 2 drops of DAB substrate. 100 µl of the DAB reaction mix was applied to each liver section and incubated for 30 s (optimal DAB reaction time was determined from optimisation experiments). Immersing the slides in tap water terminated the reaction. Sections were then counterstained with haematoxylin (Gills I, 1:2 dilution, Sigma-Aldrich, Oakville, Ontario) for 3 min and rinsed with tap water for 1 min. Sections were dehydrated through graded alcohols (one change each of 70, 80, 90, 95% ethanol for 5 min, and three changes of 100% for 5 min), cleared in xylene (three changes, 5 min each) and coverslipped using Micromount mounting medium (Surgipath Medical Industries, Richmond, IL, USA).

Fetal liver sections were used as negative controls in each assay to ensure specificity of antibody staining. Negative controls were performed by incubating Dako anti-mouse IgG1 negative control (for Ki67; Leica Novocastra, Newcastle Upon Tyne, UK) or omitting the primary antibody and only adding the antibody diluent (for caspase-3). Sections were viewed under the Leica DM IL LED light microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) and photos were captured at 40 x magnification, using QImaging MicroPublisher 5.0 RTV digital camera utilizing QCapture software (Olympus, Center Valley, PA, USA). Sections were also examined using an Olympus BX60 Light Microscope (Olympus Corporation, Lake Success, NY, USA)

3.2.8.2 Analysis

The percentage of Ki67 positively stained cells was scored in a blinded manner. A piece of white tape was placed over the slide label to de-identify the animals. The stained slides were placed in a random order and assigned a number, which was written on the white tape. A note was made of which numbers corresponded to which dietary group in a notebook. The notebook was then kept separate while analysis of Ki67 positive cells was made. During labelling of photos captured, only the assigned number on the slide was used to identify the tissue. The number of positive cells in 20 separate captured fields was counted against the total number of cells in each field. The brown peroxidase staining or the blue counterstain identified positive cells. The total number of brown cells in each field was expressed as a percentage of the total number of cells in each field. Once Ki67 percentages were calculated for each slide, the notebook was then used to identify the animals corresponding to the assigned number on the slide.

3.2.9 Reactive Oxygen Species Detection

3.2.9.1 DNA oxidation

Dihydroethidium (DHE) was used to evaluate levels of oxidative stress within DNA of fetal liver. This measure was based on a method previously published by Nin and colleagues who used DHE staining to evaluate superoxide-induced oxidative damage in fixed lung tissue (563). DHE is an indicator of intracellular superoxide production (564). It intercalates with cellular DNA and once oxidised is converted from DHE to ethidium (565). In its reduced form DHE fluoresces

blue, whereas in its oxidised form it stains the nucleus bright red following DNA intercalation. The fluorescence intensity is indicative of the degree of oxidation.

Reagent setup

DHE powder (Millipore Ltd. Mississauga, Ontario, Canada) was reconstituted to a 50 mM stock solution using dimethyl sulfoxide (DMSO) (Sigma-Aldrich Canada Ltd). Stock solutions were prepared fresh and used for a single batch of experiments. Reconstituted DHE was then diluted to a final working concentration of 5 mM in 1 X PBS. For this assay, both stock and working solutions were maintained under dark conditions.

Staining procedure

Liver sections were dewaxed in xylene and rehydrated in descending alcohol series as described above (3.2.5.1). Following this, 75 µl of DHE working solution (5 mM) was added to each tissue section. Sections were incubated for 90 min at 37 ^{o}C in a dark humidified chamber. Negative controls were performed for the whole run by omitting the DHE and incubating sections with 1 X PBS solution only. The sections were washed three times in 1 X PBS, mounted with Vectashield mounting medium (Vector, Burlingame, CA, USA) and visualised by spinning-disc confocal microscopy.

Image acquisition and analysis

DHE staining was visualised using a Leica 6000 DMI B microscope (Leica, Bensheim, Germany), and images were captured and analysed with Volocity software (Improvision, Lexington, MA). Images were captured with a 20x objective lens with modifications using the following protocol.

The section was placed on the stage and tissue was brought into focus with a 5x objective using normal light so as to avoid bleaching the DHE with the red fluorescent laser. Samples were vertically scanned from the bottom coverslip with a total depth of 20-100 μm and a pinhole diameter of 40-70 μm. The sequential images were collected at a step depth of 0.3-2.0 μm. The size of the tissue was estimated first at 5 x and then captured at 20 x magnification. The unstained slide was used to remove excess background to give a cleaner image, before deconvolution was performed in Volocity.

Volocity software was used in analysis mode to measure the intensity of the staining. The intensity was determined by counting the number of three-dimensional volumetric pixels (or voxel count). It was necessary to define a brightness threshold for analysis of the region of interest, which indicated superoxide presence, across all slides. All the captured images from the experimental groups were examined and the lasso tool in the software was used to select each the regions of intensity. Within the Volocity software the classifier tool was then used to further define cut off points for intensity for all the nuclei

to be counted. The selection lines in the classifier tool were used to define the zone for analysis. The image was checked to ensure that there was no significant loss. This process was repeated for all slides before determining an average minimum threshold that was suitable between experimental groups.

The voxels were quantified by setting an intensity minimum threshold of 20%. All voxels that had brightness lower than the threshold were assumed to be background. Once the analysis area had been selected, the data was then exported and the mean of the voxel count was taken and expressed as a percentage of voxels compared to the CON group.

3.2.9.2 Protein oxidation

The oxidation of protein in the liver was assessed by a protein carbonyl colorimetric assay. 20 mg of liver tissue was sonicated three times in 150 µl of cold 1 x PBS in 10 second bursts. All sample protein was diluted to a final concentration of 1 mg/ml. 60 µl of each sample was then pipetted into a new 1.5 ml microcentrifuge tube and labelled "S" because it contained the sample to be analysed. A repeat of the sample transfer was performed with a second 1.5 ml tube which was then labelled "C" because it contained the control sample mixture.

240 ml of 10 mM 2,4 Dinitrophenylhydrazine (DNPH) (Sigma-Aldrich, Oakville, Ontario) in 2.5 M hydrochloric acid (HCl) was then added to the S tube which

contained the sample to analysed, and concurrently only 240 µl of 2.5 M HCl was added to the C tube. Both S and C tubes for each sample solutions were then vortexed vigorously for 10 s and then covered and allowed to incubate in the dark for 15 min at room temperature, with vortexing at 5-minute intervals. Following this 300 µl of 50% (w/v) trichloroacetic acid (TCA) was added to each tube and the tube vortexed. The sample mixture was then placed at -20 °C for 30 min and then centrifuged at 9000 x g at 4 °C for 15 min. The supernatant was carefully removed and the remaining protein pellet was used for the remainder of the assay.

In order to remove excess DNPH, the pellet in the S tube was then washed three times in a cold solution of ethanol and ethyl acetate mixed in a 1:1 ratio and the added to the pellet. The C tube was processed in tandem. Following the addition of ethanol: the ethyl acetate mixture in the tube was then centrifuged at 9000 x g at 4 °C for 2 min and the supernatant removed for each round of washing. Once the pellets were washed, they were dissolved in 500 µl of 6 M guanidine-HCl solution and thoroughly mixed by vortexing. The tubes were then centrifuged one more time at 9,000 x g at 4 ^{o}C for 15 min to remove any undissolved debris.

220 µl of supernatant in tube S was then transferred to each of the first two wells of a 96-well plate. For the same sample 220 µl of supernatant was transferred from tube C to the row below. This was repeated for subsequent samples so that they were arranged in the plate as shown below (Figure 3.4).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S5	S5	S9	S9	S13	S13	S17	S17	S21	S21
B	C1	C1	C5	C5	C9	C9	C13	C13	C17	C17	C21	C21
C	S2	S2	S6	S6	S10	S10	S14	S14	S18	S18	S22	S22
D	C2	C2	C6	C6	C10	C10	C14	C14	C18	C18	C22	C22
E	S3	S3	S7	S7	S11	S11	S15	S15	S19	S19	S23	S23
F	C3	C3	C7	C7	C11	C11	C15	C15	C19	C19	C23	C23
G	S4	S4	S8	S8	S12	S12	S16	S16	S20	S20	S24	S24
H	C4	C4	C8	C8	C12	C12	C16	C16	C20	C20	C24	C24

Figure 3.4. Sample (S) and Control (C) arrangement in the 96-well plate for protein analysis.

The absorbance of the sample S and the control C in the plate was then read at a wavelength of 370 nm using a BioTek uQuant Microplate Spectrophotometer (BioTek instruments; Winooski, VT, USA) and KC Junior software version 1.31.2 (Biotek instruments; Winooski, VT, USA). The average absorbance of each sample S and control C was calculated in Microsoft Excel. To obtain a Corrected Absorbance value, the average absorbance of C was subtracted from average absorbance of S:

Corrected absorbance at 370 nm = Average Sx – Average Cx,
where x = matched sample number

Therefore, to calculate the protein carbonyl concentration, the following equation was used:

Protein carbonyl concentration (nmol/ml)
= *Corrected absorbance/extinction coefficient of DNPH at 370 nm (22,000 $M^{-1}cm^{-1}$)* * 1 x 10^6

3.2.9.3 Lipid oxidation

During periods of oxidative injury, lipids can also be oxidised via the process of lipid peroxidation (566). Lipid peroxidation leads to the development of compounds called hydroperoxides (HP), derivatives of unsaturated and saturated lipids which are generated by free radicals such hydroxyl, lipid oxyl or peroxyl radicals, superoxide, and peroxynitrite (566,567). Lipid oxidation in the liver was assessed using the colorimetric Lipid Hydroperoxide Assay Kit from Cayman Chemicals (Ann Arbor, Michigan).

HP were extracted from fetal liver tissue using a customised chloroform: methanol protocol. Prior to hydroperoxide extraction, it was necessary to remove oxygen from both chloroform and methanol using nitrogen gas. A system was devised that consisted of a nitrogen gas tank with a regulator, which was then connected to an Erlenmeyer flask containing either chloroform or methanol by Tygon tubing. The nitrogen gas was bubbled through both chloroform and methanol for 1 h before sealing the flask, which was only reopened just prior to use. During the lipid extraction steps, the deoxygenated chloroform flasks remained immersed in a Styrofoam box full of ice to keep them cold. Just prior to the beginning of the extraction, 100 mg Extract R from the kit was added to the methanol and vortexed vigorously for 2 min.

20 mg of frozen liver tissue was sonicated three times in 500 µl of ice-cold PBS in 10 second bursts. The 500 µl homogenate was then transferred to a pre-labelled glass test tube followed by 500 µl of Extract R saturated methanol. The test tube was then sealed and vortexed. 1 ml of chloroform was then added followed by another period of vortexing. The mixture was then transferred to a centrifuge at 1,500 x g at 4^0C. The bottom layer was then collected and transferred to a fresh test tube.

Deoxygenated chloroform and methanol were mixed together in a 2:1 ratio. The kit chromogen was prepared by mixing together, in a ratio of 1:1, FTS Reagent 1 and FTS Reagent 2, followed by vortexing. 500 µl of chloroform extract was then transferred to a new glass tube, followed by 450 of the chloroform/methanol mixture, and 50 µl of the chromogen. The mixture was once again vortexed and the tube capped and left to incubate at room temperature for 5 min.

An 8-point standard curve was made using concentrated HP standards ranging from 0-5 nmol (0, 0.5, 1, 1.5, 2, 3, 4, and 5). 0, 10, 20, 30, 40, 60, 80, 100 µl of each standard was added to a glass tube, followed by a corresponding volume of chloroform/methanol mixture, to make up 950 µl, which was then vortexed. The Chromogen was then added as well and the tube was vortexed. Duplicates of 300 µl of each standard were then added to the first two columns of the glass 96-well plate from the kit. 300 µl of the extracted samples was then also added to the

remaining wells in duplicate. Absorbance readings were taken at 500 nm using a BioTek uQuant spectrophotometer for microplates (Biotek Instruments; Winooski, VT, USA) and KC Junior software version 1.31.2 (Biotek Instruments; Winooski, VT, USA).

In Microsoft Excel, the absorbance readings were then re-plotted as a corrected absorbance. Subtracting the absorbance reading of the first standard from itself to obtain a zero value, and then also subtracting the readings of the remaining standards from the first one did this. The values were then re-plotted and a linear equation was obtained.

The final calculation of the HP content involved two steps. The first step used the linear equation of the adjusted standard curve of the corrected absorbance values to get the HP value in the sample tube (HPST). This was calculated using the following equation: **HPST (nmol)** = (sample absorbance - y-intercept)/slope

Finally, in order to determine the concentration of HP (in μM) in the original sample, the following calculation was used:

Hydroperoxide concentration in sample (μM) = HPST/VE x 1 ml/SV,

where VE (ml) is the volume of extract used for the assay and SV (ml) is the volume of original sample used in the extraction.

3.2.10 Localisation of shelterin protein TIN2

Immunofluorescence with primary antibodies (rabbit polyclonal; Abcam, Cambridge, MA, USA) was used to localise shelterin protein TIN 2.

3.2.10.1 Immunofluorescence protocol

Sections were deparaffinised in xylene (twice, 10 min) and washed in 100% ethanol (twice, 5 min each).

Sections were then washed twice for 5 min in 1X PBS and then blocked with Dako protein blocking solution, 1-2 drops per section (Dako, Mississauga, Ontario, Canada) for 1 h at room temperature in a humidified chamber. Protein blocking solution was removed by washing three times in 1 x PBS (5 min each) before primary antibodies or PBS (for the unstained slide) were added. TIN2 was used at a 1:100 dilution in a solution of Dako antibody diluent mixed with 1x PBS in a 50:50 ratio by volume. The primary antibody was incubated for 1 h at 37 ^{o}C. The slides were then removed and washed with 1 x PBS (three times, 5 min each) before Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L) secondary antibody (Invitrogen Canada Inc., Burlington, ON, Canada) was added. Similar to the primary antibodies, the secondary antibody added to sections with TIN2 was diluted in a solution of 50% Dako antibody diluent: 50% 1x PBS, at a dilution of 1:200. Once again, the sections were incubated at 37 ^{o}C for 1 h before being washed three times with 1 x PBS (5 min each) at room temperature. Slides were then mounted and coverslipped with Prolong Gold containing DAPI

(Invitrogen–Molecular Probes). The edges of the slide were then sealed with nail polish and the slides stored in the dark at -20^{0}C until imaging later.

3.2.10.2 Imaging acquisition and analysis

All slides were visualised using a spinning disc confocal microscope, the Leica DMI 6000 (Leica Microsystems Inc., Buffalo Grove, IL, USA) with an inverted motorised stage. Fluorescence images were captured with a mounted Hamamatsu C9100-13 EM CCD camera (Hamamatsu Photonics, Japan) with a HCX Plan Apochromat 40 x oil objective (numerical aperture 0.60), at room temperature. For the duration of image acquisition, slides were kept covered in a dark box until ready to use. Before being placed on the microscope stage, slides were gently cleaned with 70% ethanol to remove any artifacts or dust with lint free Kimwipes. A drop of immersion oil (type DF, Cargille Laboratories, Cedar Grove, NJ) was placed on the slide on the area covered by the tissue. The objective was then lowered into the oil.

Images of all probes (Alexa Fluor® 594 and DAPI) were simultaneously captured using the same illumination conditions for all slides. An automated capture of a 3-dimensional z-stack of the tissue was once again performed using Volocity software. Prior to capture, limits were set so that image acquisition started from an area of darkness above to an area of darkness below where the tissue is out of focus on either end. This limit was set initially by bringing the tissue to a central z focal point to establish a clear depth of focus, which was then set. Once this was completed, a manual scan in the z-axis direction was made to fix the initial and

final acquisition points. It was necessary to do this for every slide due to the variations in the slide glass and tissue thickness.

Capture of the z-stack was performed in 200 nm steps at 40 x magnification. An unstained negative control slide, as described above, was also captured, and used as a reference for subtraction of background noise to give a cleaner image. Once this was done the z-stack was then deconvolved using the Volocity software. The resulting images were then false-coloured.

In relation to its function in telomere maintenance, as well as its posttranslational modification and involvement in mitochondrial metabolism, TIN2 can have both a nuclear and cytoplasmic localisation (528). The ratio of nuclear fluorescence to cytosolic fluorescence was used as a measure of nucleocytoplasmic distribution to ascertain whether TIN2 was more concentrated in the nucleus or cytoplasm in response to maternal dietary restriction.

TIN2 localisation was determined using ImageJ software with the Intensity Ratio Nuclei Cytoplasm Tool (RRID:SCR_018573). Four representative images of each animal (n=3 for each dietary group) were analysed. Two input channels, one for cytoplasm and the other for nuclei, were assessed. First, an 8-bit image type was created from the image containing the cytoplasmic channel (Alexa Fluor® 594) for analysis. The image was corrected using the background correction function,

leaving zero values in blue and areas that needed to be analysed in grey. In order to segment the nuclei, the image containing the nuclear channel (DAPI) is also converted to 8-bit format. Once background correction and segmentation were complete, the intensity ratio for a single image was measured generating %nuclear and %cytoplasmic values which are corrected to give an intensity %N/%C ratio. Lower values (< 1) were indicative of higher cytoplasmic concentration of TIN2 whereas higher values (> 1) indicated showed that TIN2 was more nuclear.

3.2.11 Statistical Methods

Prior to analysis, all data were assessed for normality using the Shapiro–Wilk test. Data are represented as mean ± standard deviation. If data were parametric, a student's T-test was used to investigate for statistical differences between CON and DR groups where $P \leq 0.05$ indicated significance. Otherwise, a nonparametric Wilcoxon rank sum test was used to estimate significance differences between the two groups.

Correlations were used to determine the relationship between maternal factors on telomere lengths. If data were normal, Pearson correlation was used; Spearman correlation was used if non-normal. A linear regression model was fitted for daily gestational weight gain data in order to identify the earliest day on which daily weight gain could predict final telomere length at E18.5. The response variable in the model was TL, and the predictor was daily weight gain.

On the first day that the slope estimates significantly differed from the baseline value at E0.5, the daily weight gain value could be used to predict TL. The correlation coefficient and r^2 coefficient of determination were used to test the linear regression equation's ability to predict outcomes accurately. Statistical analysis was performed using RStudio version 2022.02.3 Build 492 (RStudio Inc., Boston, MA). Male and female fetal analyses were combined due to the small sample size and subsequent analyses showing no sex differences.

3.3 RESULTS

3.3.1 Phenotypic assessment of animals during pregnancy

3.3.1.1 Fetal measures

Statistical analysis for fetuses compared each diet group's average pup weight, placenta weight, fetal:placental ratio (FPR, a proxy for placental efficiency), and fetal blood glucose concentration. When DR fetuses were compared to CON offspring, their body weights were lower (-35%) as expected (P=0.00014; Fig 4.5A). Fetuses from dams exposed to DR also had smaller placentae (-16%) (P=0.00013; Fig 4.5B). In the calorically-challenged DR group, the mean FPR, was lower (-29%) (P=0.008; Fig 4.5C) consistent with placental inefficiency. Though it was not statistically significant, there was a trend towards higher (+42%) fetal blood glucose levels in the DR group (P=0.058). Notably, there was a sizable variation in the DR group glucose concentrations. Nested analysis did not reveal any sex effects on either body (P=0.658) or placental weight (P=0.474).

This was also the case in fetal blood glucose (P=0.3096) and FPR (P=0.853) analyses.

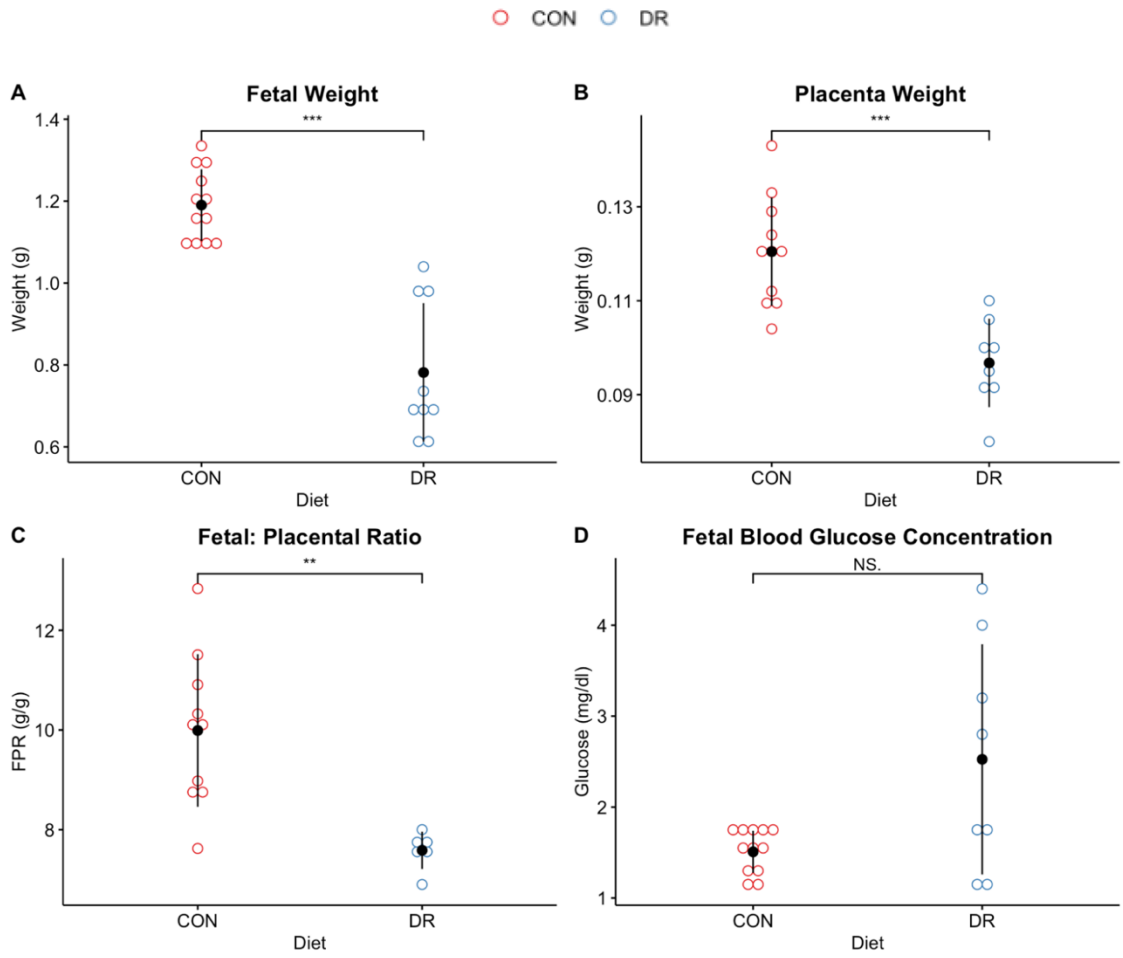

Figure 3.5. Comparison of intrauterine growth and blood glucose concentrations between CON (n=12) and DR fetuses (n=10).
(A) Fetal weight in CON and DR fetuses. (B) Placenta weight in CON and DR fetuses (C) Fetal: placenta ratio (FPR) in CON and DR fetuses (D). Fetal blood glucose in CON and DR fetuses. ** P< 0.01 ***P<0.001 NS Not significant. Data are represented as dot plots, displaying mean (represented by black central dot) and standard deviation (black lines radiating from central black dot). Males and females jointly analysed due to low n values and lack of observable sex differences.

3.3.1.2 Dam body weights

The DR dams overall were smaller, with lower body weights than CON. DR mean body weights were 8% lower (20.27±0.4g) than the CON group (22.22±0.5g) when the copulatory plug was identified at E0.5 (P = 0.014, Figure 3.6A). The DR and CON groups maintained the same weight difference at the start of the feeding regimen at E5.5. By the time pregnancy reached E18.5, the DR and CON mean weights were 27.5% higher than the DR group (P=0.001; Figure 3.6A).

After the diet was administered at E5.5, DR dams had difficulty gaining weight, experiencing significant weight loss between E6.5 and E11.5, half of the 12-day diet period, and only beginning to gain weight between E15.5 and E18.5. In contrast, in CON, E10.5 was the first day that dam daily weight gain (DWG) became significant. At the time of culling at E18.5, the DR DWG was only 4.81±0.2g (P=0.001; Figure 3.6B), which was 65% less than that of the CON mice. While the daily kilocalories consumed retained the 30% reduction from E5.5, no difference in total calories consumed or total calories per gram of body weight was observed (Figure 3.6 C,D).

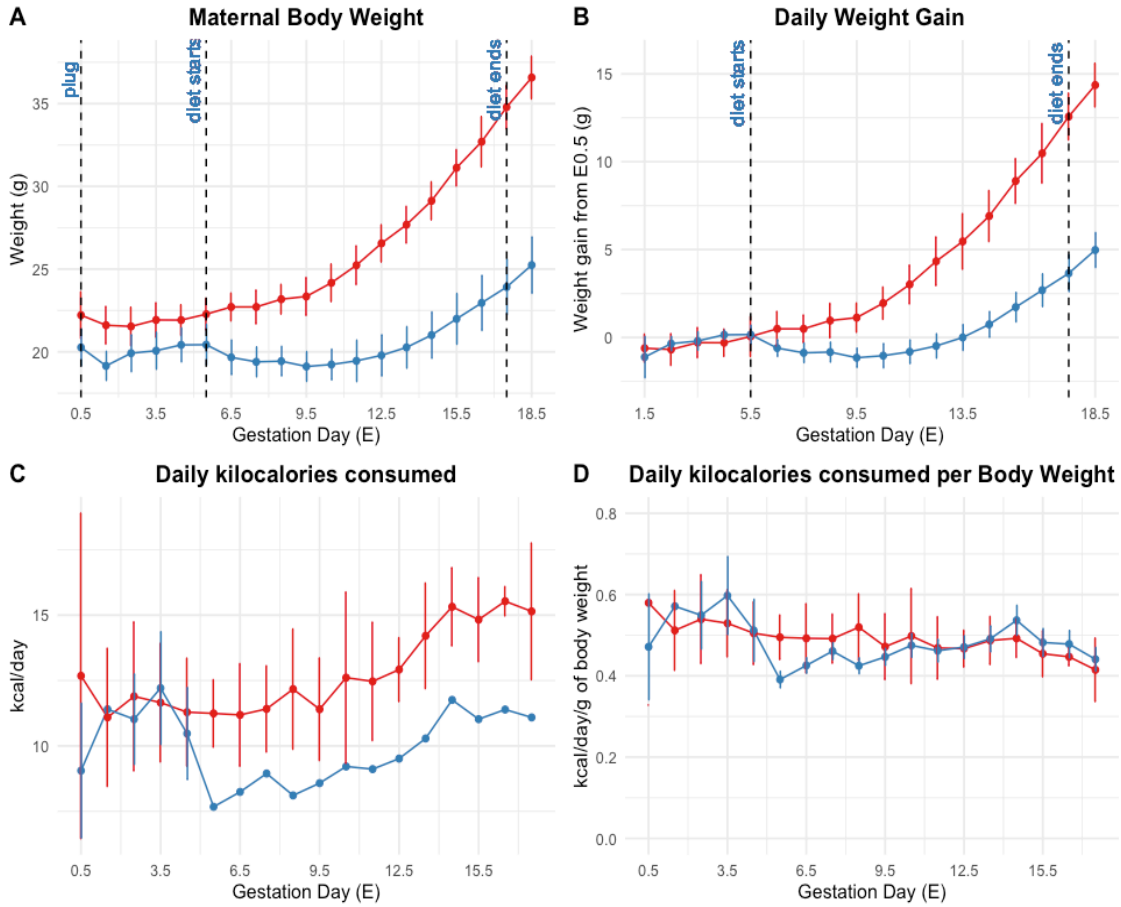

Figure 3.6 Maternal body weight gain during pregnancy in CON (n=7) compared to DR (n=7).
(A) Body weight gain. (B) Daily body weight gain relative to E0.5. (C) Daily kilocalories consumed. (D) Daily kilocalories consumed per gram of animal weight. Results are presented as means ± standard deviation. DWG became significant between dietary groups from E8.5. Red =CON (n=7). Blue = DR (n=7). Significance is at $P \leq 0.05$.

3.3.2 Telomere Length characterisation

3.3.2.1 Maternal telomere assessment

In dams, dietary restriction had the greatest effect on telomeres in whole-blood cells. Mean telomere lengths in DR dams were 62% shorter than in CON, a difference of 168 kilobases/diploid genome (P=0.0099; Figure 3.7A). Bone marrow TL was also significantly shorter in DR (P<0.05; Figure 3.7B), a source of haematopoietic stem cells for blood cell replenishment (568). Diet, on the other hand, had no significant effect on TL in the maternal gut (P=0.712; Figure 3.7C)

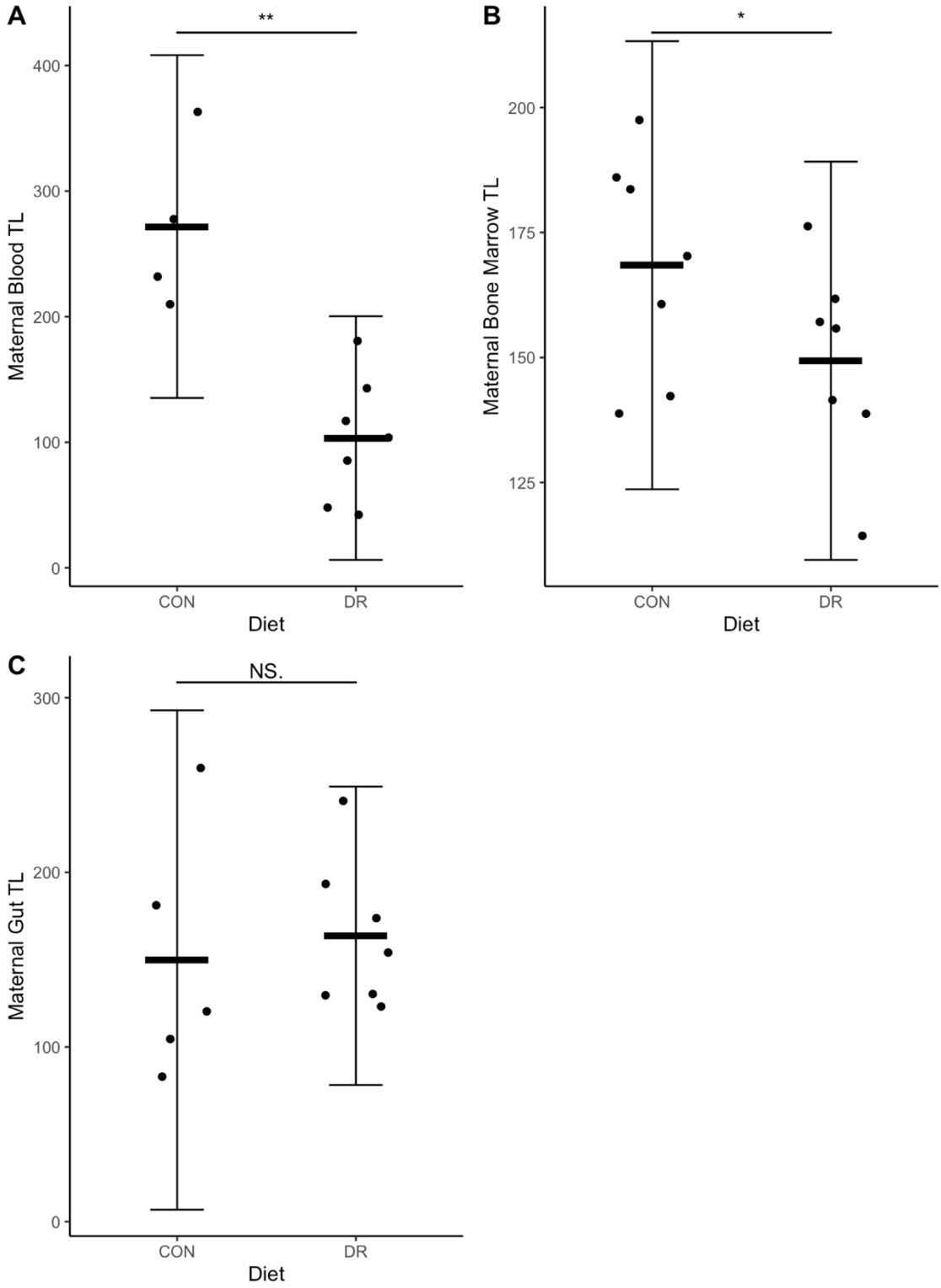

Figure 3.7. Comparison of dam TL between CON (n=7) and DR (n=7) groups. Maternal blood (A), Bone Marrow (B), and Gut (C) absolute telomere lengths (kb/diploid genome). * P≤ 0.05, ** P<0.01, NS = not significant

3.3.2.2 Fetal telomere assessment

Telomeres were shorter in DR livers than CON livers in offspring (P=0.043, Figure 3.8A). There were no significant effects of DR on telomeres in the gut (P=0.334, Figure 3.8B) or kidney (P=0.272, Figure 3.8C). In contrast to the dams, telomeres in DR fetal blood cells did not shorten, but rather were 21% longer (P=0.009; Figure 3.8D). DR had no significant effect on either cord (P=0.362, Figure 3.8E) or placenta (P=0.165, Figure 3.8F) telomeres.

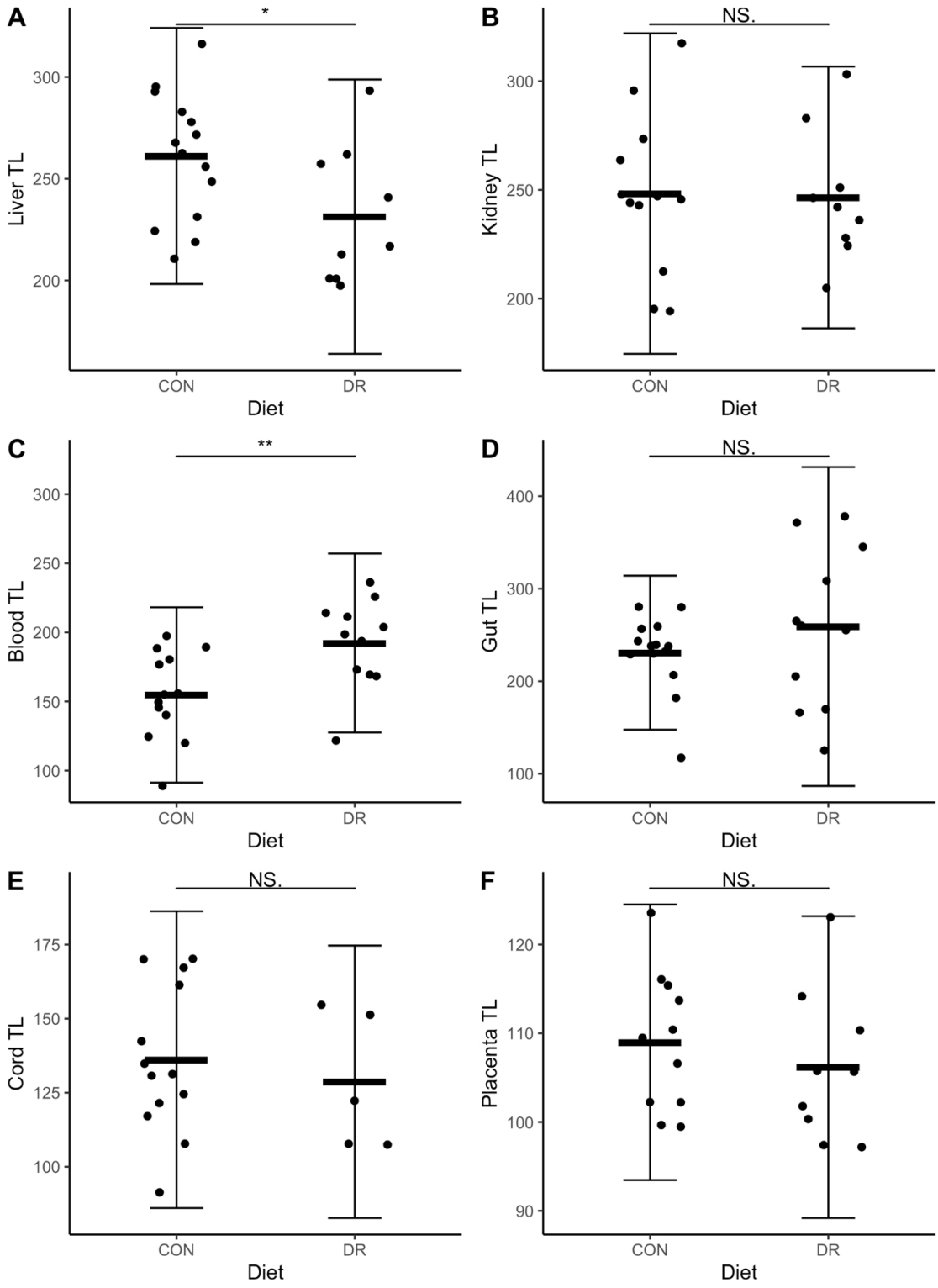

Figure 3.8. Fetal telomere lengths.
Comparison of fetal TL (kb/diploid genome) between CON (n=12) and DR (n=10) groups Liver (A), Kidney (B), Gut (C) peripheral blood (D), Cord(D), and placenta absolute telomere lengths (kb/diploid genome). * P ≤ 0.05, ** P <0.01, NS = not significant. Males and females jointly analysed due to low n values and lack of observable sex differences.

Influence of maternal factors on telomere length

Bone marrow is the primary haematopoietic organ in adult mammals (568) and a hallmark of bone marrow aging is impaired haematopoietic stem cell (HSC) DNA (569). A correlation was conducted to assess the relationship between bone marrow and blood TL. There was a positive correlation observed between bone marrow and maternal whole-blood telomere length (Figure 3.9A), suggesting that dietary restriction may be detrimental to both maternal blood and its progenitor cells.

Studies have shown that that there is a direct association between maternal weight gain and fetal birthweight (570-572). Therefore, maternal weight gain is an established predictor of fetal weight. In this study, total maternal weight gained correlated with fetal weight at E18.5 (Figure 3.9B). It was hypothesised that maternal weight gain would similarly correlate with telomere length. In dams maternal blood TL increased as the total gain in maternal body weight increased (Figure 3.9C). On the other hand, in fetuses there was an inverse association between total weight gain and fetal blood TL (Figure 3.9D).

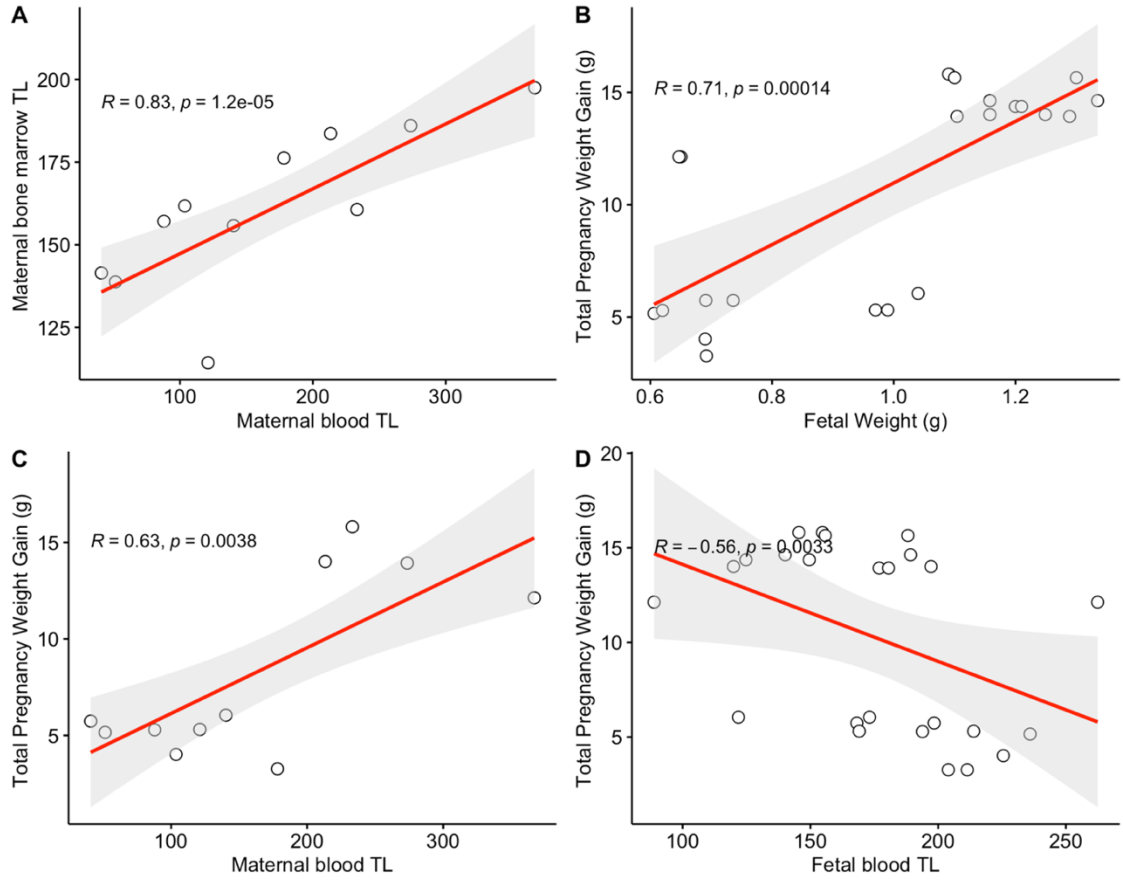

Figure 3.9. Spearman correlations of maternal and fetal outcomes.
Telomere length (TL) in maternal bone marrow compared with maternal blood (A); Maternal total weight gain compared with fetal weight (B); Maternal total weight gain compared with maternal blood TL (C); Maternal total weight gain compared with fetal blood TL (D). *R* represents Spearman's Rho, with p-value indicating significance where (P<0.05). For fetuses CON n=12 and DR n=10 with males and females combined, whereas in dams n=7 for both CON and DR.

In light of these data, it was then hypothesised that the trajectory of telomere length at E18.5 might be predicted by daily weight gained by dams (DWG). Linear regression was used to analyse maternal and fetal blood TL against DWG. The earliest day DWG could predict maternal blood TL was E13.5 (P=0.044). The equation garnered from this linear regression was: $124.30 + 21.97x$ (where x = DWG at day 13.5). DWG could predict fetal blood TL from E10.5 (P=0.0452) generating the equation: $176.89 - 8.45x$ (where x = DWG at day 10.5).

Correlation coefficients for predicted and actual TL were used to verify linear equation accuracy. The linear correlation coefficients for maternal blood and fetal blood were 0.615 and 0.377, respectively, indicating a positive relationship. For maternal blood TL, the r^2 coefficient of determination was 0.378, and for fetal blood TL it was 0.142.

3.3.3 Analysis of telomere/telomerase-related genes and cellular proliferation

The telomerase enzyme, which consists of the TERT and TR core subunits, adds repeat sequences to telomeres, stabilising TL and preventing attrition (405,406). In order to evaluate telomerase activity in offspring liver tissue, a telomerase repeat amplification protocol (TRAP) assay was attempted. The TRAP assay, however, was unable to be optimised and, as a result, direct measurement of enzymatic activity was not possible. However, increased mouse Tert (*mTert*) expression has been linked to telomerase activity in mouse embryonic stems cells prior to differentiation, and *c-myc* is known to play a role in TERT promotion (573). Furthermore, the mouse liver is known to exhibit significant hepatocyte proliferation prior to birth at E18.5, which lasts until postnatal day 9, in order to increase liver size under normal physiological conditions. (273).

It was hypothesised that, given telomere shortening in DR livers, dietary restriction would repress *mTert* expression (related to the role of telomerase in lengthening of telomeres) as well as *c-myc* expression, and decrease the ratio of proliferative cells in the liver by E18.5. To evaluate whether there was an association between expression of *mTert* and *c-myc* expression and whether it corresponded to reduced proliferation, *mTert* and *c-myc* gene expression was assessed in conjunction with Ki67 staining (a proliferation marker). DR increased *mTert* mRNA levels (P=0.01; Figure 3.10A) but there was no effect of DR on *c-myc* (P=0.855; Figure 3.10B). Upon analysing Ki67 in CON and DR livers (Figure 3.10 C,D) There was also no significant change in proliferation as determined by Ki67 staining (P=0.837; Figure 3.10E).

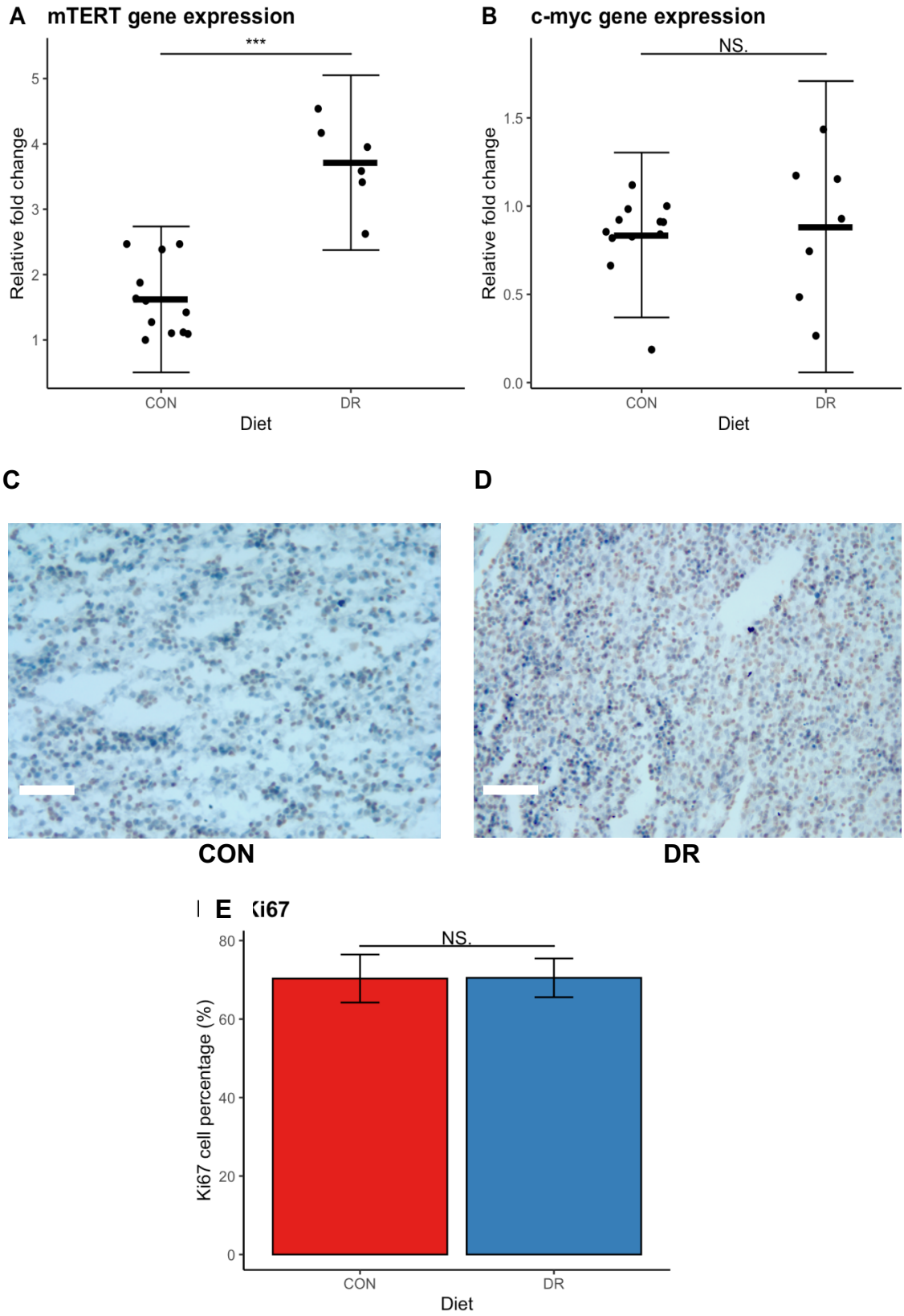

Figure 3.10. *mTert* and *c-myc* gene expression and Ki67 analysis in fetal livers. Comparison of CON (n=14) and DR (n=10) *mTert* mRNA (A) and *c-myc* mRNA levels (B); (C), (D) Ki67 staining of CON (n=8) and DR (n=8) sections; (E) Percentage of Ki67-positive cells. Males and females were analysed jointly.

3.3.4 Mitochondrial DNA and associated genes

The development of metabolic disease has been linked to perturbed liver development (239). Aging-related processes and senescence are not only associated with telomere length, but also cellular mitochondrial alterations. Because telomere and mitochondrial function overlap, as do their roles in age-related pathologies, mitochondrial copy number, a proxy for mitochondrial function, was also assessed in cells whose telomeres were most adversely affected by DR, namely cells in maternal blood and fetal hepatic cells. Mitochondria are a major source of reactive oxygen species as primary producers of superoxide ($O2^{\bullet -}$), one of the leading causes of oxidative damage in cells associated with various aging related conditions (453,574). In addition to damaging cellular structures, elevated ROS can damage mtDNA (472) resulting in significant mitochondrial genome deletions (473,474). It was hypothesised that maternal dietary restriction would cause a loss of mitochondrial DNA in maternal blood and fetal liver in response to stress from undernutrition.

Two mtDNA genes, mt-ND1 and mt-CytB were the focus of the assessment of mtDNA copies, as seen in Figure 3.10. When mt-ND1 was analysed in dam blood, the DR group had a higher copy number (P=0.035; Figure 3.11A). Furthermore, maternal mt-CytB demonstrated an identical response in the DR group (P=0.03; Fig. 3.11). However, there was no discernible difference between the CON and DR fetal hepatic mtDNA copy numbers as neither mt-ND1 (P=0.156; Fig. 3.11C) nor mt-CytB (P=0.122; Fig. 3.11B) were significantly different.

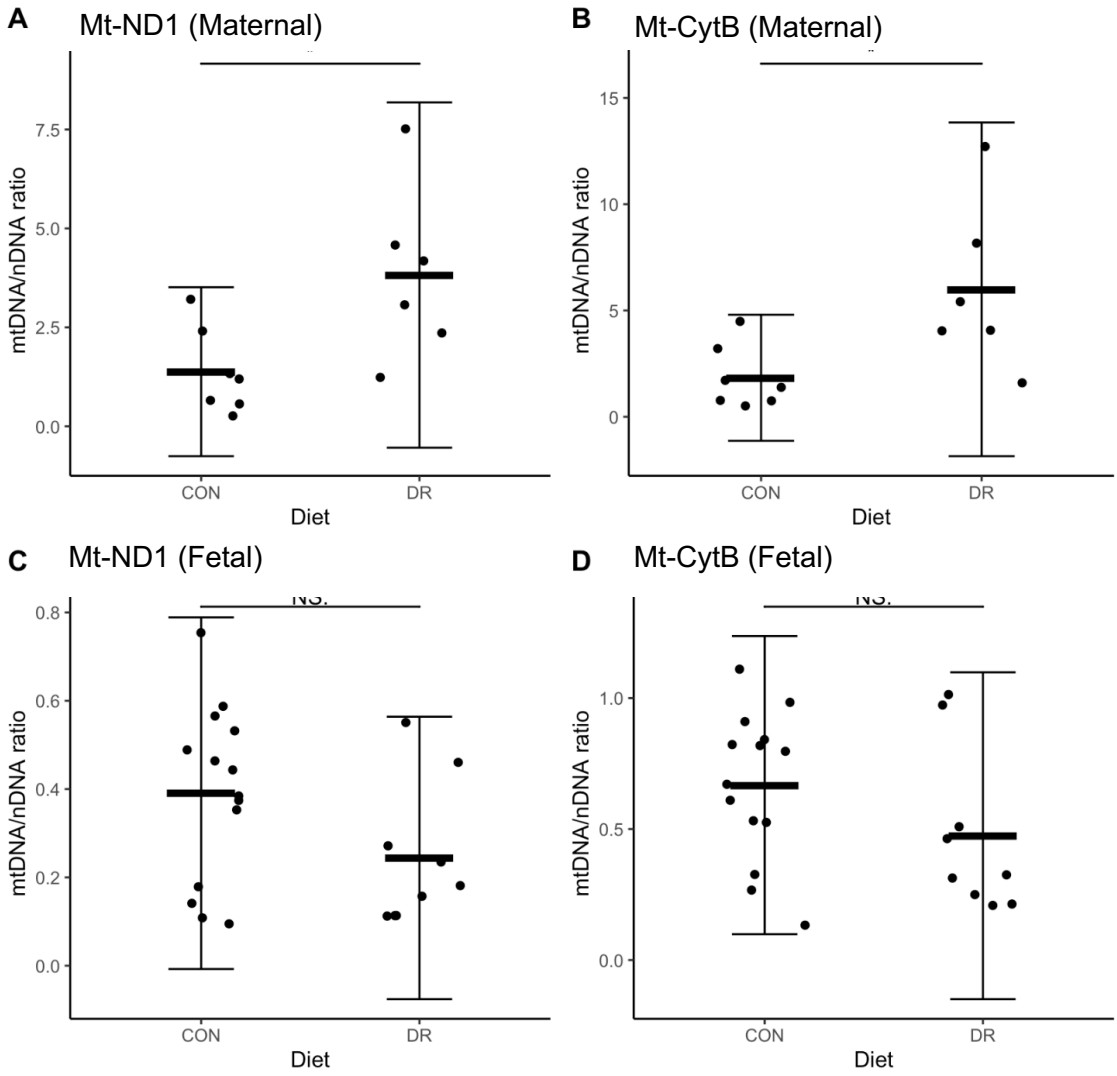

Figure 3.11. Maternal and Fetal mitochondrial DNA copy numbers
A comparison of maternal mtDNA copy numbers is shown in (A) mt-ND1 and (B) mt-CytB. Fetal hepatic mtDNA was assessed, with mt-ND1 in (C) and mt-CytB in (D). * P ≤ 0.05. For dams, n= 7 for CON and DR. For fetuses n = 14 (males and females combined) for CON, n=10 (males and females combined) for DR.

3.3.5 Analysis of shelterin protein TIN2

The shortening of telomeres and dysfunctional mitochondria are two key mechanisms related to genome instability. An earlier study by Chen and colleagues (528) illuminated a role for the shelterin protein TIN2, in the regulation of mitochondrial function and thus metabolism. TIN2 has a significant role in telomere stabilisation, acting as a linchpin molecule for the stabilisation of shelterin complex molecules TRF1, TRF2, and TPP1(373,374). However, it has also been demonstrated that TIN2 colocalises with mitochondria and inhibits ATP synthesis and oxygen consumption, resulting in hypoxic conditions and subsequently ROS (528). On the other hand, TIN2 silencing enhanced mitochondrial ATP synthesis and glucose metabolism while reducing ROS levels (528). Given that TIN2 has been shown to play dual roles in metabolism and telomere maintenance, it is therefore relevant to examine the effects of prenatal undernutrition on this protein.

It was hypothesised that DR fetuses, which had shorter hepatic telomeres, would also have increased cytoplasmic TIN2 in response to intrauterine stress from undernutrition. To see if there was a difference in TIN2 localisation in the cytosol under the stress brought on by the DR diet, immunofluorescence staining of TIN2 was performed (Figure 3.12). The intensity of nuclear and cytoplasmic localisation was compared (%N/%C ratio). There was a trend towards significance ($P = 0.06$; Figure 3.13).

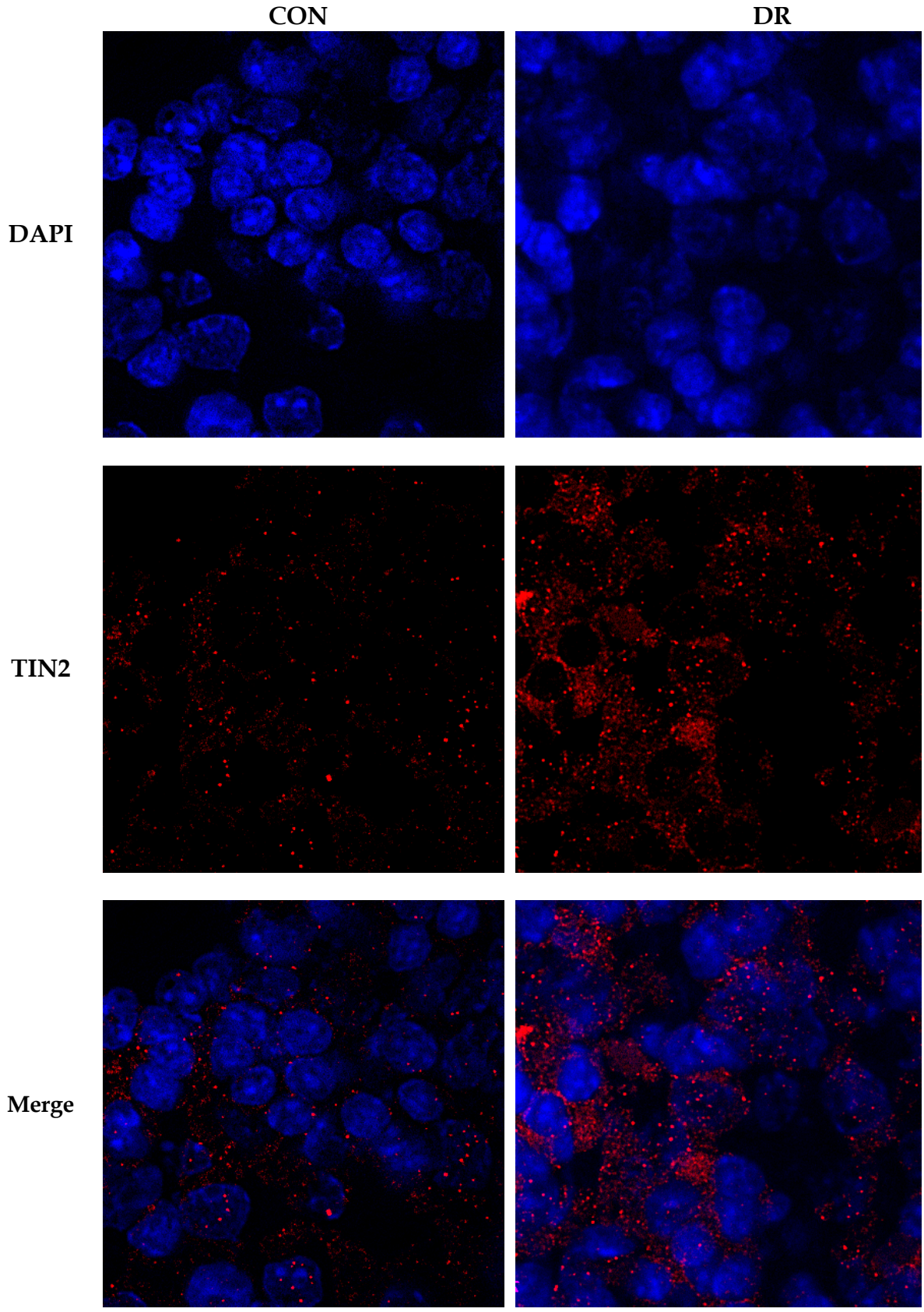

Figure 3.12 Immunofluorescence images showing fetal liver stained for Tin2 . n=3) and DR (n=3). Blue = DAPI, Red = TIN2.

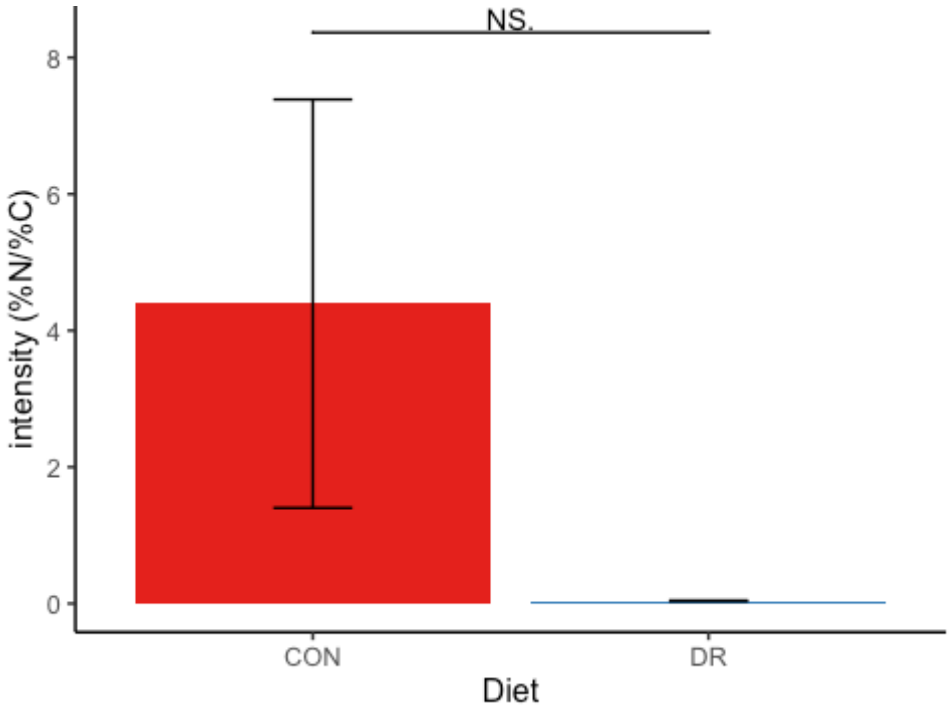

Figure 3.13. Quantification of nuclear/cytoplasmic ratio of TIN2 fluorescence intensity in CON and DR livers.
CON n = 3, DR n = 3. NS = Not significant

3.3.6 Hepatic oxidative stress in DR Fetuses

A body of research has linked increased TERT to increased oxidative stress. TERT can be upregulated and nucleocytoplasmic transport occur to bind with mitochondria and protect mtDNA from ROS-induced damage (575,576). Despite the fact that $O2^{\bullet -}$ can be converted to secondary ROS such as hydrogen peroxide, it can still damage macromolecules such as lipids and DNA (450). However, damaged or dysfunctional mitochondria can produce large amounts of superoxide, which can contribute to oxidative stress. To confirm the presence of hepatic oxidative stress, three methods were used: dihydroethidium (DHE) staining for DNA, hydroperoxide analysis for lipids, and carbonyl analysis for oxidation of proteins.

The DHE analysis (Figure 3.14A,B) was intended to serve as a proxy for superoxide oxidation of nuclear DNA in the fetal liver. This analysis was based on Nin and colleagues' methodology (563), which generated data showing a significant difference in DHE staining between groups (P=0.035; Figure 3.14C). However, it has since been established that the approach is inaccurate and inappropriate for determining DNA oxidative damage in fixed cells. This will be reviewed in greater depth in the Discussion section. The livers of DR mice had similar levels of lipid peroxidation (P=0.993; Figure 3.14D) and protein carbonylation (P=0.842; Figure 3.14E) to those of the CON group, indicating that these levels are consistent with physiologically normal levels.

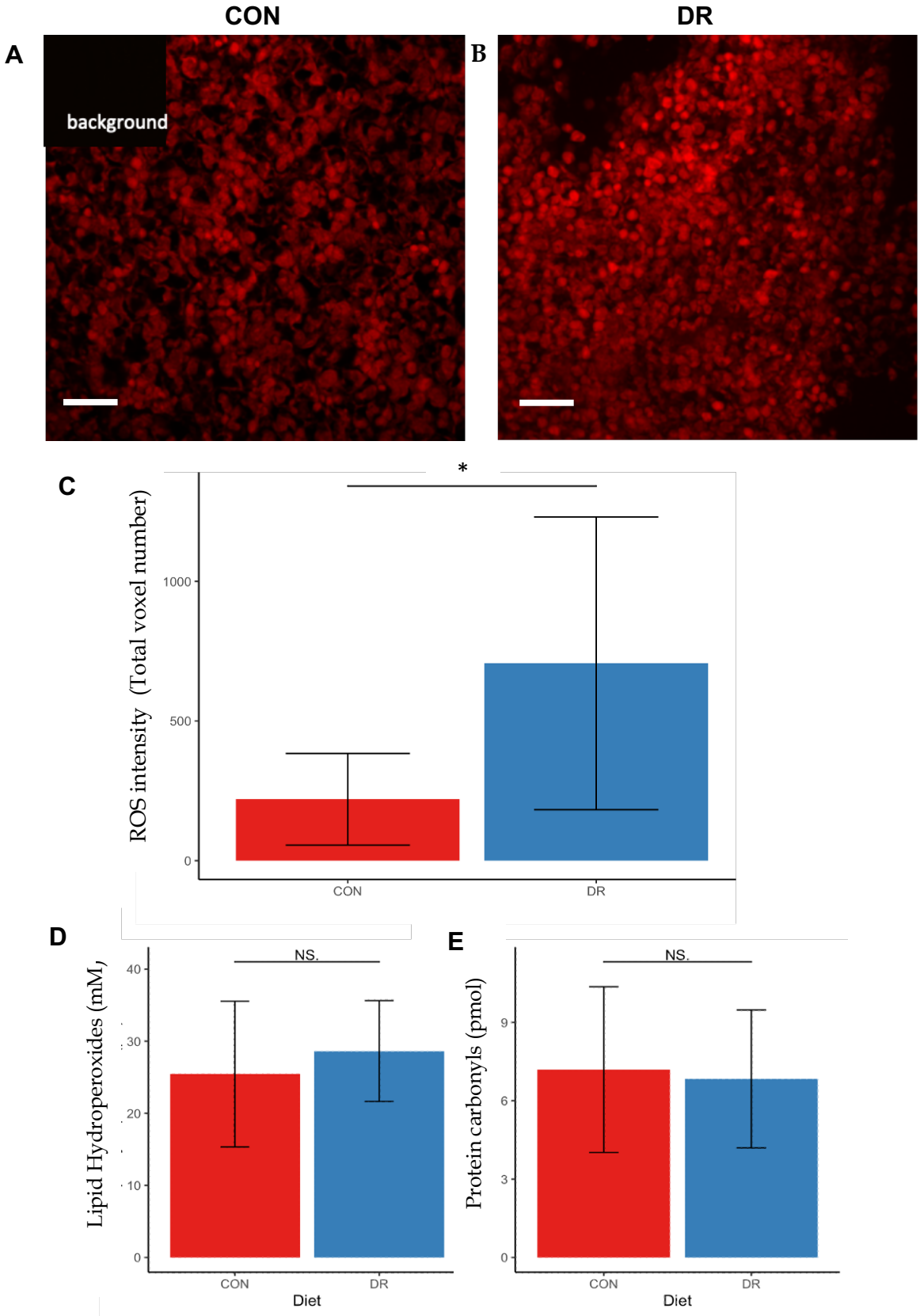

Figure 3.14. ROS detection in mouse fetal hepatocytes.
Dihydroethidium (DHE) staining of (A) Control (n=8); (B) DR (n=8) (C) Comparison of superoxide detection using DHE between dietary groups in mouse fetal hepatocytes. (D) Lipid hydroperoxide detection (E) Protein Carbonylation. CON (n=14), DR (n=10) * P ≤ 0.05. NS = Not significant. Males and females were analysed jointly.

3.4 DISCUSSION

The hypothesis for this study was that undernutrition would have a negative impact on telomeres, and that this result could illuminate a mechanism through which cellular stress contributes to metabolic dysfunction programming. The principal findings of this study concur with this. As indicated by the literature and outcomes of this investigation, dietary restriction disrupts the normal course of development. There are effects on mother and offspring alike. Early childhood nutritional insufficiency, as previously discussed, can have a considerable negative impact on organ development, impairing growth and leading to disease. The susceptibility of telomeres to poor nutrition has been described (577), though minimally examined in fetuses. It was observed that prenatal global dietary restriction selectively shortened telomeres in maternal blood cells and in the fetal liver. DR dams also had increased mitochondrial copy number with no effect observed on mtDNA in the fetal liver.

The initial objective of the study was to characterise TLs in dams and pups to see if there were any direct effects of poor nutrition that could be observed during fetal development on telomeres and whether they were tissue-specific. A restricted diet, I hypothesised, would shorten the dams' telomeres. This study showed that caloric restriction could decrease telomere length in maternal whole-blood cells and bone marrow but not gut, suggesting tissue-specific TL heterogeneity as indicated in previous studies (388-390).

Although they were only on the diet for 12 days, the mean TLs of dam whole-blood cells in DR were drastically reduced. The DR dams lost up to 6% of their body weight from baseline between E6.5 and E11.5, half of the total time spent on the diet, before starting to gain weight. It is well-established that imprinted genes expressed by the placenta are responsible for the allocation of resources for fetal growth (323,324). Imprinted pro mother and pro fetus genes exert selective pressures on maternal resources to balance the nutritional needs of the fetus against those of the mother in order to prevent maternal deterioration (326). As shown in sheep (578) and guinea pigs (579), an imbalance in imprinted genes caused by poor diets can trigger a response that favours fetal growth. As a result, nutritional resources for maternal upkeep may be strained.

The activation of a starvation response that affects the cells in maternal blood could be one explanation for such a significant reduction in TL(580). The challenges that DR dams experienced in gaining weight suggests difficulty attaining a positive energy balance, as a result of prolonged reduction in energy consumed against usage. Previous research has suggested that starvation can reduce mouse peripheral blood cellularity in a process that involves the stress hormones of the HPA axis (580). Subsequent work has shown increased cortisol/corticosterone levels in relation to telomere shortening (507-509), which may account for the drastic TL shortening. Telomere length can also vary depending on the cell types examined in blood (581-584). Given that mouse blood not only contains a mixture of white but also nucleated red blood cells (585), the TL measurements may not be indicative of a specific cell type.

The telomere shortening observed in bone marrow compliments TL in the blood and is expected. The correlation between bone marrow and blood TLs suggests that dietary restriction may be detrimental to both maternal blood and its progenitor cells. The cellularity of mouse bone marrow is higher than that of other species, including humans (586). However, the frequency of murine nucleated HSCs is only 0.01% (568). While the abundance of cells in mouse bone marrow normally does not decrease with age, clinical research has linked shorter telomeres to a reduced HSC reservoir (584). It is possible that the stress imposed by DR may have exhausted the HSC population during gestation which may explain this outcome. However, this cannot be confirmed without quantifying HSC populations at different time points throughout pregnancy, such as E0.5 (copulatory plug), E5.5, or tracking DR mice through the initial weight loss from E6.5-E11.5 and the beginnings of weight gain towards E18.5. This needs to be taken into account.

In clinical studies telomere shortening has been linked to aging and degenerative diseases in peripheral blood mononuclear cells (PBMCs) (587). PBMCs were not isolated in this experiment. However, DNA from the total pool of nucleated cells in whole blood was used. Due to sample volume constraints, purifying PBMCs from the rest of the sample proved to be a challenge. These volume restrictions also made it difficult to conduct further mRNA analyses of maternal blood. Attempts to extract mRNA from the remaining whole-blood sample yielded RNA that was not suitable for analysis.

Unlike in the dams, in fetal whole blood TL was longer. It is important to note that fetal haematopoiesis occurs in various locations; initially the yolk sac and liver in mid gestation, and the bone marrow in late gestation (588). This mixed pool of erythroid cells may have introduced variations in TL, with some cells possessing longer telomeres than others. Since the assay used cannot distinguish between TL in individual cells, it would require further examination to determine the populations of cells whose telomeres are best preserved despite dietary restriction.

In addition to having shorter hepatic telomeres, TIN2 in the livers of DR offspring trended towards an increase in cytoplasmic localisation. Further research is necessary to determine how this likely change in TIN2 localisation affects function in fetal livers. While it is involved in the telomerase recruitment process, TIN2 alone is not sufficient for control of telomere length and requires TPP1 (428). Thus, it might be necessary to also examine function of TPP1 in relation to telomerase activity. Investigating changes to TIN2 processivity in mitochondria would also be required. As Chen and colleagues demonstrated nucleocytoplasmic localisation and alteration in metabolism in response to changes in TIN2 expression (528), further studies would need to include measures of mitochondrial ATP synthesis. Despite the fact that mitochondrial copy number can change and, in this case, served as a proxy for mitochondrial function, a more thorough investigation of the respiratory complex function and dysregulation in conjunction with TIN2 may more clearly demonstrate the effects

of cytosolic TIN2, if any. Given that it has been demonstrated to be a maladaptive response to enhance metabolic function in cells in age-related pathologies (589), it is possible that the inclusion of analyses of mitochondrial mass would also lend some strength to the study.

No significant change in placental TL was observed in this study. Data from normal pregnancies suggest that while the placenta does indeed age towards term, the mechanisms of senescence are telomere-independent (590). Significant telomere shortening has been reported in placentae of intrauterine growth-restricted (IUGR) pregnancies (591,592). However, it is worth noting that these instances of placental telomere shortening in IUGR pregnancies have largely been reported in humans. The fact that TL in mouse placentae in this study remains unchanged may also highlight the differences in telomere and telomerase dynamics between humans and mice. The absence of telomere shortening in the fetal umbilical cords and placentae may be attributed to generally higher levels of telomerase retained by proliferating cells (593).

There was also no difference in kidney or gut TL. The kidney is one of the body's fastest "ageing" organs (594) and has been linked to the development of hypertension later in life (595). The finding that fetal kidney telomeres were not different between the DR and CON models contrasts with protein-restricted models of undernutrition, which show shorter kidney telomeres in rats after postnatal catch-up growth (521,522). TL was not investigated postnatally in this

study. In line with the DOHaD hypothesis regarding timing, the lack of change in fetal kidney TL may indicate that a "second hit" postnatal event is required before a kidney response can be detected.

Similarly, this may be the case in fetal gut TLs. There has been limited research into gut telomeres in mice. Fairly recent work by Chakravarti and colleagues (596) has demonstrated that shorter mouse telomeres can induce gut inflammation via a mechanism involving transcription factor YAP1. Pregnancy induces normal physiological responses that contribute to shorter telomeres such as increased oxidative stress and inflammation (597). Additional stresses may likely shift the physiological balance in a direction that exacerbates telomere damage. It is still not clear whether telomere length is the initiator or the target of oxidative stress and inflammation. Furthermore, the inflammatory response targets may be tissue and organ specific, demonstrating an organ-sparing effect (598), preserving the gut epithelia at the expense of the liver in this case.

Amongst all fetal tissues assayed, telomere shortening was only observed in the DR livers, despite increased *mTert* mRNA levels. Telomerase activity in mouse embryonic stem cells has been linked to increased *mTert* gene expression (573). While this is true, telomerase activity cannot be inferred directly from gene expression because both *mTert* and the RNA template *mTR* must be present (417) and interact within the enzyme structure (427). The fact that DR fetuses had shorter hepatic TL and higher *mTert* levels than CON fetuses may suggest that *mTert* assayed is not working in tandem with telomere elongation mechanisms.

The ability to correlate *mTert* mRNA data with a direct telomerase measure, such as a TRAP assay, would have clarified these analyses. In the absence of data from a TRAP assay or other telomerase activity assays, elucidating the function of increased *mTert* becomes challenging.

In humans, TERT is regulated by alternative splicing of the gene, which can transcribe more than 20 isoforms associated with cellular proliferation but not enzyme activity (599). The *mTert* gene alternative splicing process differs from that of the human TERT gene and can result in isoforms that are either telomerase inactive or associated with low telomerase activity (600,601), with some thought to be involved in tissue-specific telomere lengthening (602). Changes in *mTert* mRNA levels may be related to the generation of different isoforms, which may play roles that are independent of telomere maintenance. This cannot be confirmed in this study, given that the *mTert* PCR primers used do not completely cover the region where alternative splicing occurs. An alternative primer design might begin to shed some light on *mTert* function.

The lack of change in Ki67 and *mTert* regulator *c-myc* mRNA levels, may suggest that *mTert* contributes to proliferation maintenance occurs via alternative pathways such as the canonical Wnt/β-catenin pathway (603). Proliferation maintenance may not involve direct regulation by *c-myc* as has been shown prior (604). However, this requires further exploration to also investigate changes at the protein level. The observed telomere shortening in the absence of inhibition of hepatic cellular proliferation is consistent with the literature, which has

reported that telomere shortening can occur independently of effects on cell division (605,606). One possibility for the elevated *mTert* may be linked to oxidative stress challenges.

Given the known association between increased rates of telomere shortening and elevated ROS (605), I originally attempted to assess the levels of hepatic oxidative stress using DHE staining. While the results appeared to show that the level of nuclear ROS in hepatocytes was higher in DR fetuses than CON, the use of DHE fluorescence to assess oxidative stress in fixed cells was subsequently shown to be flawed. The methodology was based on peer-reviewed work published by Nin and colleagues (563) who demonstrated the use of DHE on formalin-fixed paraffin-embedded sections of lung tissue. Several more recent studies have also used this technique on paraffin-embedded sections (607-611). These studies mention the use of DHE in the detection of oxidative stress.

In the literature, the use of DHE (also called hydroethidine) as a probe is well documented for the detection of superoxide, and its use in numerous *in vitro* and *in vivo* studies is well-reviewed (612,613). However, the important distinction between those studies and this one is the viability of the cells or organs which allows for active DHE uptake and hence detection of superoxide activity *in situ*. The use of DHE as a measure of superoxide oxidation of DNA in this study and aforementioned studies in paraffin-embedded tissues relies on several

assumptions. Firstly, the product 2-hydroxyethidium is only formed due to a superoxide-hydroethidine reaction. Secondly, 2-hydroxyethidium will remain at the locus of it is formation (612), likely the nucleus. Thirdly, 2-hydroxyethidium is preserved throughout the fixation process.

From my examination of the literature these assumptions appear to be inaccurate when applied to this work. Firstly, hydroethidine conversion to 2-hydroxyethidium, the fluorescent product analysed in DHE staining, is not solely produced by superoxide, but can also be made by reacting with other molecules such as with Nox proteins (614). It is not confirmed in this study, or the others listed, whether Nox proteins are upregulated in the treatment groups; this is a limitation of the study. Secondly, products of hydroethidine oxidation can translocate and not only be found in the nucleus (615), but also mitochondria in the cytosol (616). The staining I have observed is concentrated in the nuclei which may not be a true reflection of 2-hydroxyethidium presence. Lastly, the presence of superoxide ion is short-lived (scavenged by superoxide dismutase), and occurs in aqueous solution (617). During the process of tissue preparation for paraffin-embedding, superoxide may have been degraded. Furthermore, the dehydration of tissue during fixation would likely remove traces of superoxide and products of oxidation from the tissue.

The use of DHE as a ROS-specific probe was rightly challenged in a previous draft of this thesis. While superoxide-related damage to DNA may indeed be apparent, it has not been confirmed in this study. This analysis may be remedied through the use of 8-Oxo-2'-deoxyguanosine (8-Oxo-dG) staining, a widely accepted measure of oxidative damage to DNA. Lipid peroxidation and protein carbonylation did not change in the fetal DR groups. It is, however, possible that the more extreme effects of oxidation on those macromolecules were present in the dams, not fetuses, given the already highly inflammatory state of pregnancy.

This study also found a link between gestational weight gain and absolute telomere length in response to undernourishment. Gestational weight gain is the sum of several factors, including maternal body composition, and placenta weights and is a positive pregnancy indicator in mice (618,619). From the data in this study, maternal DR presents significant challenges to pregnancy, as evidenced by lower DWG and the accompanying growth-restricted fetus and placental inefficiency. Interestingly, a relationship between maternal weight and TL is only present in maternal and fetal blood.

In dams, the amounts of weight gained between E0.5 and E18.5 correlated with greater blood cell TL; the inverse was observed in fetal blood. There is a paucity of comparable findings in animal studies across the literature. However, Maugeri *et al.* (620) recently reported an association between higher gestational weight

gain and longer telomeres in humans, in maternal leukocytes and amniotic fluid TL. They did not establish mechanisms or implicate diet as a factor.

Body weight is a crude measure for the assessment of pregnancy wellbeing. So, this study also evaluated its effectiveness in forecasting TL at E18.5 by using the DWG as a determinate of eventual TL. E10.5 for fetal and E13.5 for maternal TL were the earliest days DWG was a significant predictor. The relatively small r^2 value may indicate that the DWG is not the only factor exerting effects on telomeres. The addition of potential confounders to the model may result in stronger associations and improved predictive power. Dam levels of stress could have been assessed as extraneous variables in these analyses by measuring blood cortisol, and including ROS, and inflammation. However, further experiments would be required. If the gestational daily weight gain observed in this case can be extrapolated to a clinical setting, maternal TL measurements may be a useful clinical indicator of fetal well-being.

There was an unexpected increase in mtDNA content in maternal circulating blood cells. While unconfirmed in the analyses, oxidative stress may be a likely mechanism for triggering an increase in mtDNA synthesis to compensate for damaged mitochondria as well as meet metabolic requirements for the removal of excess ROS (621,622).Once again this provides another clinically relevant use

for this model for a more in-depth examination of the effects of undernutrition on maternal mitochondria in pregnancy using an easily accessible tissue.

Dietary restriction had not measurable effect on fetal hepatic mtDNA in this study. Given the absence of significant ROS oxidation of protein and lipids, this may suggest that there is protective effect on mitochondria which does not trigger the biogenesis response of cellular distress. Mitochondrial function can be maintained and mitochondrial replication can continue in a limited capacity even if mtDNA copy numbers are low. (623). As previously reported by Larsson *et al*. (624), Tfam protein, which was not examined in the study, may be preferentially upregulated to initiate the process of increasing mtDNA independently of mtDNA transcription and mitochondrial respiratory chain function (624).Similarly, these findings could point to the emergence of other compensatory mechanisms involved in mitochondrial renewal and mass restoration that are not dependent on the PGC-NRF-Tfam pathway. More studies, however, are required to fully comprehend them.

In conclusion, this study has demonstrated that poor intrauterine nutrition prematurely ages fetal and maternal cells. This is evidenced by shorter maternal and fetal telomeres and also with differential effects on mitochondrial number, suggesting that maternal and fetal compartments are affected differently by dietary restriction.

Importantly, both telomeres and mitochondria in maternal blood are directly impacted by dietary restriction, providing a clinically relevant model for a more exhaustive study of undernutrition effects on maternal telomeres and mitochondria in pregnancy. And specifically, being able to predict the trajectory of telomere length during a specific timeframe during pregnancy may prove useful to a clinical setting for assessing fetal outcomes. This model further provides evidence of interaction between gene and environment in programming phenotypes.

The limitations of the study must be taken into account when interpreting the results of this work. Within the framework of the Developmental Origins of Health and Development hypothesis, timing, dose, and duration are factors that have varied impacts on outcomes in the maternal and fetal milieu. This study does not examine the exact timing that dietary restriction may have generated these effects in telomeres.

Dietary restriction was implemented between E5.5 and E17.5. While this confines the study to the desired fetal development period, it is possible that the findings would have been enhanced if the effects of early and late gestation nutritional deprivation had been studied separately. Manipulation of the time of restricted food exposure may aid in identifying critical periods that would be ripe for intervention so early in development. However, this was not feasible due to cost considerations.

Given that the telomeres of the liver are affected at E18.5, it would be ideal to narrow down the window of vulnerability in order to establish when telomeres are first impacted.The data from linear regression analysis is only predictive and not causative. According to Dutch and Chinese famine research (46,625) disease outcomes in progeny can change depending on when they were exposed to intrauterine nutritional deprivation. In the Dutch study, famine was linked to higher levels of glucose intolerance, particularly in the middle and late stages of pregnancy (46) but obesogenic BMI was highest when exposure happened early in the pregnancy. Furthermore, in both famines, early gestation exposure was linked to poor liver function later in life (46,625).

Several questions arise from these observations: Is it more likely that telomere lengths are altered by periconceptional conditions and start from E5.5 through post-partum, or is this only a mid to late-gestation effect? Further, is there a period of recovery for telomeres in the other fetal tissues? Conversely, in this instance, one could also suggest that there is a “low power-low responsibility" situation. As a result of the modest size of the animal cohort, statistical power to detect these effects across treatment groups in diverse organs may have been insufficient.

The mouse model itself is also in question. Telomere shortening is not a significant cause of senescence in mouse cells due to significantly longer

telomeres and constitutively expressed telomerase (397). This enzyme is expressed in a broader range of tissues and maintained at higher levels throughout life (389,432), and thus does not serve the same role in mice as it does in humans (626). Therefore, any physiological alterations observed in this study may not directly apply clinically. Further compounding this is the fact that this work focuses on fetuses prior to disease onset. These results must be interpreted with caution since many of the physiological effects associated with disease pathogenesis are more commonly seen in adult animals. The persistence of the observed outcomes of dietary restriction effects into adulthood is uncertain from these findings. The critical developmental windows may either produce long-term phenotypic and genotypic outcomes, or they may imbue the sensitive periods with changeable and reversible effects manifested by epigenetic modifications (578).Following up on this work by tracking the maturation of the fetal liver alongside telomeres from birth to maturity could more thoroughly reveal mechanistic effects during developmental windows as well as changes in telomere signalling and postnatal life.

CHAPTER 4

EFFECTS OF OVERNUTRITION ON TELOMERES AND MITOCHONDRIA

4.1 INTRODUCTION

The preceding chapter established that maternal global-calorie deficiency, persisting throughout gestation, resulted in distinct effects on telomeres and mitochondria, accompanied by significant outcomes for fetal and maternal weights. While the exact mechanisms that lead to metabolic disease still require clarification, earlier work has made a strong case for the role of undernutrition in programming of metabolic disease (9,11,13). Previously, malnutrition in the form of protein restriction has been shown to shorten telomeres and impair mitochondrial function in offspring pancreatic islets, suggesting a mechanistic link between inadequate early-life nutrition and the development of metabolic disease (519,520). This finding is in agreement with work in the previous chapter, showing a similar response, but exclusive to the fetal liver. The work presented in this chapter aims to examine the effects of nutritional excess, in the form of a maternal HF diet, throughout pregnancy on telomeres. Further, this study also examines whether, similar to the undernutrition model in the previous chapter, there may also be a corresponding effect on mitochondria that is also detrimental to telomeres.

Several studies of diet-induced obesity using HF feeding have demonstrated that offspring exposure from gestation until weaning results in metabolic disease

phenotypes in offspring, including hyperlipidaemia, hypertension and insulin resistance (128-132). Moreover, excessive consumption of nutrients, such as fat, increase risk for cardiovascular disease and other aging-related conditions is suggested to have equally detrimental effects on TL. However, despite attempts in various studies to investigate the effect of consuming large quantities of fat on TL, clinical evidence of this interaction is still sparse.

Epidemiological studies have been able to link high total fat consumption with shorter leukocyte telomeres in the elderly (514,627). Studies in animals have complemented this finding, showing shorter telomeres in both peripheral skin cells as well as circulating leukocytes in HF-fed rats (628,629). Indeed, feeding a high fat-high sucrose diet to telomerase-deficient mice with already drastically shortened telomeres exacerbates insulin resistance and promotes greater inflammation in adipose tissue (630). Furthermore, a HF diet affects mitochondria by reducing copy number during development, providing a potential mechanism for future mitochondrial dysfunction and, eventually, disease (132). Based on these findings, there appears to be a link between HF diets, metabolic disease and telomere length.

Notably, research to date has largely focused on the postnatal outcomes of HF diet, with very limited exploration of effects on offspring whilst still *in utero*. More specifically, the effects of maternal HF diet on offspring telomeres and mitochondria before birth has not been previously investigated. It is hypothesised that maternal overnutrition will adversely affect telomeres and

mitochondria, suggesting a mechanism by which cellular stress leads to metabolic dysfunction. It is proposed that these changes in telomeric and mitochondrial phenotype are apparent *in utero*, long before onset of metabolic disease. Further, the interaction between telomere and mitochondria highlights a possible mechanism by which stress in early life leads to accelerated cellular aging/dysfunction and the development of adverse metabolic phenotypes in later life.

4.2 METHODS

4.2.1 Animals

4.2.1.1 Ethics, Animal care and treatment

As described in Chapter 3, all animal procedures were performed at the Lunenfeld-Tanenbaum Research Institute at the Toronto Centre for Phenogenomics (TCP) animal facility, in Toronto, Canada with requisite approvals.

4.2.1.2 Pregnancy

Nulliparous C57Bl/6J female mice were randomised into two groups, control (CON) (n=7) and high fat (HF) (n=8), and fed two separate diets, which were not isocaloric. The One-gram *Dustless* Precision *Pellets*®, (diet # S0173; Bio-Serv, Frenchtown, New Jersey) were used to feed the CON group *ad libitum,* as described in Chapter 3. The same CON group used in the previous DR study were also used here. Conversely, the HF group consumed Bio-Serv diet #F3282, a HF diet containing 60% of calories from fat (lard), 27% sucrose and 13% from

protein. Compared to the Dustless pellets described, the HF diet contained 20.5% protein, 36% fat, 35.7% carbohydrate, with the remainder comprised of 3.5% ash with a less than 10% moisture content. Upon arrival, 5-week-old virgin females were placed on the HF diet and consumed diet until mating at 10 weeks. The HF diet was then administered *ad libitum* in the same manner as the CON group with 10 grams maintained on a daily basis throughout gestation (Figure 4.1). All feeding conditions in the CON group are as previously described in section 3.2, with 2 pups (1 male, 1 female) taken from each dam.

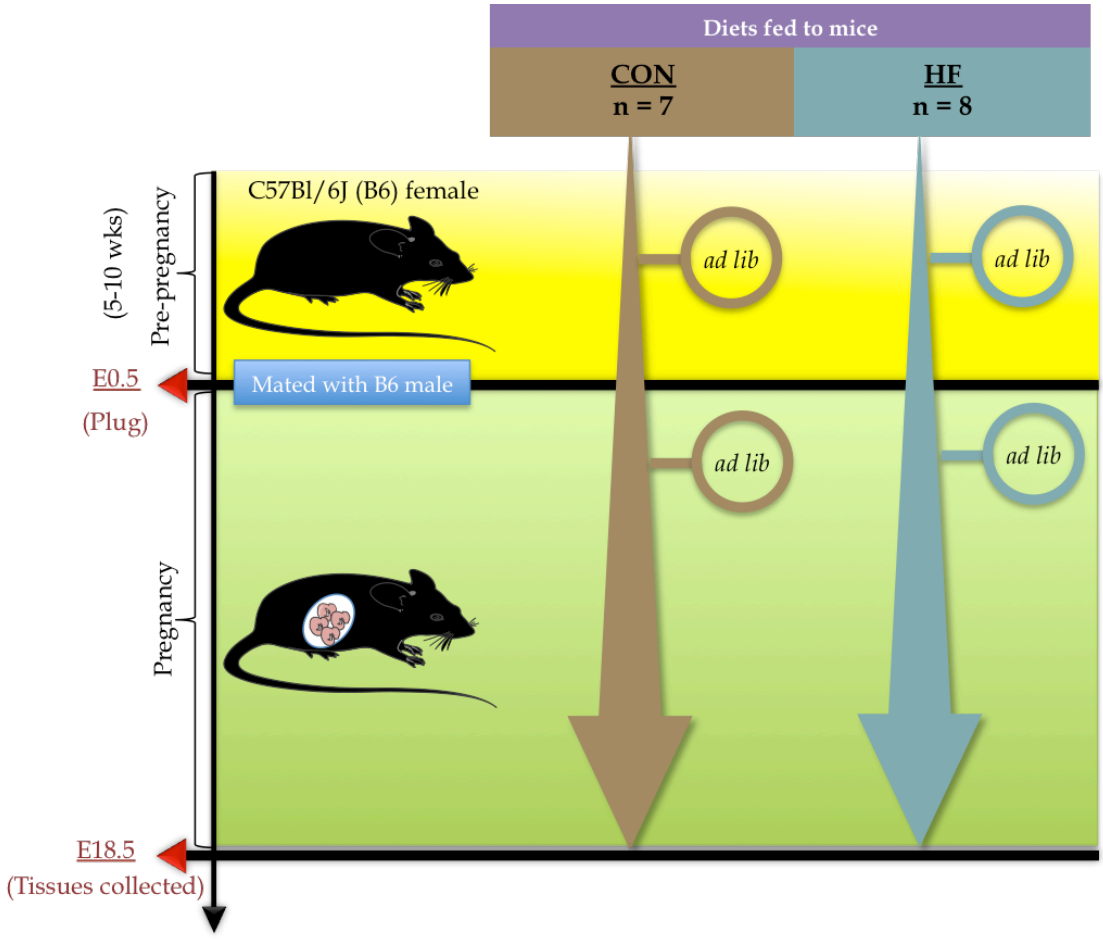

Figure 4.1 Experimental design of the HF diet study.
Ad lib represents the *ad libitum* feeding in both experimental groups.

4.2.1.3 Tissue collection, molecular and cell biology

All procedures for collection as well molecular biology and cell staining protocols are the same as described in Chapter 3 (Refer to section 3.2 for detailed methods), with the exception of the methods described from Section 4.2.3 onwards. In addition to analysis of *mTert* and *c-myc* mRNA levels as described in Chapter 3, qPCR was used to examine the gene expression of the senescence marker p21 as well as that of the hepatic fatty acid metabolism genes carnitine palmitoyltransferase 1a (Cpt1a)(631) and fatty acid transport protein 2. (FATP2 or Slc27a2). FATP2 is a fatty acid transporter that is highly expressed in the liver, and is involved in the uptake of long chain fatty acids to the liver which can lead to steatosis (632). The primer sequences are listed in Table 4.1.

Table 4.1 PCR primers for mRNA quantification

`	Sequence (5′ → 3′)	Amplicon Size (bp)	Reference
Cpt1a	Fwd 5′- CCAACACGTCAAGGACAGCA-3′ Rev 5′-CCCGTCATGGTAGAGCCAGA-3′	79	NM_013495.2
FATP2	Fwd 5′-GAAGTCGCTGACATCGTGGG-3 Rev 5′-GCCATCCCAATTCGACCCTC-3	95	NM_011978.2
p21	Fwd 5′-CGCTTCTCACCTCGCTTGTC-3′ Rev 5′-GTGACCAAGAACCTGCGACC-3′	78	NM_007669.4

4.2.2 Western Blot Analysis

A total of 20 mg of frozen fetal liver tissue from each of the dietary groups (CON, HF) was weighed. The tissue was then disrupted using the Qiagen Tissuelyser II (Qiagen Canada, Qiagen Inc., Mississauga, Ontario, Canada) in 250 µl of chilled protein lysis buffer, containing 80mM Tris-HCl (pH 6.8), 2% (v/v) sodium

dodecyl sulphate (SDS), 10% (v/v) glycerol and water, supplemented with 100 μM sodium orthovanadate (Sigma-Aldrich, Oakville, Ontario) and protease inhibitor cocktail tablets (Roche, Mannheim, Germany). The homogenate was then placed on ice, transferred to fresh 2 ml microcentrifuge tubes and sonicated three times for 10-second bursts to further disrupt the tissue. Between each round of sonication, samples incubated on ice for 20 s. The sonicated samples were then centrifuged at 12000 rpm for 30 min at 4°C and the supernatant transferred to fresh tubes. Storage of extracted protein solution was at -80°C for later use.

The concentration of protein in lysates was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), using a colorimetric 96-well microtiter plate assay with slight modifications. An 8-point standard curve was made using concentrated bovine serum albumin (BSA) standards ranging from 0-10 mg/ml (0, 0.5, 1, 2, 4, 6, 8, and 10). In duplicate, 3 μl of each BSA standard was added to the first two columns of a 96-well plate. Duplicates of 3 μl of each of the extracted protein samples were added to the remaining wells. A mixture of BCA Reagent A and Reagent B was created (50:1, Reagent A: B) to make the working reagent. Each well received 200 μl of working reagent. The plate was then covered and placed on a shaker for 30 s, and then incubated at 37 °C for 30 min. Once the plate was cooled, absorbance readings were taken at 562 nm using a BioTek uQuant spectrophotometer for microplates (Biotek Instruments; Winooski, VT, USA) and KC Junior software version 1.31.2 (Biotek

Instruments; Winooski, VT, USA). Protein concentrations were calculated in Microsoft Excel.

75 µg of each protein sample was suspended in NuPAGE 4X LDS sample buffer (Invitrogen Canada Inc., Burlington, ON, Canada) containing 10% v/v β-mercaptoethanol (Sigma-Aldrich, Oakville, Ontario) and boiled at 95°C for 10 min. The boiled protein was then resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 100V for 2 h using a 12% gel. The protein on the gel was then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Canada, Mississauga, Ontario, Canada) using an 8-minute-high molecular weight protocol on the Trans-Blot Turbo Transfer System (Bio-Rad Canada, Mississauga, Ontario, Canada) with Trans-Blot Turbo Midi Transfer Packs (Bio-Rad Canada, Mississauga, Ontario, Canada).

The PVDF membranes were rinsed for 10 min with 1 x Tris-buffered saline with Tween 20 (1 x TBS-T) on a shaker, and incubated for 1 h at room temperature in blocking solution consisting of 5% (w/v) skim milk powder in TBS-T. The membranes were then probed with FATP2 (SLc27A2) antibody (rabbit polyclonal; 1:500, Thermo Scientific Pierce, Rockford, Illinois, USA) overnight at 4°C in the dark, with gentle shaking. PVDF membranes were then washed in 1 x TBS-T (three changes, 10 min each), with gentle shaking, and incubated with biotinylated affinity purified goat anti-rabbit IgG (1:2000; Vector, Burlingame,

CA) for 1 h at room temperature. The protein loaded on the membrane was normalised by actin as described below.

Once the membranes were imaged, they were then washed in 1 x TBS-T (three changes, 10 min each), with gentle shaking. Membranes were then blocked in 5% (w/v) skim milk powder in TBS-T for 30 min at room temperature, before addition of primary actin antibody (goat polyclonal; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. This was followed by washing in 1 x TBS-T (three changes, 10 min each) as before and the addition of a secondary donkey anti-goat IgG-HRP antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). The blots for FATP2 and actin proteins were then visualised using SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Quantitation of the chemiluminescent signals was performed using the VersaDoc 5000 MP molecular imaging system with Quantity One software version 4.6.7 (Bio-Rad Canada, Mississauga, Ontario, Canada) by densitometry. To account for differences in exposure time between membranes, a corrected intensity value for each band was used. This was obtained by taking the sum of the intensities of all bands on the blot. The intensity of each band was then divided by the obtained sum total and expressed as a percentage. This was done for both FATP2 and actin bands. Using the corrected percentage values, the data were then expressed as a ratio of FATP2 to actin relative optical density.

4.2.3 Assay for inflammatory marker Interleukin-6

Studies have shown that exposure to HF diets during gestation increases inflammation. Fetal liver interleukin-6 (IL-6) concentration was determined using enzyme-linked immunosorbent assay (ELISA). The liver lysate used for this analysis was isolated and quantified as described in Section 4.2.2. The lysate was assayed in duplicate for IL-6 levels using the eBioscience mouse IL-6 Ready-SET-Go kit (eBioscience, San Diego, CA, USA).

Each well of the 96-well ELISA plate provided in the kit was coated with 100 µl of Capture Antibody, and the plate sealed and left to incubate overnight at 4 ^{o}C. The wells were then aspirated and washed three times with 300 µl of Wash Buffer (1xPBS containing 0.05% Tween-20). The Assay Diluent provided in the kit was diluted 1:4 in deionised water and then 200 µl was added to each well and left at room temperature for 1 h to block the wells. Wells were then washed once with Wash Buffer. An 8-point standard curve was then made from 2-fold serial dilutions of the IL-6 standards provided in the kit. 100 µl of each standard on the curve was added to the first two columns of the 96 well-plate. 100 µl of the sample containing 800 µg of total protein was added to the remaining wells in duplicate. The plate was then sealed and incubated at room temperature for 2 h.

The standards and samples were then aspirated and the plate washed with Wash Buffer (300 µl, five times). 100 µl of Detection Antibody from the kit, diluted 1:250 in Assay Diluent, was then added to the plate and incubated for 1 h at room

temperature followed by aspiration and plate wash with Wash buffer (300 µl, five times). Following this, 100 µl of Avidin-HRP, also diluted 1:250 in Assay Diluent, was added and the plate sealed and incubated for 30 min at room temperature followed by a further wash as before (300 µl wash buffer, 5 times). The wells were then soaked in Wash Buffer to further wash the wells (5 times, 2 min each), before 100 µl of Substrate Solution was added to each well. The plate was left to incubate for 15 min. Thereafter, 50 µl of 2N sulphuric acid (H_2SO_4) was added to each well and used as a Stop Solution for the reaction. The plate was then read at a wavelength of 450 nm using a BioTek uQuant Microplate Spectrophotometer (Biotek instruments; Winooski, VT, USA) and KC Junior software version 1.31.2 (Biotek Instruments; Winooski, VT, USA). The obtained wavelength values were then used to determine the concentration of IL-6 in the samples in a Microsoft Excel datasheet.

4.3 RESULTS

4.3.1 Phenotypic assessment of animals

4.3.1.1 Fetal measures

At E18.5, fetal and placental weights were not affected by maternal HF-feeding. In addition, examination of FPR also did not show diet differences close to term for either group (Figure 4.2). There were no sex effects on either body (P=0.603; Figure 4.2A) or placental weight (P=0.607; Figure 4.2B). This was also the case in fetal blood glucose (P=0.279; Figure 4.2C) and FPR (P=0.833; Figure 4.2D) analyses.

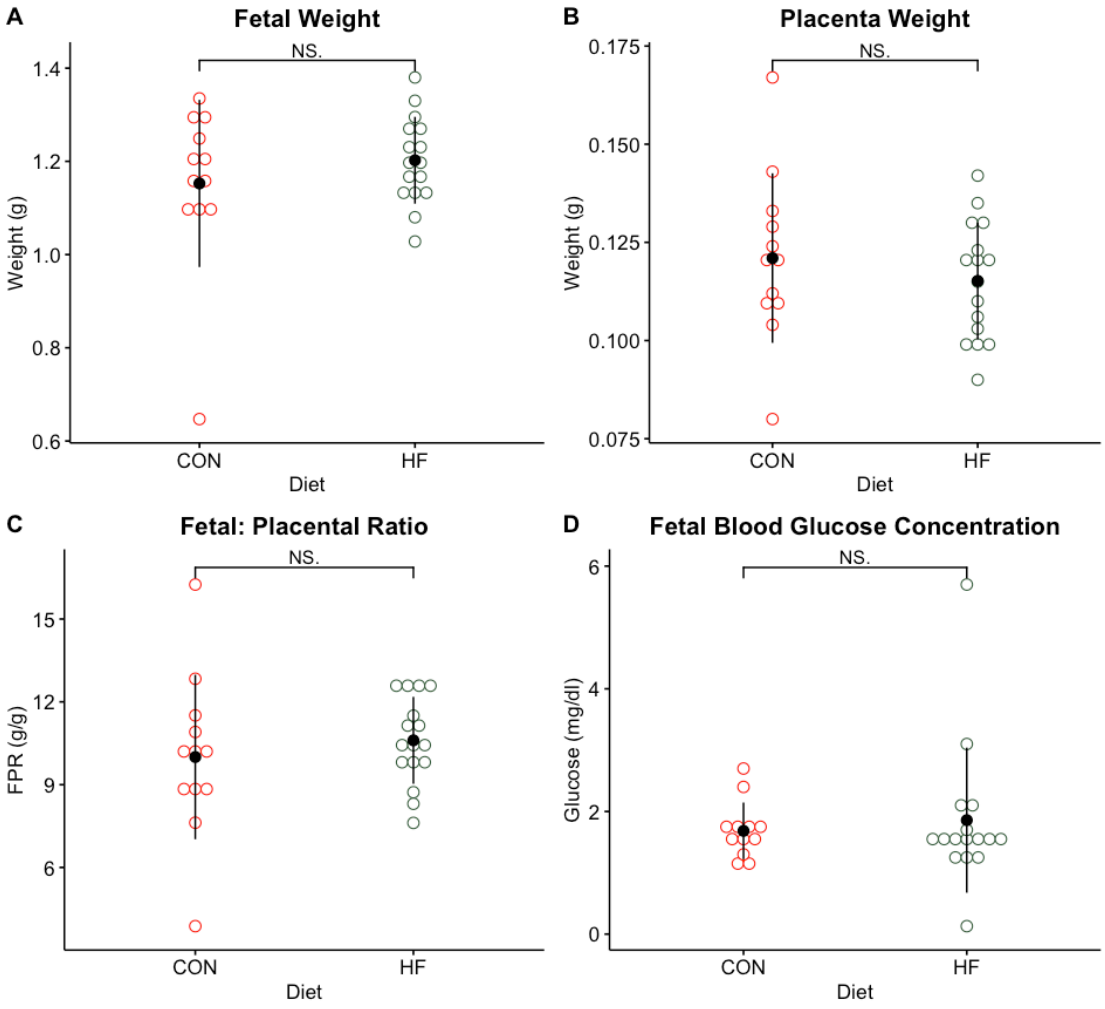

Figure 4.2 Comparisons of fetal phenotype assessments.
Comparison of intrauterine growth and blood glucose concentrations between CON (n=12) and HF (n=14) fetuses. Male and female fetal analyses were combined due to the small sample size and subsequent analyses showing no sex differences. Fetal body weight (A) and placental weight (B) with Fetus: Placenta weight ratio (C) and (D) Fetal blood glucose concentration at E18.5 where significance is at $P \leq 0.05$. Data are represented as a dot plot displaying mean (represented by black central dot) and standard deviation (black vertical lines radiating from the centre).

4.3.1.2 Dam body weights

Exposure to a 60% HF diet did not significantly affect final E18.5 body weight in dams, compared to the CON group (Figure 4.3A). While mean dam body weight values tended to be higher in the HF groups, they were not significantly different from CON and remained on par for the duration of the pregnancy (Figure 4.3A). E9.5 was the first day that weight gain for CON was significant from baseline, whereas E13.5 was the first day that body weight became significant for HF. Similarly, daily weight gain was not significantly different between the two groups (Figure 4.3B).

No difference in the groups was noted in the amount of energy consumed per day (Figure 4.3C). However, significant variation was observed with some HF dams demonstrating hyperphagia whilst other animals consumed similar amounts of energy to the CON group. Likewise, energy consumed per body weight was not different between the groups (Figure 4.3D).

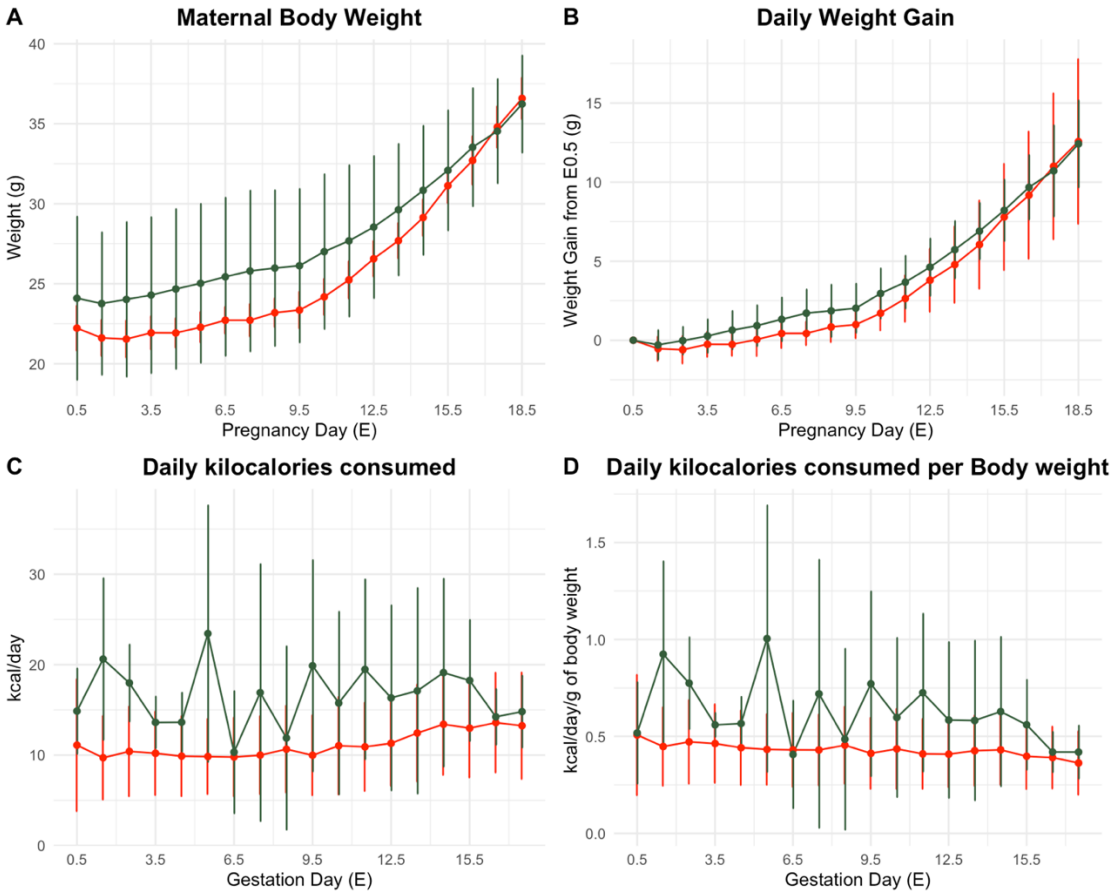

Figure 4.3 Maternal body weight gain during pregnancy.
(A) Body weight gain. (B) Daily body weight gains relative to E0.5. (C) Daily kilocalories consumed. (D) Daily kilocalories consumed per gram of animal weight. Results are presented as means ± standard deviation. DWG became significant between dietary groups from E8.5. Red =CON (n=7). Green = HF (n=8). Significance is at $P \leq 0.05$.

4.3.2 Telomere Length characterisation

As in the studies in the preceding chapter, maternal and fetal TLs were analysed between the CON and HF-fed groups by qPCR method as described in Chapter 3.

4.3.2.1 Maternal telomere assessment

It was hypothesised that a HF diet would adversely affect telomeres in dams due to excessive nutrient intake. In order to assess the impact of HF diet during gestation on maternal telomeres, TLs in maternal whole blood were examined (Figure 4.4). Maternal TL length was not affected in any of the assayed tissues, blood (P=0.2118, Figure 4.4A), bone marrow (P=0.2094, Figure 4.4B) and gut (P=0.9424, Figure 4.4C).

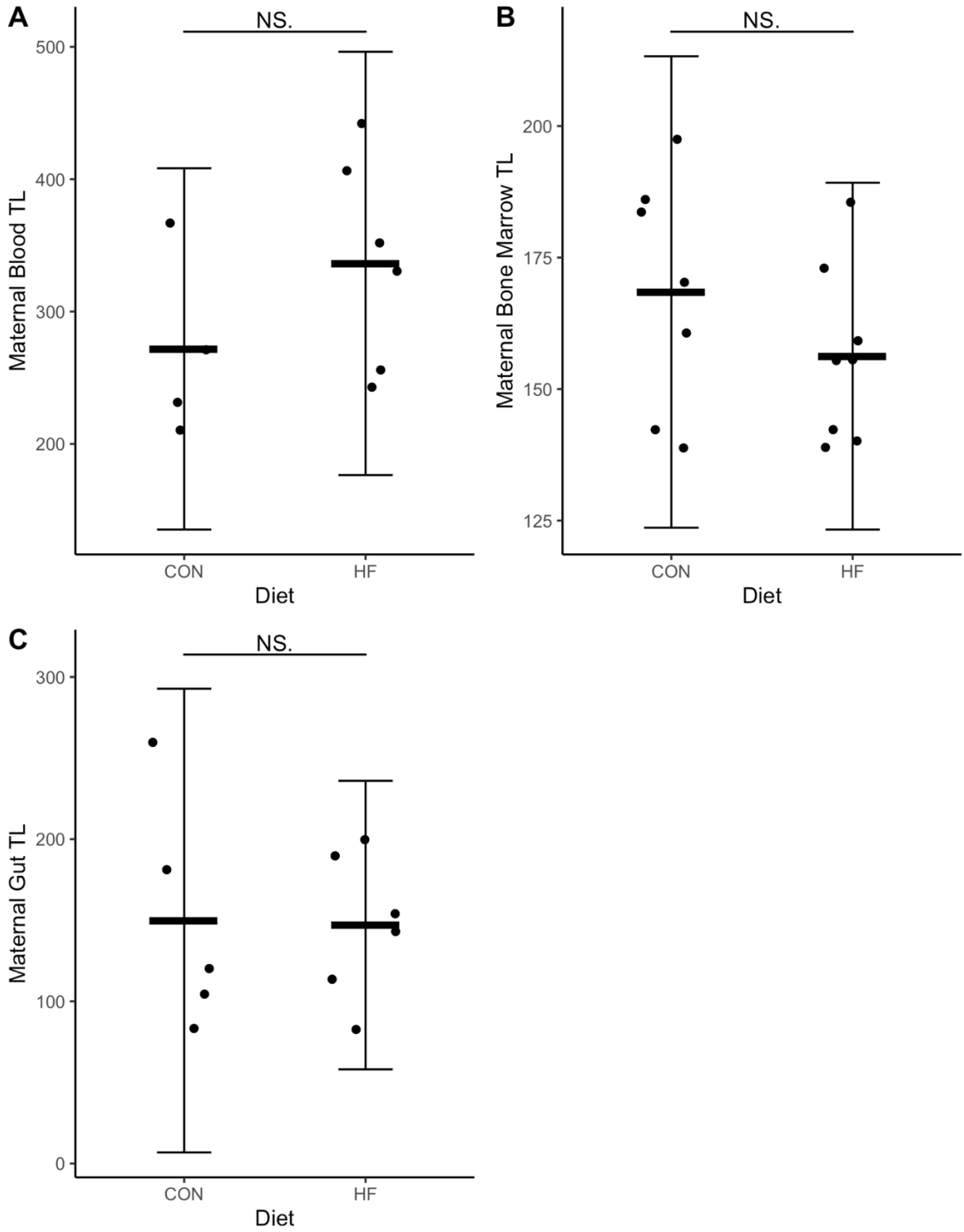

Figure 4.4 Maternal telomere lengths.
Maternal TL (absolute telomere lengths (kb/diploid genome) was not different in blood (A), bone marrow (B) and gut (C) at E18.5 CON (n=7), HF (n=8). NS = not significant where significance is at $P \leq 0.05$

4.3.2.2 Fetal telomere assessment

It was hypothesised HF feeding would shorten fetal telomeres which may contribute the development of disease in later life. Indeed, TL was shorter in livers of HF dam offspring (P=0.01, Figure 4.5A) and, surprisingly longer in the gut (P=0.024, Figure 4.5B). There was no statistically significant difference in TL in fetal kidneys (P=0.15, Figure 4.5C) or blood (P=0.802, Figure 4.5D). Furthermore, no differences were observed in the placenta (P=0.103, Figure 4.5E) or umbilical cord TLs (P=0.09, Figure 4.5F).

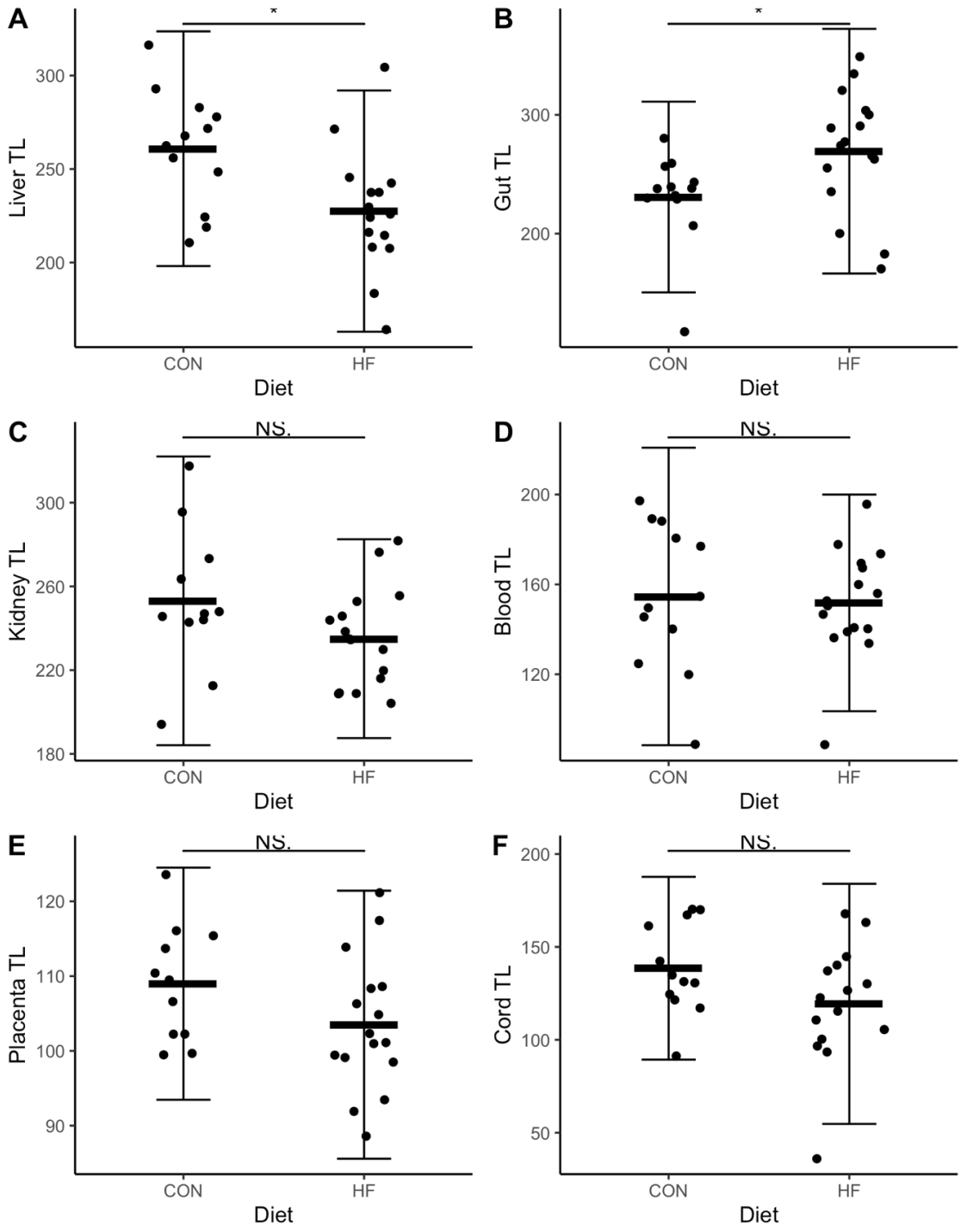

Figure 4.5 Fetal telomere lengths.
TL (absolute telomere lengths (kb/diploid genome).in liver (A), kidney (B), gut (C), blood (D), placenta (E), cord (F) at E18.5. * P≤ 0.05. NS = not significant CON (n=14), HF (n=14). Male and female fetal analyses were combined due to the small sample size and subsequent analyses showing no sex differences.

Maternal gut TL correlated with maternal blood TL and maternal body weight

A correlation was conducted to assess whether there was any relationship between the maternal TLs and maternal or fetal TLs or growth parameters. A positive correlation was observed between maternal body weight at E18.5 and maternal gut telomere length (Figure 4.6A). Maternal gut TL also strongly correlated with maternal blood TL (Figure 4.6B). In both cases the correlation was driven by HF telomere length values.

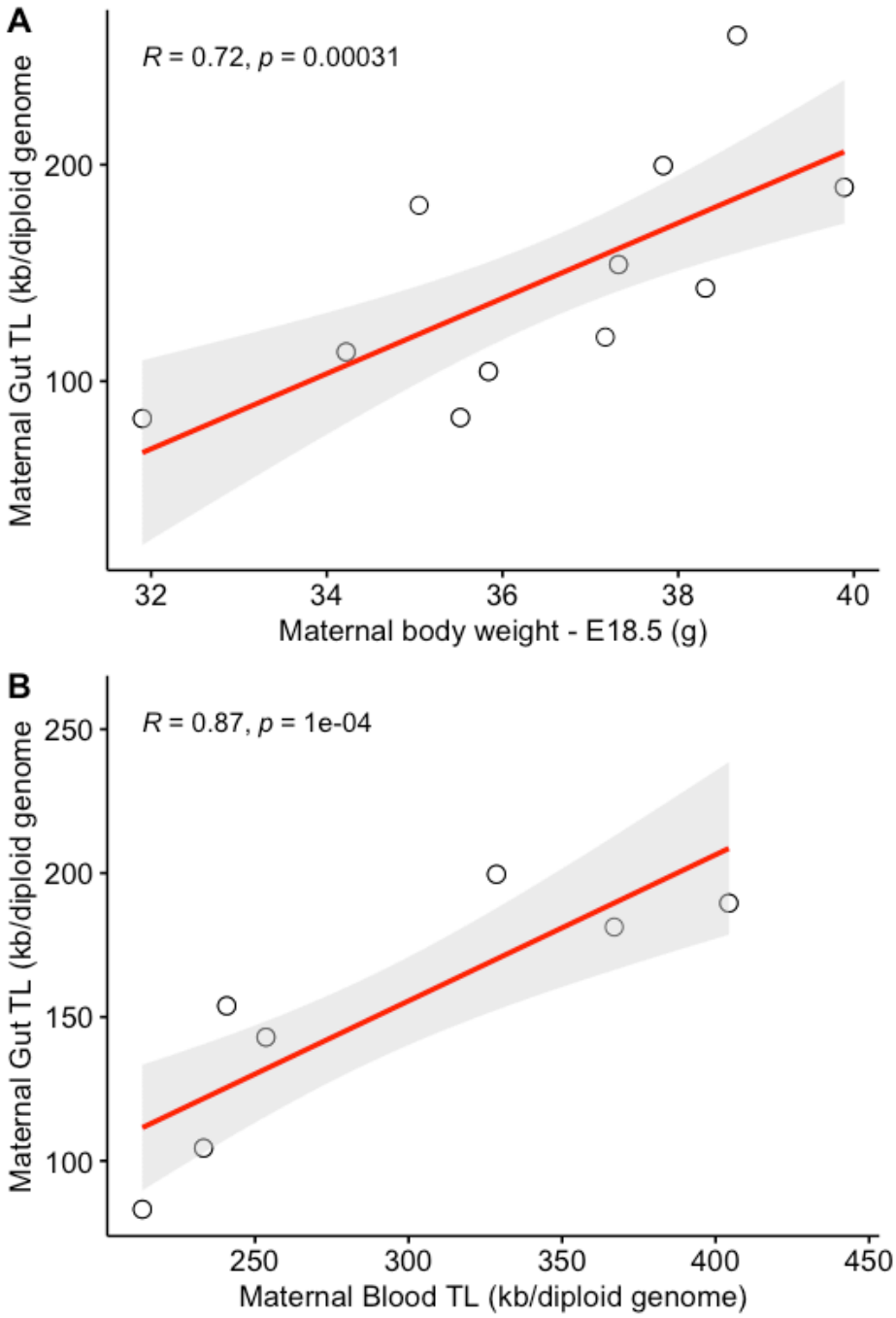

Figure 4.6 Correlations of maternal telomere lengths and maternal phenotype (A) Pearson correlation of maternal gut TL and maternal body weight at E18.5. *R* represents correlation coefficient. (B) Pearson correlation of maternal gut TL and maternal body weight at E18.5.*R* represents Spearman's Rho. P-value indicates significance where (P≤0.05). CON n=7, HF n=8 for dams

4.3.3 Effects of a maternal HF diet on telomere and senescence-related genes in the fetus

Once again, due to the fact that a TRAP assay could not be optimised, mRNA levels of the telomerase-associated gene *mTert* were analysed (Figure 4.7A). Offspring of dams that were fed the HF diet demonstrated greater hepatic expression of *mTert* (P=0.003). To assess whether there was a relationship between mTert and its known *mTert* transcription factor c-myc, gene expression of both was compared. The difference in expression of *c-myc* was not significant in the liver between dietary groups (P=0.542; Figure 4.7B). Given the elevated *mTert*, proliferation was also assessed with Ki67 staining. Ki67 maintained a high percentage of actively proliferating cells (Figure 4.7 C, D), and there was no difference in overall percentage of stained cells between CON and HF groups (Figure 4.7 E).

Senescence gene p21 has been demonstrated to suppress TERT expression and repress the mRNA levels of numerous cell cycle and mitosis genes, thereby inhibiting cell cycle progression (633,634). When present, p21 can establish or accelerate the onset of cellular senescence (633). Given the elevated *mTert* mRNA levels, and the maintenance of cellular proliferation despite shorter telomeres, it was predicted that p21, which is transcriptionally regulated (635), would have lower mRNA levels in the HF group. Interestingly, *p21* gene expression increased (P=0.043; Figure 4.7F).

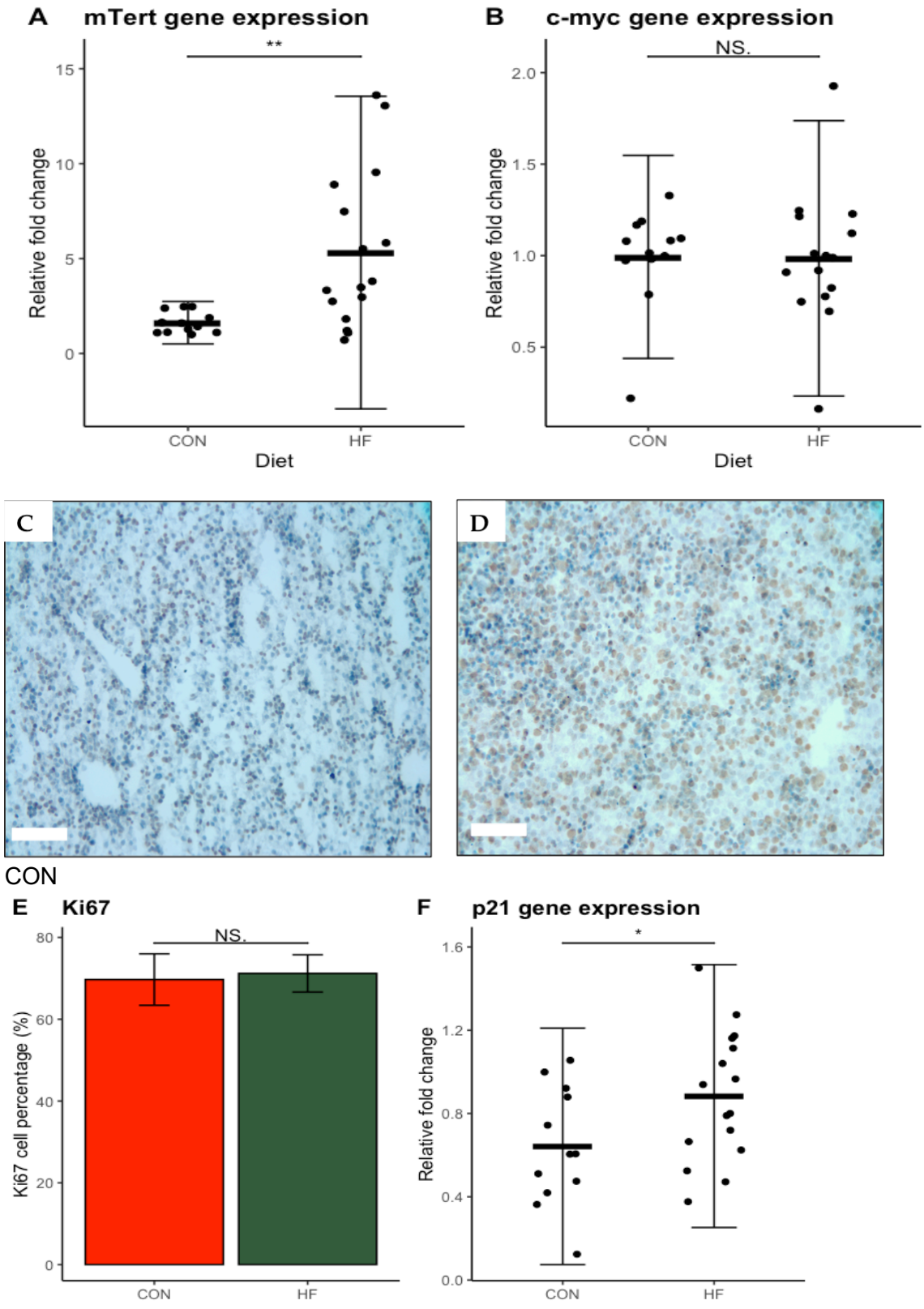

Figure 4.7 Gene expression of (A) *mTert*, (B) *c-myc*, (C), (D) Ki67 immunohistochemistry staining (E) Ki67 analysis and (F) *p21* gene expression.

* P≤0.05, ** P< 0.01, NS = not significant. CON (n=14), HF (n=16). Data are represented as an error plot with mean and standard deviation. Male and female fetal analyses were combined.

4.3.4 Analysis of fetal hepatic oxidative stress and inflammation

It was hypothesised that the high-fat diet would increase oxidative stress and inflammation in fetal livers, accompanying shorter telomeres observed. As in Chapter 3, DHE staining was used to investigate whether there was an increase in oxidative damage to DNA which may be a contributing factor to cellular physiological stress. However, as described in Chapter 3, inaccuracies in this method were apparent in its use in fixed cells. Despite providing significant evidence of nuclear oxidation by superoxide, all DHE staining results have not been included in these analyses and have been removed. In examining macromolecular oxidation, HF feeding did not increase lipid hydroperoxide formation (P=0.083, Figure 4.8A), but it did trend towards a reduction in protein carbonyls (-34%) (P=0.051; Figure 4.8B). In order to assess inflammation, IL-6 protein ELISA was carried out. According to these findings, there is no statistically significant intracellular ROS damage to lipids in the fetal liver from maternal high-fat feeding during gestation. And there is also no increase in inflammation, as measured by IL-6, in this instance (P=0.508 ; Figure 4.8C).

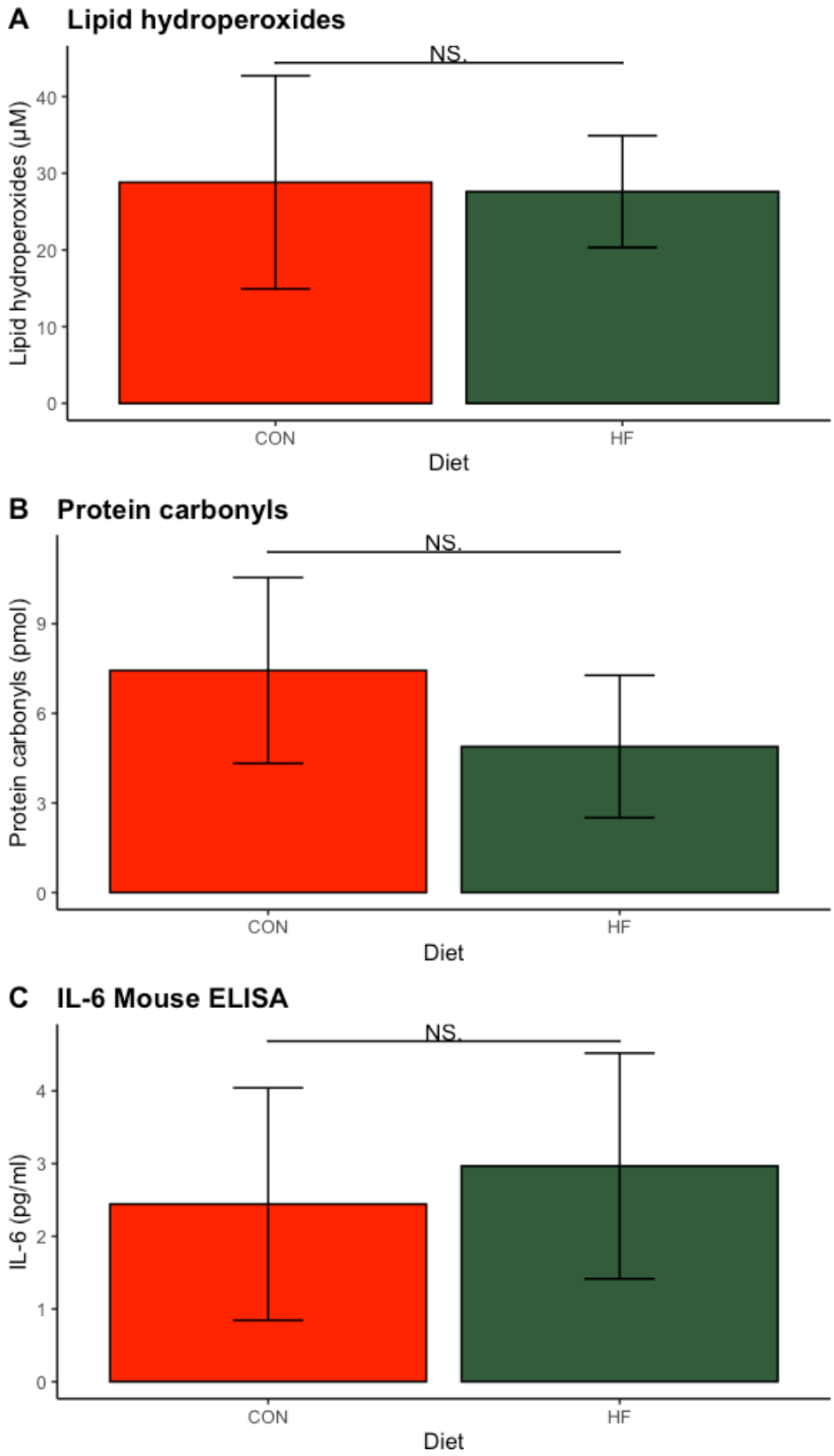

Figure 4.8 ROS and inflammation detection in mouse fetal hepatocytes.
(A) Lipid hydroperoxide detection, (B) Protein Carbonylation. (C) IL-6 ELISA.
* P ≤ 0.05, NS = not significant. CON (n = 12), HF (n= 14) with males and females combined.

4.3.5 Analysis of fetal hepatic mitochondria

Given the shortening of telomeres, it was hypothesised that the HF group would decrease mitochondrial DNA copy number in cells as a precursor to metabolic dysfunction. Surprisingly, the mt-CytB qPCR assay revealed a significant increase in mitochondrial DNA content in livers of HF-fed offspring (P=0.0282; Figure 4.9A), as did the mtND1 qPCR assay, which also trended towards significance (P=0.0526; Figure 4.9B). This inferred mitochondrial biogenesis. To confirm this, the expression of the mtDNA transcription genes *Pgc-1α*, *Tfam* and *Nrf1/2* was investigated. The increase in mtDNA was accompanied by increases in *Pgc-1α* (P=0.01; Figure 4.9C) and *Tfam* mRNA (P=0.024; Figure 4.9D). *Nrf1* (P=0.251; Figure 4.9E) and *Nrf2* (P=0.944; Figure 4.9E) showed no change in gene expression.

Studies have reported that HF-fed mice have higher *Cpt1a* enzyme activity in their livers (636). As a result of increased *Cpt1a* activity, which is regulated by *Pgc-1α*, this adaptation to a high fat diet increases fatty acid oxidation (631). It was hypothesised that the increased mitochondrial DNA would coincide with an increase in *Cpt1a* mRNA suggesting an increase in the use of fatty acids, mitochondria's preferred fuel, in metabolism. Therefore, *Cpt1a* gene expression was assayed. The increased mtDNA copy number was indeed accompanied by increases in *Cpt1a* (P=0.009; Figure 4.9G) mRNA levels.

To further assess whether HF fetal livers might be adapting to fat exposure *in utero, Fatp2* mRNA and protein were assessed. It was hypothesised that an increase in FATP2 might be observed, suggesting increased uptake of fatty acids as a means of fetal adaptation to maternal HF diet during gestation. There was an increase in the *Fatp2* mRNA levels (P=0.031; Figure 4.9H). However, the difference in FATP2 levels between CON and HF mice was not replicated in the Western blot protein analysis (Figure 4.9I).

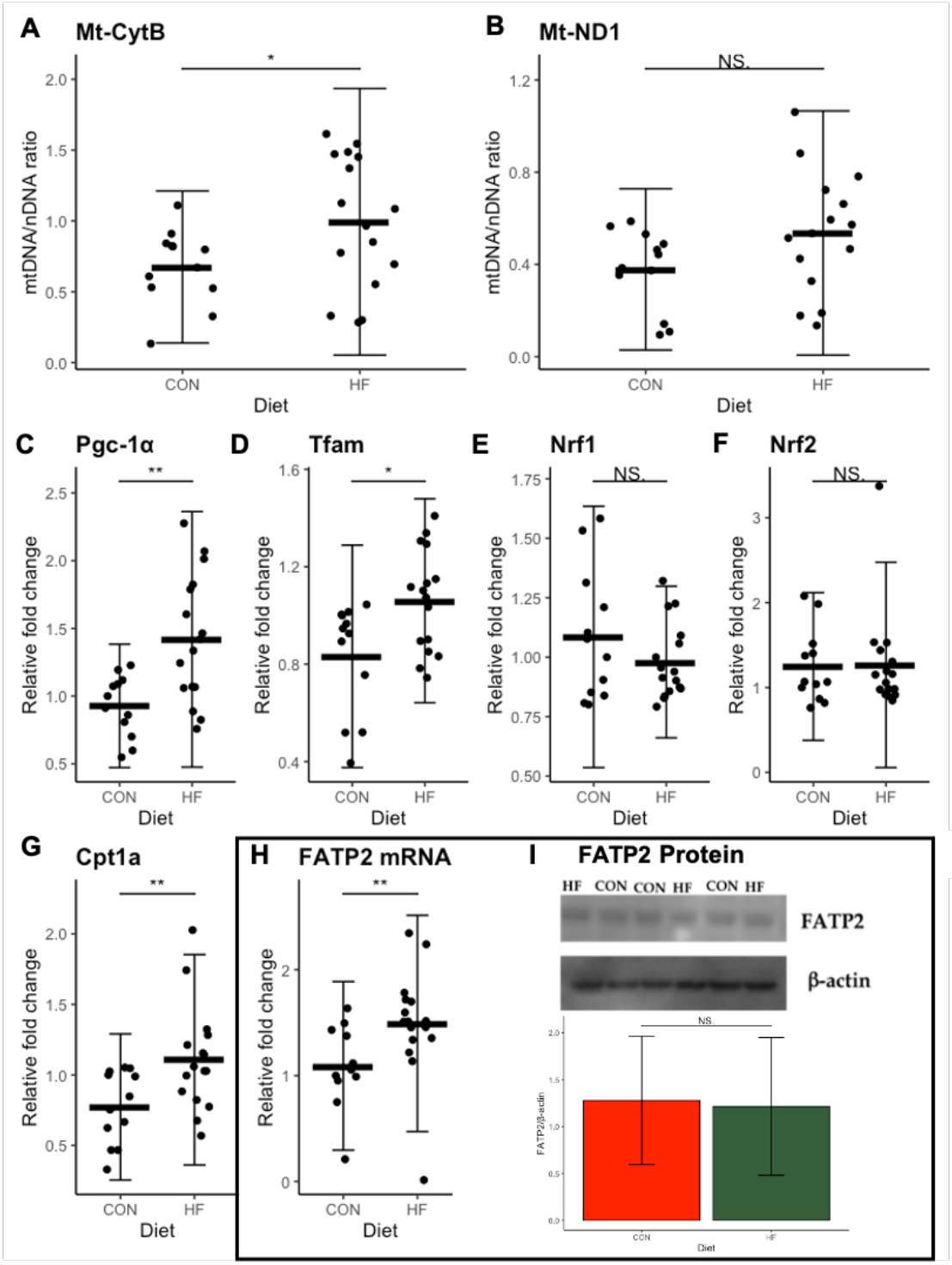

Figure 4.9 mtDNA content, biogenesis and lipid metabolism genes and protein.

Fetal mt-ND1(A) and mt-CytB (B), gene expression of mitochondrial biogenesis genes *Pgc-1α* (C) and *Tfam* (D), lipid metabolism genes *Nrf-1* (E), *Nrf-2* (F). (G) Maternal mtDNA content. FATP2 mRNA and protein (H), (I). * P ≤ 0.05, ** P< 0.01. CON (n = 12), HF (n= 14) with males and females combined.

4.3.6 Analysis of shelterin protein TIN2

The telomere protein TIN2 is critical to the shelterin complex's overall integrity. The presence of TIN2 in the nucleus has long been associated with improved telomere function (637,638). The loss of TIN2 from telomeres in mice results in the loss of TPP1 and POT1 subunits and the induction of telomere-adverse phenotypes such as fusions and activation of ATM kinase signalling cascades (639). It was hypothesised that shortening of telomeres in response to HF feeding may also result in a concurrent reduction of TIN2 in the nucleus. Hepatic cells were stained for TIN2 (Figure 4.10). TIN2 localisation in the HF cytoplasm and nuclei were comparable to the CON group (P=0.376; Figure 4.11).

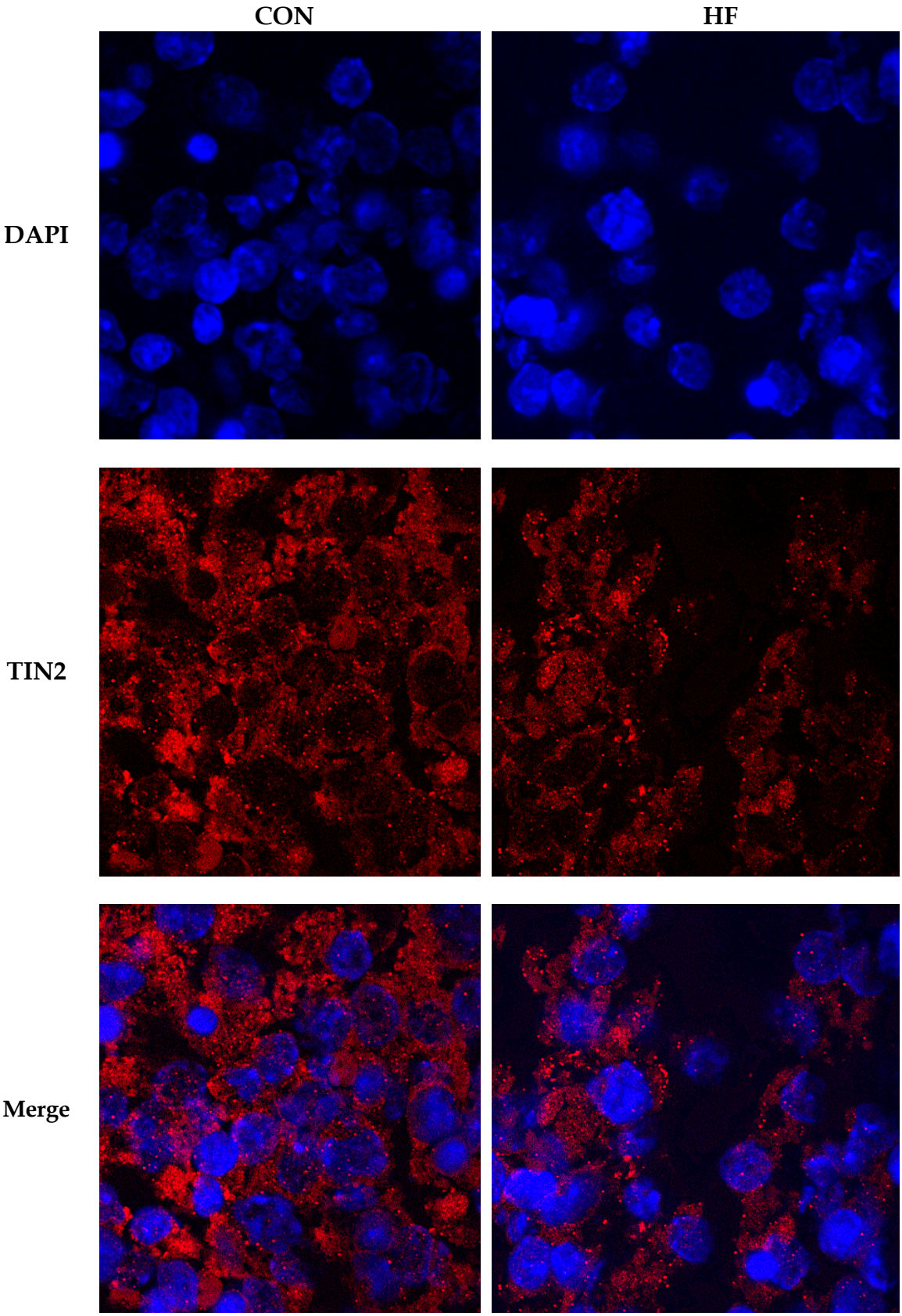

Figure 4.10 Immunofluorescence images showing localisation of fetal liver stained for TIN2 in CON and HF. Blue = DAPI, Red = TIN2

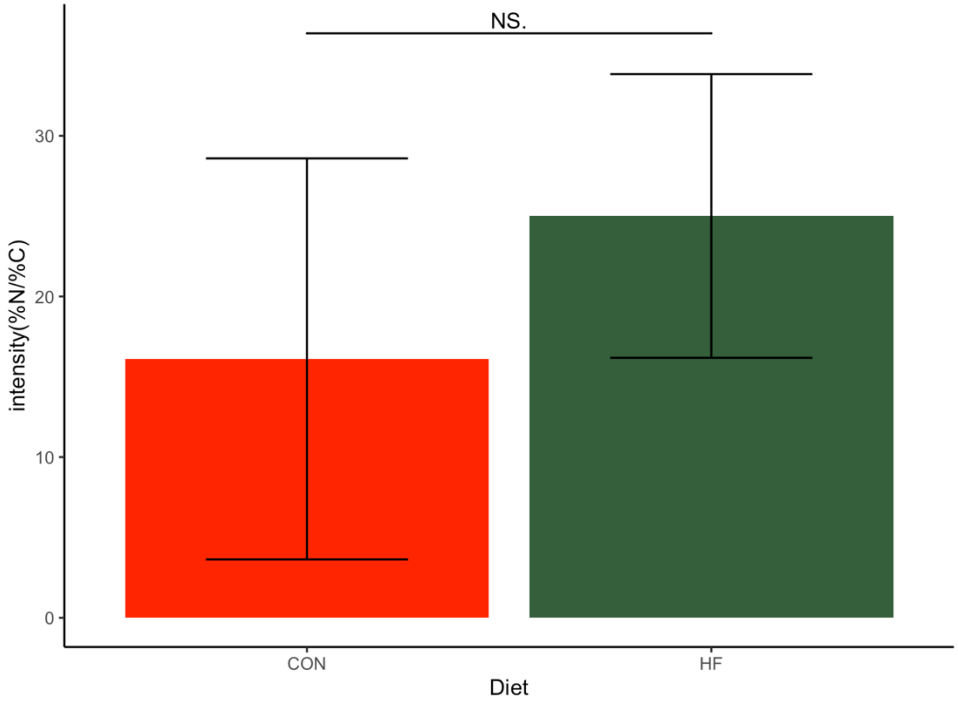

Figure 4.11. Quantification of nuclear/cytoplasmic ratio of TIN2 fluorescence intensity in CON and HF livers. CON n = 3, HF n= 3. NS = not significant.

4.4 DISCUSSION

In this study, HF feeding during pregnancy expedited offspring aging in a tissue-specific manner, with effects seen only in the liver. Furthermore, these findings suggest a mitochondrial adaptation in the fetal liver that promotes fatty acid metabolism. While the HF diet does not appear to be not functionally detrimental to the liver during this stage of development, it may program adverse phenotypic outcomes later in life.

The findings in this study do not directly point to oxidative stress as a significant mechanism through which HF diet might program metabolic disease in later life. Higher superoxide and lipid hydroperoxide concentrations in the HF group were anticipated, given the high-fat diet exposure *in utero*. Direct measurements of superoxide presence in nuclei, however, were not possible in this study. As a result, confirming superoxide/ROS production in relation to DNA damage becomes challenging. In light of the fact that the levels of lipid hydroperoxides and IL-6 were not increased in HF livers and that there was a tendency for a decrease in protein carbonylation, these data do not support the hypotheses and instead suggest the involvement of alternative mechanisms. Studies suggest that dysregulation of the hepatic immune response (640) and epigenetic modifications to the fetal genome (641,642) are likely mechanisms relating to the programming of metabolic disease. Future research may need to investigate how

these variables affect fetal telomeres and whether studies in older animals can shed more light on how the obesity phenotype develops.

During pregnancy, maternal body composition is linked to fetal growth restriction (643) as well as overgrowth (107). Rodent studies in offspring born to dams fed high fat and high fat-high carbohydrate diets like the one used in this study have also shown the former (644-647) and latter (648,649) outcomes. Since the fetuses in this study were neither macrosomic nor growth-restricted, the effect of the diet may have been minimal and not severe enough to result in significant negative outcomes related to DOHaD.

Despite the higher caloric content of the diet, it was interesting to observe that HF dams gained almost identical amounts of weight as the CON dams, and fetus body weights were not significantly higher in the HF group at E18.5. It is possible that the mice adjusted their intake and activity to compensate for the hypercaloric HF diet. One prominent alteration in rodents exposed to high-fat diets is change in feeding behaviour, as well as decreased activity. (650,651). Prior research has demonstrated that even a brief exposure to a high-fat diet can alter eating habits and increase visceral adipose tissue deposits, even in the absence of significant weight gain (652). It is unclear how much dams may have adjusted their intake since analysis of feeding periods was not considered in this study. A further limitation is the lack of measurements of ambulatory activity and total energy

expenditure between the CON and HF dams. Even though the HF diet had significantly more fat per unit of energy, it is possible that both groups' energy expenditure was comparable. It may be necessary to conduct additional studies on energy consumption and intake, in addition to macronutrient and micronutrient analyses of specific HF diet components, to determine why the weights of the fetus and the dams did not significantly increase.

This impact of high-fat feeding might also be due to concentration, timing, and length of exposure. In earlier studies, pregnant rats fed HF diets with concentrations of 20 and 30% had offspring with higher birthweights(653). In contrast to controls, the effect was lessened when given a diet higher in fat (40%), and the birthweights were normal (653). These findings indicate that HF feeding had a plateauing effect on body weight. A similar plateau effect might also be present in response to the 60% HF diet, which ultimately resulted in the absence of macrosomia or obesogenic effects at E18.5. However, a study of the effects of the same diet with lower fat concentrations would be required.

In contrast, Sferruzzi-Perri *et al*. (654) previously demonstrated a high fat-high carbohydrate effect on offspring growth *in utero*. When exposed to the obesogenic diet, fetal body weights were reduced at E15.5; however, by the time they reached term, their weights had returned to normal compared to controls (654). Their data raises the possibility in this study that the greatest effect of HF exposure *in utero*

may not be seen near term, but rather earlier in gestation. Future studies may need to accommodate analyses at various gestational timepoints and may also need to consider various fat intakes in order to pinpoint specific developmental windows when the HF diet has the greatest impact on growth.

While maternal weight gain is an acceptable indicator of the obesity phenotype, it is limited in that it does not take adiposity or total body composition into account. A secondary measure, such as the Lee obesity index (655), or a magnetic resonance scan to determine total body composition would have lent more precision to the measures (656). These alternative measures produce assessment criteria that are comparable to BMI (655,657) and visceral adipose tissue deposits in humans (658), associated with insulin resistance and metabolic disease. The absence of either of these measures limits the ability to accurately assess the whether the HF diet promoted adiposity and obesity in dams, and to confirm whether maternal body composition may have contributed to the fetal phenotype.

The HF-fed dams produced offspring with shorter hepatic telomeres and increased mtDNA content ratios. However, there was no direct evidence implicating increased oxidative stress levels or inflammation in telomere shortening. While these data may suggest that increased p21 gene expression is evidence of the activation of a senescence pathway, and thus premature aging of

the liver organ as a result of HF diet exposure, this may only be a partial explanation.

It is very likely that a DNA damage response (DDR) activates p21 in response to telomere damage (659). However, proliferation, as estimated by Ki67 staining, remains uninterrupted in the HF groups. Higher gene expression of *mTert* contrasted with shorter telomeres and was not linked to the expression of regulator c-myc; this may indicate a role for *mTert* that is not directly related to telomere maintenance. As previously reported, *mTert* may play an important role in proliferation regulation (660,661), which may be the case here. Studies using overexpressed TERT have found that it has a positive effect on mitochondria, including maintaining mitochondrial integrity (662), as well as improvements in overall function and inhibition of ROS-induced autophagic (663,664) and apoptotic (575,576) cell death. This would require further investigation to elucidate the cell pathways which are inhibited and which aspect of mitochondrial respiratory chain might be affected. The proposed positive effects on hepatic cellular physiology in HF fetuses may not only apply to increased expression of *mTert*, but also p21.

Previous research indicates that silencing p21 in mice reduces rather than increases hepatocyte proliferation, implying that p21 is required for DNA-damaged cell proliferation (665). It was also discovered that the dual roles of p21

were determined by the intensity of p21 upregulation, which was determined by the degree of liver injury in mice (665). The increase in p21 mRNA levels observed in this study could be due to liver cells recovering from a previous insult, such as hypoxia. More research into fetal hepatocyte DNA damage earlier in pregnancy, however, may be required. This could explain why the TL was shorter, as it could reveal the gestational age at which the most significant DNA damage occurred, allowing for potential therapeutic intervention. Furthermore, incorporating data from a TRAP assay could help to better understand telomerase activity in the tissue.

The fact that the HF group's fetal gut TL was longer is also noteworthy. This could be due to changes in the cellular composition of gut epithelium as a result of the HF diet. Functional maturation of the gastrointestinal tract occurs both before and after birth in rodents, and is influenced by maternal nutrition (666,667). The number of intestinal stem cells and other progenitor cells increases in mice fed an HF diet (668). This was also confirmed in piglets, where a previous study of gestational overnutrition resulted in taller villi and heavier intestines in the small intestine (669). The strong positive correlation between increasing dam gut TL and dam weight at E18.5 could be explained by this change in gut physiology. In the gut, increasing stemness could be a significant contributing factor. According to a study by Maysam and colleagues, the positive correlation between gut TL and blood TL could be explained by increased amounts of leukocytes in mouse blood as a result of extended HF feeding (670). However,

more studies are required to quantify cell type populations in dams, as well as to determine structural changes to villi and increased stem cells, which could account for telomere lengthening in fetuses despite similar body weights.

The expression of several mitochondrial biogenesis genes, including Pgc-1α, Nrf1, Nrf2, and Tfam, was studied. Notably, Pgc-1α. and Tfam mRNA levels increased, which regulate mtDNA content. However, there was no change in Nrf1 or Nrf2 gene expression. Nrf1 regulation may occur at the posttranscriptional level. While not detected at the mRNA level, oxidative stress has been shown to increase Nrf2 protein levels, indicating that it is not transcriptionally regulated in response to elevated ROS (671), and thus protein analyses would be required to confirm this.

Previous research has shown that an increase in mtDNA copy number can be a feedback response to excessive ROS (672,673). This feedback response may be engaged as a compensatory mechanism to replace dysfunctional mitochondria (674). Elevated ROS may have been present earlier in gestation to trigger these responses. However, this was not evident from these data at the timepoint of E18.5. It is also possible that the increased transcriptional regulation of Tfam by the HF diet may be due to factors independent of Nrf1/2. Other regulators of Tfam include p53 (675) as well as microRNAs which regulate Tfam levels by

interfering with mRNA translation (676,677). Further studies would be required to assess the effect of HF on p53 protein levels and microRNAs.

Studies have also shown that extended periods of HF feeding in rodents elevates Pgc-1α and TFAM, which increases mtDNA content (490), as in this study. An increase in Pgc-1α expression is suggested to be an adaptation to enhance tissue oxidative capacity (490), which may also apply in this instance. The increase in mtDNA, and thus mitochondrial content, may be an adaptation of the tissue to a new respiratory substrate following exposure to large amounts of free FAs (492). Indeed, FAs produce significantly higher levels of hepatic oxidative stress when used as substrates (678). So, it may only be a matter of time before levels of ROS are increased and oxidative stress overwhelms hepatic cells.

Intensification of mitochondrial biogenesis may also be a preventive adaptation against future mitochondrial function impairment caused by oxidative stress and toxic lipid accumulation (493). It has also been reported that significant mtDNA increases can occur during aging and is accompanied by significant alterations in the form of deletions of the mitochondrial genome (674,679). Consequently, while these mtDNA adaptations may be advantageous to offspring at this early age, they may be a preliminary step for an aging-related disease phenotype.

Although earlier studies have demonstrated increased mtDNA in older animals in response to a HF diet (492), to date no studies have shown this effect in fetal livers following HF diet exposure during pregnancy. There is a greater tendency to report the postnatal mitochondrial consequences ensuing from HF nutritional excess. For instance, in offspring of female rats fed a HF diet, there was a decline in hepatic mtDNA in young adulthood (132). This decrease was in parallel with impaired glucose detection by pancreatic β-cells, which in turn compromised the release of insulin (132). Other studies concur with this, showing a reduction in a mitochondrial electron transport chain activity and elevated oxidative stress post-weaning, in 15-week-old offspring of HF-fed dams (680). In addition, 8-week-old obesogenic diet-fed mice have reduced mtDNA content, Tfam expression, as well as lower ATP release (681).

These later life effects of HF diet on mtDNA run contrary to these observations *in utero*. This discrepancy may indicate that the observed mtDNA increase in this study is temporary. As is evident in the highlighted animal work (132,680) as well as in clinical studies (682,683) the loss of mtDNA and mitochondrial dysfunction is well associated with insulin resistance and impaired lipid metabolism. When mitochondria are exposed to excess fatty acids, they can be markedly polarised, resulting in incomplete metabolism and the generation of large amounts of ROS which in turn results in cellular metabolic dysfunction (684). The outcome of increased mitochondrial biogenesis *in utero* may in fact suggest an adaptation

designed to protect the cell against the effects of FA accumulation. This finding is perhaps further justified upon examining the likely role of Cpt1a.

Greater gene expression of Cpt1a in this study concurs with previous work showing higher Cpt1a enzyme activity in livers of HF-fed mice (636). Increased Cpt1a which is regulated by PGC-1α, enhances β-oxidation of fatty acids (631). The concurrent increase in fetal PGC-1α mRNA may suggest a preference for fatty acid metabolism over glucose (631). The purpose of this increase in Cpt1a-induced β-oxidation may be to prevent adipocyte hypertrophy as well as hepatic lipid accumulation, in addition to restricting storage in adipocytes (685). As such, Cpt1a has been shown to lower the concentration of hepatic TG and reduce inflammation as well as oxidative stress (636). This protective role may explain why protein levels of the inflammatory marker IL-6 were not significantly different in the HF group.

The concurrent increase in Cpt1a and FATP2 mRNA levels in this study is also evidence of mitochondrial adaptation to high levels of FFAs. Increased FATP2 mRNA expression is associated with liver steatosis as well as oxidative stress (686). Evidence from mouse studies has shown that hepatic FATP2 is transcriptionally regulated by insulin signalling via insulin receptor substrate (IRS), and if this signalling is perturbed, lipid accumulation may occur (687). This accumulation of fat in the liver is characteristic of the disease non-alcoholic fatty

liver disease (NAFLD), which is also noted for greater expression of FATP2 in disease sufferers (688). Therefore, elevated FATP2 mRNA in the HF fetuses suggests an increase in FFA uptake. Notably, maternal high fat feeding during gestation has been shown to prime future NAFLD steatosis in mice (680). These data suggest this might be the case. The discordance observed between mRNA and protein may be due to FATP2 not being fully stabilised at the protein level, despite increased transcription in response to stimuli.

TIN2 nuclear localisation of HF was comparable to CON. The observed telomere shortening in HF was initially thought to be attributed to decreased nuclear TIN2, which stabilises the shelterin complex (373,374). However, direct staining for TIN2-telomere immunostaining would need to be carried out to reveal whether the nuclear localisation observed in these data translates to localisation at telomeres and would be a key component of future analyses. Notably, the degree of localisation to the nucleus does not predict activity. TIN2 in the nucleus may have significant activity even in limited concentration. Chen at al. (528) proposed that TIN2 functions as an alternative regulator of glucose metabolism. Knockdown of TIN2, and hence diminished import into mitochondria, improved ATP synthesis and energy metabolism, but repressed glycolysis (528). However, it is not clear what degree of cytoplasmic TIN2 was localised to mitochondria, whether there were alterations to mitochondrial function. The lack of difference in TIN2 immunofluorescence in the HF group despite shorter telomeres suggests that other mechanisms, such as disruption of binding of other shelterin proteins

or elevated ROS, may be involved in the telomere outcomes. This, however, would necessitate further investigation. The increased expression of genes involved in fatty acid uptake and metabolism may provide insight into how mitochondrial function is affected. If there is a link between TIN2 and mitochondrial metabolic adaptation for high-energy ATP production from fatty acid oxidation, more research is needed to determine TIN2's role. In addition, studies would need to look into whether TIN2 localisation contributes to mitochondrial adaptations to high fat feeding or whether mitochondrial adaptations contribute to changes in TIN2 expression. **These data bring to light the fact that hepatic TIN2 may be diet-sensitive *in utero* and may play a significant role in programming of metabolic disease, depending on the nature of the maternal diet. This is the first time that this role for TIN2 has been proposed in a model of nutritional excess**.

While the main objective of the research was to look at the effects of HF feeding on fetal telomeres and mitochondria, this study might have benefited from extending the timeline to early adulthood. This would aid in determining whether the telomere effects and mitochondrial metabolism adaptations persist and contribute to a metabolic syndrome-like phenotype after HF exposure.

There are similarities in the outcomes of prenatal undernutrition and overnutrition in terms of changes in TL and *mTert*. The greatest differences, however, are in mitochondrial adaptations to the prenatal diet and their potential role in disease programming, which provide alternative therapeutic targets

unique to each diet. According to the findings of this study, there may be protective mechanisms in place in early life to protect against nutritional excess. More research is needed to determine how and when these mechanisms are overwhelmed, resulting in future disease development. These findings suggest that excess prenatal fat exposure may program future metabolic dysfunction.

CHAPTER 5

EFFECTS OF EARLY LIFE EXPOSURES ON TELOMERE LENGTH IN LATE ADOLESCENCE

5.1 INTRODUCTION

Altered intrauterine and postnatal growth is associated with the later development of cardiovascular and metabolic diseases, notably obesity (29). Such impairment of metabolic function is thought to be programmed through adverse periconceptional and early life environments, such as physiological stress or by way of fetal/infant malnutrition (51). In fetuses, programming of metabolic disease may occur as a result of an inadequate maternal diet or overnutrition during gestation (13,689). In infancy, breastfeeding is believed to have beneficial programming effects due to the lower risk of overweight and obesity observed in later life in breastfed individuals (144,690). The presence of breast milk may interact with genetic and other environmental factors to modify development (294).

Whilst the global incidence of obesity in adulthood has almost doubled in the last three decades (95), more concerning is the fact that overweight/obesity in pregnant women and in children is on the rise (96). Estimates suggest up to 40 million children worldwide are overweight/obese (691). Furthermore, childhood obesity, principally during adolescence, is a strong predictor of overweight and obesity in adulthood (692-694). The increasing prevalence of obesity in early life sets children on adverse health trajectories towards comorbidities such as

diabetes, hypertension, and hyperlipidaemia (695,696) - the clustering of conditions that comprise the metabolic syndrome (2).

Despite the relatively infrequent occurrences of metabolic syndrome and related pathologies in children or adolescents, some studies have linked childhood obesity to the early onset of diabetic, hypertensive, and hypercholesterolemia phenotypes (697-699). These conditions can lead to long-term morbidity. It is, therefore, critical that any interventions to lower the incidence of obesity and other chronic disorders are targeted to early life. Such interventions would need to be based on a full understanding the mechanisms behind the development of the metabolic syndrome, which have yet to be fully clarified.

As discussed in previous chapters, telomeres are DNA-repeat complexes located on the ends of chromosomes that progressively shorten as a result of repeated cell division (361,700). When telomeres shorten below a critical length, cells enter senescence and programmed cell death mechanisms are initiated (380). Telomere length (TL) is an inherited genetic trait; genotype may account for up to 70% of the variance (541-543). Telomeres naturally shorten with increasing age (361). TL serves as a marker for the replicative history and capacity of cells, and is therefore indicative of cellular biological age (378,701). The rate of telomere shortening accelerates with exposure to oxidative stress and inflammation (702), both of which are also present in obesity (703,704). Furthermore, shorter leukocyte TL has previously been associated with childhood and adolescent obesity (504,505). A link has also been shown between shorter telomeres and exposure to high

levels of psychological and social stress (499,705-707) suggesting a relationship between stress-related alterations to telomere structure and the development of age-related disease. Therefore, in addition to aging, TL may be a surrogate for altered metabolism and cellular dysfunction. Indeed, evidence of the past decade has implicated telomeres directly in altered cellular metabolism (523,708). Studies have clearly established a link between suboptimal antenatal/early postnatal environments and the development of metabolic syndrome (39). Moreover, in both adults and children, shorter telomeres have been associated with risk factors for metabolic disease (501,504,505).

It is likely that factors *in utero* and in early postnatal life may influence oxidative stress pathways and hence, telomere length, impacting normal cellular metabolism. Thus, it is hypothesised that early life environments and risk factors associated with the development of metabolic disease are associated with TL and measures of adiposity in late adolescence. These associations may be apparent before the onset of metabolic disease, suggesting a mechanism by which stressors in early life may accelerate cellular aging/dysfunction and the onset of adverse metabolic phenotypes in later life.

5.2 METHODS

5.2.1 Participants

The Western Australia Birth Cohort (Raine) study, as described in Section 2.2 was used as the data source for this research. The children from this on-going longitudinal study in Perth, Western Australia, have been comprehensively

followed up and phenotyped at the ages of 1, 2, 3, 5, 8, 10, 14, 17, 20, 22 and 26 years. (540,709-711).

During the 17th year of cohort review, anthropometric, cardiovascular, and biochemical assessments were conducted. The Raine Executive Committee approved access to participant DNA. The University of Western Australia Human Research Ethics Committee approved ethics and experiments. This study was conducted with institutional ethics approval from King Edward Memorial Hospital, Perth, Western Australia, with written informed consent from all the mothers.

5.2.2 Sample collection

Adolescent venous blood samples were collected with the assistance of phlebotomists and nursing staff at King Edward Memorial Hospital (KEMH), in Subiaco, Perth, Western Australia. Blood was collected into 4 ml Vacutainer® Plus plastic K2-EDTA tubes (BD biosciences) either on site at KEMH or at participants' homes, and then stored at -80°C until later analysis.

5.2.3 Measures

Maternal medical history variables were included in the measures of analyses: maternal pre-pregnancy BMI calculated from measured body weight in kilograms (kg) and height in metres squared (m^2), maternal age at birth, maternal pregnancy hypertension (as yes/no) and history of maternal diabetes (as yes/no).

Childhood nutritional information was established from records of duration of breastfeeding. The measures examined in this study incorporated two enquiries into the participants' records: firstly, whether the child was breastfed and when breastfeeding was stopped, and secondly, when other foods or milk types were introduced into the child's diet. This information gave us two measures, duration of breastfeeding and period of exclusive breastfeeding, recorded in months.

Family socioeconomic status (SES) at birth was determined using the Index of Relative Socioeconomic Advantage and Disadvantage (IRSAD) as determined by the Australian Bureau of Statistics. The index assigns Socioeconomic Indexes for Areas scores, which are used to identify and rank areas in terms of relative socio-economic advantage and disadvantage (712). Higher scores are indicative of greater socioeconomic advantage and a relative lack of disadvantage overall (712). In addition, parents completed a Depression Anxiety Stress Scale (DASS-21) questionnaire, consisting of 3 scales each containing 7 self-report to give a total of 21 assessments for depression, anxiety and stress symptoms (713). This generated a score to determine familial psychological stress at the 17-year follow-up.

For adolescents, BMI at 17 was calculated similarly to maternal BMI, and waist-to-hip ratio was generated from participant waist and hip measurements in cm.

5.2.4 DNA isolation

Genomic DNA was extracted manually from peripheral blood mononuclear cells (PBMCs) in whole blood frozen at -80 ^{o}C using the Gentra Puregene Blood kit

(Qiagen, Chadstone, VIC, Australia) or with the AutoPure LS robotic workstation (Qiagen, Chadstone, VIC, Australia). Both manual and robotic DNA extraction protocols utilise the same Gentra Puregene Blood kit reagents (Qiagen, Chadstone, VIC, Australia) and protocol for DNA isolation. Samples were thawed and warmed to 37 ^{o}C in a water bath. The tube of thawed blood was inverted gently 5-6 times to mix. Blood was transferred into a 50 ml tube containing 12 ml of RBC lysis buffer to lyse the erythrocytes. The tube was then inverted gently 3 times to mix. This blood: buffer mix was then incubated for 5 min at room temperature to lyse the erythrocytes. The mixture was then inverted 3 more times and placed in a centrifuge. Samples were centrifuged 2000 x g for 2 min to pellet the white blood cells. The red cell supernatant was poured off and the tube with the remaining cell pellet inverted onto absorbent paper for 10 s to drain. Less than 200 µl of residual liquid was left in the tube. The tube was vortexed vigorously with the remaining liquid for 10 s.

1.87 ml of Protein Precipitation Solution from the Puregene Blood kit was added to the centre of the sample followed by another 4 ml of Cell Lysis Solution. Samples were then vortexed vigorously for 20 s to completely lyse the white blood cells and to precipitate any proteins. Samples were centrifuged at 2000 x g for 6 min. Precipitated proteins were pelleted and the resulting supernatant contained the DNA was used for subsequent steps.

The supernatant was poured into a new 50 ml tube containing 4 ml of 100% isopropanol. The tube was capped and inverted 50 times to precipitate the DNA, visible as a large white pellet. The tube was centrifuged to at 2000 x g for 3 min

and the supernatant was decanted. The tube was left inverted and then left to drain for 1 minute on clean absorbent paper.

4 ml of 70% ethanol was added to the tube containing the pellet and centrifuged at 2000 x g for 1 min to pellet the DNA. The ethanol was decanted and the tube left to drain for 15 min on absorbent paper. 300 μL of DNA hydration solution was then added to the tube to rehydrate the DNA pellet. The tube containing the DNA was initially transferred to an incubator to rehydrate at 65 ^{o}C for 1 h and then placed at room temperature overnight on a rotating mixer.

The resultant DNA from each sample was assessed for quantity using a BioTek Epoch Microplate Spectrophotometer (Biotek, Winooski, VT). DNA quality was determined from the 260/280 and 260/230 nm ratios calculated by the spectrophotometer. Only 260/280 nm and 260/230 nm ratios of between 1.8-1.95 were used for analysis (554). Moreover, DNA integrity was checked by loading on a 1% (w/v) agarose gel stained with GelRed (Biotium, Hayward, CA 94545) (Figure 5.1). Samples were run with the HyperLadder™ 100bp molecular weight marker at 80 volts for 1 h, and imaged using the ImageQuant 350 imager (GE Healthcare). Intact genomic DNA was indicated by the presence of a single high molecular weight (MW) band (Figure 5.1). DNA that appeared as a smear was not used for analysis, and the sample was re-extracted.

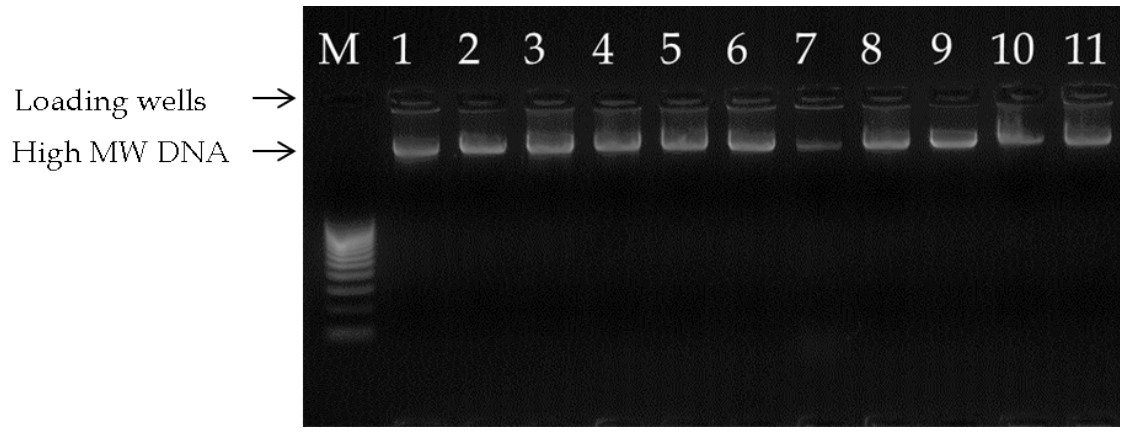

Figure 5.1. Representative image of isolated DNA on a 1% agarose gel. Single bands indicate intact genomic DNA. Lane M: HyperLadder 100bp marker. Lanes 1-11: High MW DNA bands

5.2.5 Telomere length measurements

PCR primers used for this study are shown in Table 5.1. Telomere (Tel) primer sequences were previously published (562). TATA-box binding protein (TBP) was selected as the reference gene after reaction efficiencies for suitable reference genes were evaluated. TBP primers were designed using NCBI Primer Design and checked by *in silico* PCR (http://genome.ucsc.edu/cgibin/hgPcr?command=start).

Table 5.1 Primers used for telomere length analysis.

Gene	Sequence (5′ → 3′)	Source/ Accession number	Amplicon size (kb)
Tel	F 5′-CGGTTTG(TTTGGG)$_5$TT-3′ R 5′-GGCTTGCC(TTACCC)$_5$T-3	Cawthon (2002)	~76
TBP	F 5′-CCACAGCTCTTCCACTCACA-3′ R 5′-CTGCGGTACAATCCCAGAAC-3′	NM_003194	138

5.2.4.1 qPCR to determine TL measurements in adolescents

All qPCR reactions for adolescent samples were performed at KEMH in Perth, Western Australia. A total of 1385 adolescent samples were analysed. All DNA was normalised to 15 ng/µl concentration using the Qiagility automated liquid-handling system (Qiagen, Chadstone, VIC, Australia). PCR mastermix composition was identical for Tel and TBP. All PCR primers were obtained from Geneworks (Adelaide, Australia). qPCR was performed in a Rotorgene 3000 cycler (Qiagen, Australia) in a 10 µl reaction volume. Each 10 µl PCR reaction contained 2 µl of DNA template. 1 µl of 10x ImmoBuffer, 0.05 µl IMMOLASE™ DNA Polymerase, 2 mM MgCl2, 0.2 µl of 10 mM dNTP mix, 0.3 µM of each primer, 0.1 µl of 20x EvaGreen dye and nuclease-free water. All reagents were obtained from Bioline (Alexandria, NSW, Australia), with the exception of EvaGreen dye which was purchased from Biotium (Hayward, CA, USA). PCR cycling conditions were: initial denaturation at 95 ^{o}C for 10 min, followed by 28 cycles of at 95 ^{o}C for 15 s, at 56^{o}C for 10 s and 72 ^{o}C for 5 s.

1:3 serial dilutions of pooled DNA from the Raine cohort were used to create a standard curve to determine the amplification efficiency of each PCR reaction. All reactions were carried out with samples in triplicate with a non-template control and a reference DNA sample. The reference DNA sample was a pooled sample of Raine genomic DNA, diluted in nuclease-free water. The same pooled DNA sample was used alongside unknown samples in every reaction plate, for each of the two (Tel and TBP) genes of interest. All Raine PCR samples were normalised to the reference DNA sample values.

5.2.4.2 Calculation of relative telomere length

Relative telomere length was calculated following qPCR as described by Cawthon (562). This procedure determines a ratio of telomere repeat copy number to a single copy gene (T/S ratio) in experimental samples as compared with a reference sample. In brief, T/S ratios (or ΔCq) for each sample were calculated by subtracting the mean of the triplicates for TBP quantification cycle (Cq) number from the mean of the triplicates for Tel Cq for each unknown sample. The same calculations were performed to determine ΔCq of the reference DNA sample:

$$\Delta Cq_{(sample)} = Cq_{(Tel, sample)} - Cq_{(TBP, sample)}$$
$$\Delta Cq_{(reference\ DNA)} = Cq_{(Tel, reference\ DNA)} - Cq_{(TBP, reference\ DNA)}$$

The relative T/S ratio (ΔΔCq) was determined by subtracting the ΔCq ratio of the reference DNA sample from ΔCq of each unknown sample.

$$\Delta\Delta Cq = \Delta Cq_{(sample)} - \Delta Cq_{(reference\ DNA)}$$

Finally, the normalized relative T/S ratio was then calculated using $2^{-\Delta\Delta Cq}$.

5.2.6 Statistical Methods

Analyses were conducted on 1385 adolescents from singleton pregnancies, free of congenital anomalies and of European descent (Figure 5.2). TL data were log-transformed to improve distribution of normality. Analyses used backwards elimination in multiple regression models to correlate TL, age 17 body mass index (BMI) and age 17 waist-to-hip ratio (WHR) with prenatal and postnatal factors such as maternal pregnancy hypertension, maternal age at birth, maternal pre-pregnancy BMI, duration of breastfeeding, duration of exclusive

breastfeeding, socioeconomic status and psychological stress as described above. Duration of breastfeeding was defined as the period during which any breastfeeding took place, and did not exclude infants that had solid foods or other types of milk as part of their diet (714). Alternatively, duration of exclusive breastfeeding was defined as the breastfeeding period from birth until other milk was introduced into the infant's diet (715).

TLs were divided into quantiles, splitting the data into 5 segments q10, q25, q50, q75, and q90. Mixed-effects quantile regression was then employed to determine longitudinal relationships between BMI from infancy to adolescence, for each TL quantile.

Other covariates that were considered in these analyses included maternal and paternal age in years and BMI at birth, pregnancy health conditions such as preeclampsia (yes/no), and birth measures such as birth weight in grams (g), birth length (cm), head circumference (cm), ponderal index and abdominal circumference (cm). Adolescent physiological measures at the 17-year follow-up such as serum glucose and insulin concentrations (mmol/L) used to generate homeostatic model assessment for insulin resistance (HOMA IR) values in mg/dL, blood triglycerides (mmol/L), total cholesterol (mmol/L), and smoking, blood pressure (mm Hg) and LDL:HDL ratio were also investigated. While included in the initial analyses, these variables were not significant in the modelling. Therefore, data presented herein represents the significant outcomes of multivariate analysis of the risk factors for metabolic syndrome and associated

telomere length. A p-value of less than or equal to 0.05 was considered statistically significant. Statistical analysis was performed in statistical software system R (version 3.0.2).

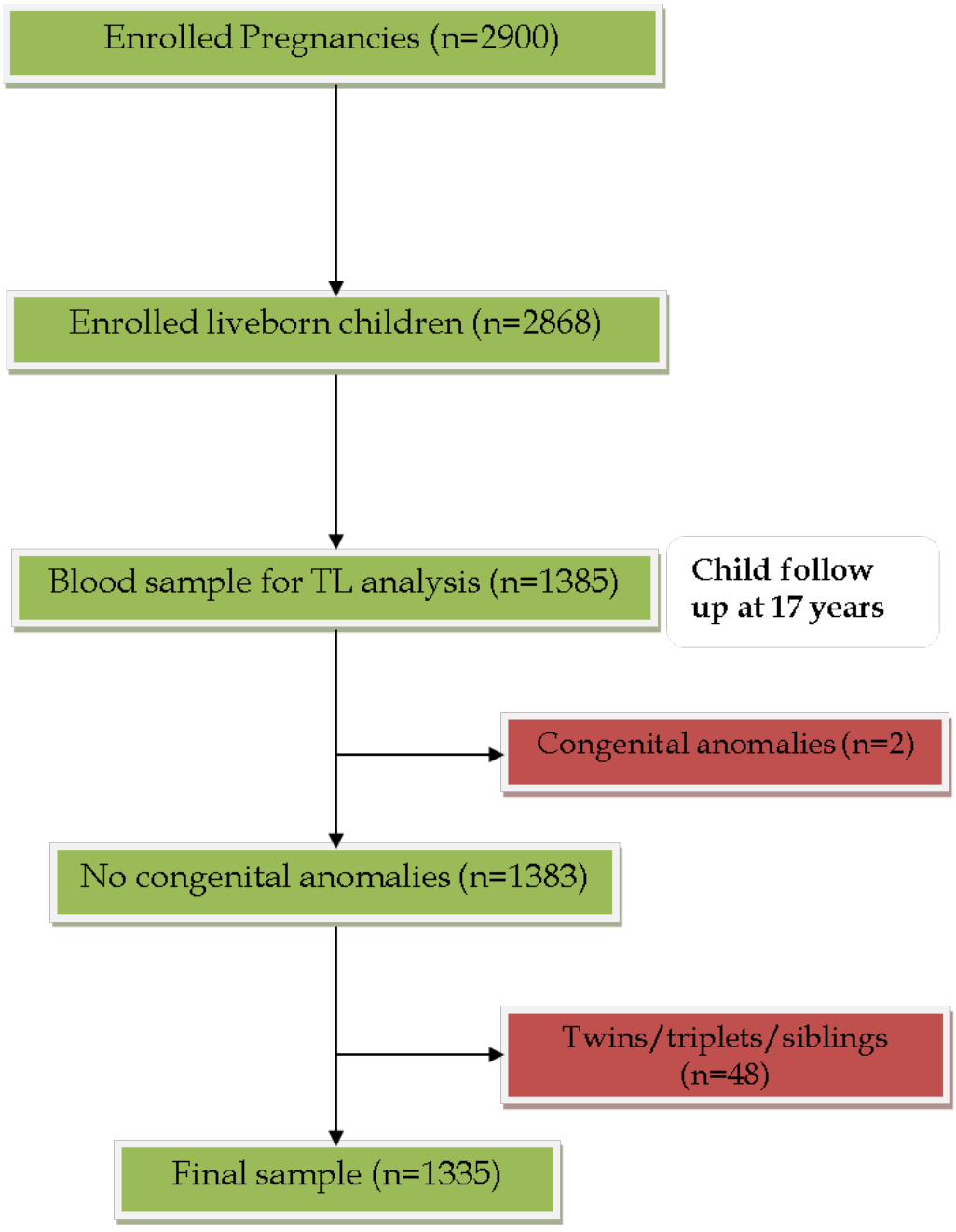

Figure 5.2. Adolescent study population for telomere analysis - West Australian Pregnancy (Raine) Cohort

5.3 RESULTS

Cohort characteristics

Data are presented on 1335 cohort participants (Table 5.2). Table 5.2 is composed of parental, pregnancy and child early life characteristics, as well as traits at age 17. The median age of the participants was 17 years with interquartile range (IQR) 16.9-17.1. Paternal and maternal BMI was on average normal for the Australian population (716), with 36% of fathers and 18% of the mothers classified as overweight or obese. At birth, girls were smaller and lighter than boys ($P<0.0001$). There was no difference in the length of time male and female offspring were breastfed. At age 17 boys and girls had similar BMIs with no difference across WHO-classified groups, falling into healthy (18.5 to < 25), underweight (< 18.5), and overweight and obese categories (BMI ≥ 25). Due to sample size limitations, the proportion of participants was largely skewed towards the healthy BMI range at 69%, followed by overweight and obese at 23%, and 9% underweight. As such WHR, a stronger indicator of poor metabolic status and health risk than BMI (717,718), was also largely within the healthy range for both sexes, based on healthy adult values (male WHR ≤0.95, female ≤0.88)(719). Global cut offs for adolescent WHR have been difficult to define because the degree of the measure sensitivity and specificity are not only affected by both age but also ethnic group (720,721). Moreover, while WHR percentiles have been used to define obesity in children in different countries, the cut off values are not consistent (722-725). Boys had greater WHR ($P<0.001$), LDL:HDL cholesterol ratio ($P=0.03$) and systolic blood pressure ($P=0.002$) than girls. Adolescent girls

had greater amounts of abdominal subcutaneous fat ($P<0.001$), serum total cholesterol ($P<0.001$) and serum triglycerides ($P=0.04$) than males, and had higher levels of the cardio protective cholesterol HDL-C than boys ($P<0.001$). Sensitivity analysis was performed to investigate gender differences in TL; sample size constraints limited the analysis and joint analysis of male and female participant TL data was performed.

Table 5.2 Cohort parental, family, early life and adolescent characteristics

	Mean ± SD or median (IQR)	**Mean ± SD or median (IQR)**	**P-value (Boys *vs.* girls)**
	Boys	Girls	
Participant Age (y)	17 (16.9, 17.1)	17 (16.9, 17.2)	
n (%)	688 (51.5)	647 (48.5)	
PARENTAL & FAMILY			
Paternal age (y)	30.8 ± 6.7	30.8 ± 6.7	0.68
Maternal age (y)	28.5 ± 5.8	28.5 ± 5.8	0.92
Paternal BMI (kg/m^2)	24.3 ± 3.3	24.5 ± 3.4	0.25
Maternal BMI (kg/m^2)	22.3 ± 4.3	22.4 ± 4.4	0.93
Maternal pregnancy hypertension (Y/N)	190 (Y), 498 (N)	164 (Y), 483 (N)	
Parental history of diabetes (Y/N)	11 (Y), 95 (N)	6 (Y), 90 (N)	0.93
IRSAD†	1077 (998, 1116)	1073 (1007,1111)	
BIRTH & NEONATAL			
Birth weight (g)	3386.2 ± 591.8	3276 .4 ± 574.4	<0.001
Birth length (cm)	49.4 ± 2.8	48.7 ± 2.6	<0.001
Head circumference (cm)	34.9 ± 1.5	34.2 ± 1.5	<0.001
Ponderal index (g/cm^3)	27.8 ± 2.9	28.2 ± 3.1	0.015
Abdominal circumference (cm)	31.0 ± 2.4	31.0 ± 2.3	0.88
Period of exclusive breastfeeding (months)	4 (2, 7)	4 (1.5, 7)	0.68
Duration of breastfeeding (months)	6 (2, 12)	6 (2, 11)	0.56
ADOLESCENCE			
Body Mass Index (BMI, kg/m^2) < 18.5	17.5 ± 0.7	17.6 ± 0.8	0.66
Body Mass Index (BMI, kg/m^2) 18.5 to < 25	21.4 ± 1.7	21.7 ± 1.7	0.07
Body Mass Index (BMI, kg/m^2) ≥ 25	29.7 ± 4.0	29.8 ± 4.3	0.94
Waist-to-hip ratio (WHR)	0.84 ± 0.1	0.79 ± 0.1	<0.001
Abdominal skinfold thickness (mm)	14.1 (9.4,22.7)	23.8 (18.1,30.3)	<0.001
Total cholesterol (mmol/L)	3.9 (3.5,443)	4.3 (3.8,4.8)	<0.001
Triglycerides (mmol/L)	0.92 (0.7,1.3)	0.93 (0.7,1.2)	0.04
HDL-C (mmol/L)	1.2 (1.0,1.4)	1.4 (1.2,1.6)	<0.001
LDL:HDL ratio	1.9 (1.4,2.3)	1.7(1.4,2.3)	0.03
Systolic blood pressure (mm Hg)	124.6 ± 60.2	113.1 ± 51.2	0.002
Diastolic blood pressure (mm Hg)	64.3 ± 64.4	63.2 ± 53.9	0.18
HOMA IR (mg/dL)	1.6 (1.0,2.3)	1.6 (1.1,2.3)	0.71
Relative telomere length	1.87 ± 1.9	1.93 ± 2.2	0.62

IRSAD† Calculated from year 16 follow-up. Index scores ranges from 844 to 1221. Higher scores reflect a relative socioeconomic advantage.

Adolescent TL is associated with early life factors and metabolic syndrome precursors

Multivariate modelling showed that telomere length at age 17 tended to be shorter in adolescents whose mothers had hypertension during pregnancy (P=0.053, Table 5.3) and was significantly shorter in adolescents with greater WHR (P=0.015). Consistent with the known association between aging and TL, as adolescent age increased, TL decreased (P=0.023). Importantly, adolescents, who as infants were breastfed for longer, had significantly longer TL at age 17 (P=0.004). While 12 months was deemed optimal univariate modelling showed that a minimum of 2 months exposure to breastfeeding was sufficient to begin to observe positive effects on TL (P=0.020).

Table 5.3 Multivariate modelling of correlations between telomere length and early life and adolescent characteristics

Variable	β	Std error	p-value
Maternal pregnancy hypertension (Y/N)	-0.115	0.059	0.053
Waist to hip ratio (WHR) at 17 years	-1.032	0.422	0.015
Participant age (years)	-0.251	0.110	0.023
Duration of breastfeeding (non-exclusive) (months)	0.011	0.003	0.004

Adolescent anthropometry is associated with maternal body composition, stress, and early life nutrition

Despite the relatively healthy body composition of this cohort of adolescents, BMI was greater in adolescents whose mothers had been hypertensive during pregnancy (P=0.001, Table 5.4). Interestingly, this was not the case for offspring born to mothers with familial history of diabetes, where a significant association with lower adolescent BMI was observed (P=0.023). IRSAD analyses showed that BMI was also greater in adolescents who came from more disadvantaged socioeconomic backgrounds (P=0.001), and adolescents whose parents reported higher current psychological stress on the Depression anxiety and stress scale (P=0.009). In contrast, higher HDL concentrations at age 17 were associated with lower adolescent BMI (P=8.60e-10). Importantly, early life nutrition modified adolescent BMI, where adolescents who were exclusively breastfed as infants had significantly lower BMI at age 17 (P=0.016), whilst duration of breastfeeding did not impact adolescent BMI. Analysis also showed that female sex was correlated with BMI values (P=9.19e-05) (See Table 5.4).

Table 5.4 BMI multivariate regression correlations with early life and adolescent characteristics.

Variable	β	Std error	P-value
Maternal pregnancy hypertension (Y/N)	1.122	0.340	0.001
Maternal history of diabetes (Y/N)	-0.174	0.076	0.023
IRSAD	-0.019	0.006	0.001
Depression anxiety stress score	0.022	0.008	0.009
Adolescent serum HDL-C	-3.410	0.549	8.60e-10
Exclusive breastfeeding duration (months)	-0.112	0.046	0.015
Duration of breastfeeding (non-exclusive) (years)	0.036	0.026	0.166
Sex (1=M, 0=F)	-1.271	0.323	9.19e-05

Adolescents with higher WHR were born to mothers who had higher BMI during pregnancy (P=4.1e-07, Table 5.5) or to younger mothers (P=0.035, Table 5.5). In addition, higher WHR was observed in adolescents from disadvantaged socioeconomic backgrounds (P=0.015, Table 5.5), much like those with high BMI. The duration of breastfeeding was not associated with WHR. Independent of the previous models with TL analysis, a different model of linear regression was used to examine associations between WHR and early life and adolescent characteristics. The results of the initial data analysis by correlation revealed several covariates correlated with WHR. The covariates with the strongest correlation were then added to the linear regression model. In this model higher WHR trended with shorter telomeres (P=0.076, Table 5.5). Unlike BMI, analysis also showed that male sex was correlated with increased WHR (P<2e-16, Table 5.5).

Table 5.5 Waist to hip ratio correlations with early life and adolescent factors

Variable	β	Std error	P-value
Maternal pre-pregnancy BMI (kg/m²)	2.913e-03	5.694e-04	4.1e-07
Maternal age at birth (years)	-9.582e-04	4.535e-04	0.035
IRSAD	-6.645e-05	2.731e-05	0.015
Duration of breastfeeding (month)	6.429e-04	3.643e-04	0.078
Relative Telomere Length	-5.561e-03	3.123e-03	0.076
Sex (1=M, 0=F)	4.606e-02	4.649e-03	<2e-16

TL associations with BMI change from infancy to adolescence

Previously in this cohort, our research group has demonstrated that complex gene-early life environment interactions influence childhood obesity risk by impacting BMI growth trajectories from infancy to adolescence (294). However, the mechanisms underlying these interactions are unknown. Given the link between TL and childhood obesity (504,505) and the findings in the present study, it is therefore hypothesised that growth trajectories, as determined by changes in BMI over time, may be related to differences in TL at age 17. Quantile regression mixed-effects analysis was used to assess the relationship between BMI from infancy through adolescence and TL at 17 years of age. TL data were divided into quantiles based on the distribution of telomere lengths in the study population (q10, q25, q50, q75, q90). Growth trajectories were determined by comparing changes in BMI over time for adolescents in the upper TL quartiles relative to those in lower TL quantiles.

BMI declines from ~1 year of age until ~5 years of age at the time of adiposity rebound, after which BMI increases again into adolescence (294). The association between BMI and TL in the present study changed during infancy and childhood (Figure 5.3). In both boys and girls across early life, BMI increased as TL quantiles decreased. The quantile in which TLs were shortest (q10) was associated with the highest BMI and the quantile in which TLs were longest (q90) was associated with the lowest BMI (Figure 5.3). At the age of one year for each quantile, shorter TL was associated with higher BMI. This relationship reversed from about 2-3 years of age. At the time of adiposity rebound for each quantile, shorter TL was again associated with higher BMI. After age 10 and as the BMI trajectory approached age 15, the lines of the upper (+2 SD) and lower (-2 SD) bounds of TL for each quantile began to diverge. After age 15, in each of the lower four quantiles (q10-q75) there was an association with shorter TL and *higher* BMI. However, in the highest TL quantile (q90) beginning from about age 10 to age 17, there was an association with shorter TL and *lower* BMI. These dynamic relationships were observed in both boys and girls.

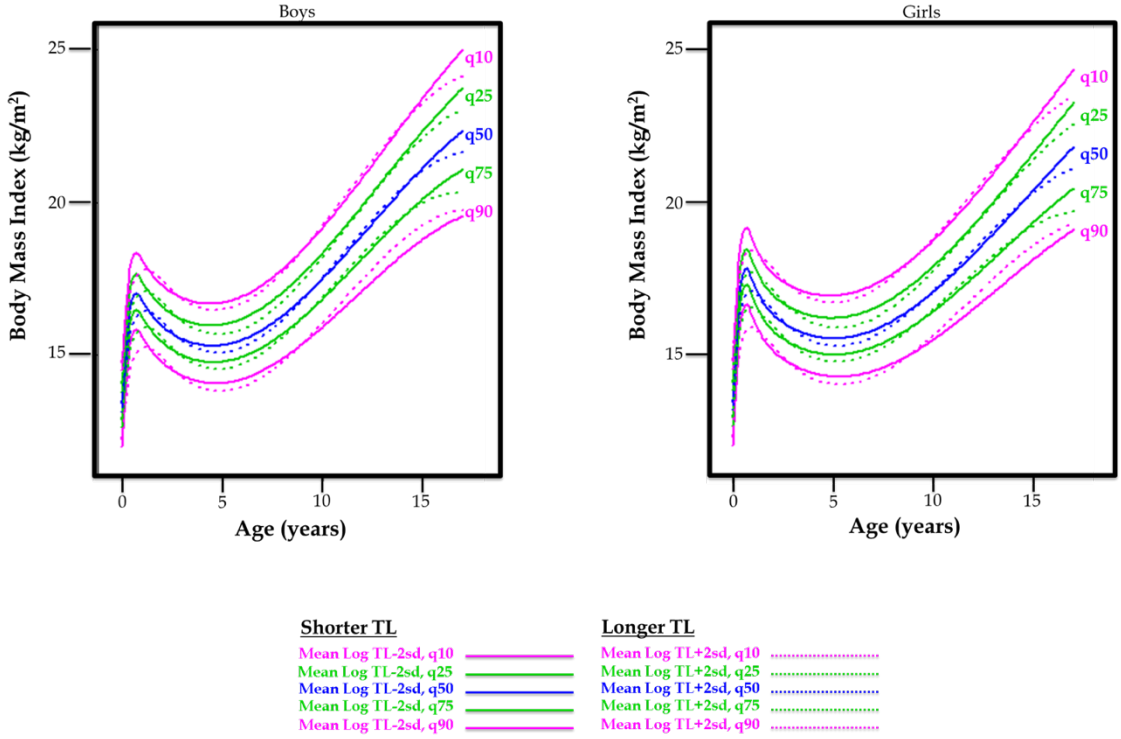

Figure 5.3 Mixed-effects quantile analysis of BMI versus age for Log TL quantiles from birth to 17 years in the Raine Study.

5.4 DISCUSSION

The results of this study show that early life environments (including gestational hypertension and duration of breastfeeding) and risk factors of the metabolic syndrome (BMI and WHR) associate with TL in a healthy adolescent population prior to onset of disease. Not only do these metabolic risk variables correlate with early life environmental exposures in healthy adolescents, but psychological and social stressors may play a role.

TL was shorter in children born to mothers who were hypertensive during pregnancy. These results suggest that hypertension during pregnancy may adversely affect offspring health and longevity through mechanisms that promote cellular dysfunction, and increasing rates of telomere shortening may reflect this. In adulthood, hypertension has been associated with vascular endothelial cell dysfunction, which is characterised by a pro-inflammatory/pro-thrombic phenotype associated with adverse cardiovascular outcomes (726). Exposure to hypertension during pregnancy has been linked with the early development of hypertension in children and increased risk of adult stroke (727-729). It is possible that hypertension during pregnancy may compromise growth and development of organs and tissues in the fetus leading to perturbed metabolism, which may ultimately contribute to a hypertensive phenotype. However, the underlying mechanisms involved in disease pathogenesis are unknown.

Studies in neonates have shown that TL at birth does not differ between preeclampsia/hypertensive and normotensive pregnancies (730,731). Shorter TLs in adolescents of hypertensive pregnancies observed in this study suggests that selective pressures occur postnatally which interact with factors reprogrammed *in utero* in response to adverse conditions such as hypertension. The consequence may then be altered cellular function in the offspring, as indicated by shorter TL. One likely cause of this is exposure to higher levels of oxidative stress in the early postnatal environment.

Oxidative stress is believed to be a key mechanism linking early life adversity to the development of metabolic disease (449). In normal pregnancy at birth the fetus migrates into a hyperoxic environment with greater oxygen tension, which results in increased oxidative stress (732). Infants then endure a prolonged period of increasing oxidative stress which declines to normal adult levels by 6 months, as they adapt to a more oxygen-rich environment (733). To counter the increase in oxidative stress, there is an accompanying increase in plasma antioxidant free radical scavengers for the first 3 months which also declines as oxidative stress levels decrease (733). Yet, in hypertensive pregnancies, the fetus may be exposed to additional intrauterine hypoxemic and oxidative stress conditions (734-736). This in turn may result in a deficient antioxidant defence system and vulnerability to oxidative stress at birth (737).

Animal and human studies have linked prenatal hypoxia to the development of metabolic disease in later life (199). It is well documented that telomeres are

sensitive to oxidative stress (605), and *in vitro* hyperoxia-induced oxidative stress shortens telomeres more rapidly and promotes premature cellular senescence (738,739). Consequently, at the start of postnatal life an overabundance of oxidative stress coupled with limited antioxidant defences may have a lasting detrimental effect on TL and cellular physiology. Notably, shorter telomeres in adults and children are associated with risk factors for metabolic disease (501,504,505). It has been demonstrated that exposure to intrauterine hypertension promotes oxidative stress and DNA damage (740). The shorter telomeres observed in this study complement these earlier findings.

In further agreement with this concept, there was an inverse association between TL and age 17 WHR. WHR is a proxy measure for abdominal visceral fat mass, which, in excess, is associated with greater risk of obesity and its comorbidities (741). Furthermore, higher WHR also positively correlates with markers of systemic oxidative stress (742,743). Furthermore, more studies have demonstrated shorter TL in PBMCs and reduced mtDNA associated with increased adiposity in later life (744). This would be an interesting facet to examine in this cohort once metabolic dysfunctions have taken hold. Given that the shortest TLs were observed in offspring who were exposed to pregnancy hypertension and had higher WHR at age 17, it is very likely that enhanced oxidative stress could be a significant mechanism contributing to the development of metabolic disease. An investigation of mtDNA copy number could be informative of future hyperoxidative state.

In concert with other findings (745), these data have shown a positive association between duration of breastfeeding and TL. However, specifically, findings from the Raine cohort demonstrate that exposure to a minimum of 12 months breast milk feeding may be beneficial, again suggesting that the postnatal environment plays a role in modifying prenatal setpoints in a beneficial manner (715,746,747). The positive effects on TL from the second month only serve to highlight the importance of exposure to breastmilk in the first year of life.

Studies have demonstrated that breastfeeding for longer periods is protective against overweight and obesity both in adults (142,143) and children Furthermore, we have previously demonstrated an interaction between single nucleotide polymorphisms in the fat mass and obesity (FTO) gene and the postnatal nutritional environment, where exclusive breastfeeding was protective against increased BMI associated with the risk allele (294). Collectively, these studies of the Raine cohort suggest that a longer period of breastfeeding, whether exclusive or non-exclusive (mixed with other foods), has beneficial outcomes for offspring late into adolescence both at the level of cellular function and somatic growth. Longer duration of breastfeeding may modify the observed associations between TL and pregnancy hypertension or adolescent WHR discussed above. There may be a dose-response protective effect for the duration of breastfeeding against obesity risk, which is in agreement with previous studies (145,748). This longer exposure to breastfeeding may also contribute to protection against against excessive telomere loss in infancy, and prevent cellular dysfunction.

How longer periods of breastfeeding may protect against cellular dysfunction and hence, telomere shortening, may be a result of the antioxidant capacity of breastmilk (749-751). Antioxidants contained in breastmilk are believed to protect neonates against increasing oxidative stress experienced in early postnatal life (733,749,752). The activity of antioxidants is greatest in colostrum (751,753), the first milk the neonate consumes (754) which is produced and stored in the mammary gland in late pregnancy, and is present for up to to four days postpartum (754). As transitional milk replaces colostrum between days 5 to 9, there is a gradual decline in antoxidant activity until the final mature milk replaces it from day 10 (751,753,754). Clinical studies have shown the beneficial effect of breastfeeding over formula in alleviating oxidative stress in compromised pregnancies (750,755,756). However, there appears to be no difference in antioxidant content of breast milk following normal and compromised births (750). This may apply in the present study, where TLs were shorter in children of hypertensive mothers but were longer overall if the children breastfed for longer. These data suggest that while breast milk antioxidants effectively counter oxidative stress, the hypertensive damage to offspring DNA, evidenced by shorter TL, might be minimised but cannot be reversed. Shorter telomeres have been associated with a smaller haematopoietic stem cell pool (584), which may be a result of hypertension. The long-term effects of microchimerism, in which maternal cells are transported into the organs and tissues of the foetus, cannot be discounted. Increased cell trafficking between the mother and fetus is observed in hypertensive conditions such preeclampsia (757). However, in terms of telomeres, more research in this area would be required.

Furthermore, one cannot discount the probable role of leptin in this telomere dynamic. As previously stated, in various species, adipocyte-derived leptin plays a prominent role in promoting energy expenditure and reducing intake of food (83). This hormone is also abundant in breast milk (758,759). It has already been proposed that leptin consumed by breastfeeding not only plays a role in programming of food intake and appetite in infants but also later in life (760,761). Notably, like antioxidants, leptin concentration is also considerably higher in colostrum then in transitional milk (762). There is also evidence that leptin promotes telomerase activity in a dose-dependent manner (763), which may suggest that antioxidants work in concert with leptin to provide a protective effect. However, only cancer cells such as breast cancer and hepatocellular carcinoma cells have shown this modulatory effect of ectopic leptin expression on telomerase (763,764) and it has not yet been highlighted in the early development studies. This effect could be associated with *de novo* synthesis of telomeres or conferring a protective effect on mitochondria, which in turn, also protects telomeres from ROS damage. Whether continued leptin exposure through longer breastfeeding has a direct impact on telomeres and telomerase is not certain, but warrants further investigation.

The relationship between shorter TL and obesity risk was further demonstrated in the longitudinal modelling of BMI. As previously stated, the quantile in which the telomeres were shortest had the highest BMI (q10), and the quantile in which the telomeres were longest had the lowest BMI (q90). This highlights an inverse

relationship between TL and BMI, and complements several studies linking shorter telomeres to higher BMI, and therefore obesity risk (765-767). Also strengthening this association is the fact that offspring that had the highest BMI values as infants, in each of the lower four quantiles (q10-q75), maintained higher BMIs throughout childhood until adolescence. It has been shown that heavier infants are at an increased risk of becoming obese in childhood, adolescence and adulthood (768). Therefore, the highest BMI infants in the lowest quantiles may be at greater risk of developing obesity in the cohort. Since offspring in q90 have a lower BMI, and hence are smaller in size in infancy, they may be more protected against the development of obesity. In addition, the fact that there is an inverse correlation between TL and BMI from childhood through adolescence may indicate that the effects of harmful early life exposures on telomeres persist and that changes to telomeres are maintained over the course of an individual's lifetime as a marker of obesity. If BMI tracks into adulthood for each quantile, adolescent TL may potentially be an indicator of future obesity in our cohort.

The pattern of divergence in q90 not only demonstrates an association between shorter TL and lower BMI, but also a difference in the age at which the divergence occurs compared to the lower four quantiles (q10-q75). This may be because the q90 lower bound comprises of a portion of the cohort that is underweight (BMI<18.5). Despite having BMI values largely in the healthy range, 6% of the cohort were classsified as underweight at age 17. Being underweight has been associated with shorter TL in instances of disease and depression (769,770). The divergence of the bounds in q90 begins after the age of 10, as adolescence begins,

which may be a critical developmental window, particularly in girls, where altered behaviours towards food, body image, and psychosocial stress, may influence weight trajectories in the peripubertal periods (771-773).

In an attempt to understand which factors may link telomere shortening and the development of metabolic disease, the obesity markers BMI and WHR in adolescents were examined. A positive association was found between age 17 BMI and pregnancy hypertension. This finding is in agreement with previous work showing a positive association between exposure to the hypertensive condition preeclampsia and the BMI in pubertal girls (774). However, what must be noted from the previous study is that this outcome was only apparent in offspring of hypertensive mothers who were obese. While there was no association between maternal obesity, determined by pre-pregnancy BMI, and adolescent BMI, maternal obesity was associated with adolescent WHR. Maternal obesity is a strong predictor of overweight and obesity risk in offspring (775,776), and the association with WHR supports this notion. Given that WHR is a measure of abdominal fatness specifically, this is also in agreement with other findings (777). The link between BMI and hypertension may be explained by exposure to maternal obesity. Maternal obesity is associated with increased risk of several pregnancy complications including GDM and preeclampsia (778) and is the strongest predictor of obesity in children (106). There was a positive correlation between maternal prepregnancy BMI and pregnancy hypertension in the cohort (data not shown). Therefore maternal obesity might be a significant contributing factor to this outcome.

Interestingly, adolescent BMI was inversely associated with family history of maternal diabetes. This may be because mothers had a less severe form of diabetes or were able to manage the disease either through dietary alterations or with pharmacological agents (779,780) which may lead to better to glycemic control. This may be protective in that it could prevent fetal overnutrition by exposure to maternal nutrient excess, and prevent fetal hyperinsulinemia which is associated with future obesity, as described by the fuel-mediated teratogenesis concept (92).

Further analyses of early life factors showed that BMI correlated inversely with exclusive breastfeeding duration. This inverse association compliments a previous study by our research group (294), signifying a protective effect against obesity risk. The interaction between exclusive breastfeeding and the FTO gene variants (294) may provide a likely mechanism by which this occurs. Longer exclusive breastfeeding may also account for the inverse association between BMI and HDL-C, a type of cholesterol that correlates with cardiovascular health (781). The high density lipoproteins in HDL-C have been shown to have a wide range of anti-atherogenic properties, including removing cholesterol from circulation (782) and anti-inflammatory actions (783). This finding is supported by earlier observations made by Parikh *et al.* (784), who also report lower BMI and increasing concentrations of HDL-C in adulthood following breastfeeding in infancy.

The trend towards an association between increased WHR and increased duration of non-exclusive breastfeeding is in agreement with earlier findings within the Raine cohort, which demonstrated increased adolescent obesity risk following non-exclusive breastfeeding (747). Exposure of the infant to complementary feeds during breastfeeding can result in increased food and energy intake, and elevate the risk of childhood obesity (785). Further, while exposure to breast milk may confer some protection against obesity, the protective effect may be much smaller in mixed feeding (786). Therefore, it may be necessary to breastfeed for a longer period when complementary feeds are simultaneously given, to achieve the same degree of beneficial effect as seen with shorter periods of exclusive feeding (787). The World Health Organisation recommends a minimum of 6 months of exclusive breastfeeding for optimal health outcomes (788). The median 6-month duration of non-exclusive breastfeeding in the cohort may not be adequate to fully protect against disease or modified growth trajectories. While, longer non-exclusive breastfeeding can protect against childhood obesity (789) and may be beneficial for telomere maintenance, postnatal exposure to increased energy feeding may increase risk of disease into adulthood.

The study also examined the effects of psychological and social wellbeing on obesity risk and found a positive association between BMI and psychological stress. In addition, higher BMI and WHR correlated with lower socioeconomic status (SES) at 17 years. Lower SES is associated with higher levels of the hypothalamic pituitary adrenal axis (HPA) stress hormone cortisol (790).

Elevated cortisol is linked to greater accumulation of fat in the abdominal region (791) indicated by higher WHR (792). The accumulation of body fat has been shown to also correlate strongly with BMI in childhood and adolescence (793). Since both WHR and BMI correlate with low SES, potentially this may indicate a role for cortisol in pathogenesis of metabolic disease in the cohort. Although I did not uncover an association between TL and SES or psychological stress (data not shown), studies have shown that psychosocial stress exposure (33-35) is linked to shorter TL and concurrently increases oxidative stress (507). Animal studies have shown that prenatal stress as such can reduce the HPA axis function in response to stressors if they had previously been exposed to prenatal stress (794). Findings in the cohort have demonstrated a reduced response to stress challenges, estimated by increases in cortisol, in adolescents who had been exposed to prenatal stress. In addition, reduced cortisol was also associated with obesity and BMI (unpublished work). It is likely that exposure to hypertension *in utero* may have compromised the HPA axis, elevating cortisol which contributed to telomere shortening, but blunting the postnatal HPA response such that other stressors such as SES or psychosocial stressors might not have as significant an effect. A diminished HPA axis may be indicative of aging (795).

The finding of an inverse association between adolescent WHR and maternal age may be consistent with the positive association between adolescent WHR and SES, as younger maternal age has been linked to indicators of lower SES such as lower education and income, and poorer health in offspring (796,797). Hence,

greater maternal age may indicate higher SES, and therefore better health outcomes in children.

Previously it has been reported that TL is longer in females than in males, both in adolescence (29) and in adulthood (798,799). While work does highlight increased susceptibility of male telomeres to early life stress (800), there were no sex differences found in adolescent TL. It is possible that this finding may not reflect the rates of telomere shortening between boys and girls, which differ due to various factors including higher female estrogen levels. Estrogen more easily activates telomerase, the enzyme that synthesises telomeric repeats to maintain telomeres (801), and acts as an antioxidant to protect female TL (802). Also, as this was the first time that TL was measured in the Raine cohort, it was not possible to evaluate the rate of telomere shortening. It was, however, possible to observe that TL was negatively correlated with age, as shown in previous studies (394,395).

In conclusion, this study has identified novel associations between a child's early-life environmental exposures and the length of telomeres in peripheral blood cells at 17 years of age. These data show that adverse early life environments are associated with shorter TL and measures of adiposity in adolescence. Conversely, this study also demonstrates beneficial effects in that longer periods of breastfeeding may be protective against telomere shortening by potentially by helping to counter increasing levels of oxidative stress. Thus, breastfeeding may be a significant contributing factor, reducing prevalence of

obesity and metabolic disease risk in adolescence via mechanisms associated with cellular metabolism and aging.

CHAPTER 6

FINAL DISCUSSION

I have observed the effects of both prenatal undernutrition and overnutrition on telomeres in this thesis, as well as the interaction between maternal physiological state and telomere length (TL). Two hypotheses were tested using a complementary approach of early-life studies in both mice and humans. First, it was proposed that unfavourable early-life environments (antenatal, postnatal, or a combination of both) would affect telomere length before metabolic disease manifested itself. Second, it was proposed that a telomere-related mechanism underlies how a suboptimal early environment impairs metabolism in offspring.

Fetal hepatic telomeres did indeed shorten in the case of dietary restriction and high-fat diet exposure during gestation. On the other hand, mouse fetal gut and blood telomeres were spared despite exposure to HF and DR during pregnancy. This is significant because it shows that telomeric DNA damage is linked to both under- and over-resourced intrauterine environments and that there is a heterogeneity attached to the telomeric response. The shelterin protein TIN2 had a trend towards increased localisation to the cytoplasm in the livers of DR offspring, which is an interesting complement to the loss of telomeres. The TIN2 localisation between the cytoplasm and nuclei in the HF livers is comparable to that in the CON group. The direct identification of TIN2 protein whose behaviour varies depending on the prenatal diet consumed makes this finding interesting.

The role of TIN2 in telomere maintenance is to prevent incorrect identification of telomeric DNA damage (639) as well as recruit telomerase for telomere lengthening (428,429). TL can be negatively regulated by TIN2, an important member of the shelterin complex that interacts with TRF1, TRF2, and POT1 (803) and plays a telomerase-independent role in embryonic development (804). Conversely, TIN2 has also been shown mediate telomere length by recruiting telomerase, in a different role from its protective effects on telomeres (805). This provides an interesting contrast for examination in future studies. How this shift in localisation of TIN2 affects its function in fetal livers warrants further investigation.

Not limited to the animal studies, shorter telomeres in peripheral blood cells were linked to previous presence of maternal hypertension in the Raine cohort. The late adolescent Caucasian population studied in the cohort is healthy and on par with populations free of disease in other developed countries. Despite this, telomere shortening was already evident in those whose BMIs were higher despite not falling into overweight or obese categories. Overall, these results support the first hypothesis and demonstrate the significant influence of early environments on highly adaptable telomeres.

Furthermore, the plasticity of telomeres seems largely dependent on developmental window. It may be the case that telomeres in some organs and

tissues are only impacted *in utero*, while the effects on others only present postnatally. These differences in the timing of telomere effects implies that it may be possible to pinpoint interventional timepoints. The Raine Study presents a period that may be ripe for an intervention to occur in humans, the first 12 months of life. Infants who breastfed for longer periods of time (at least 12 months) in early postnatal life had longer telomeres. Additionally, these findings imply that breastfeeding for at least 12 months may mitigate the negative effects of gestational hypertension.

While 12 months was optimal, even an exposure of 2 months to breastfeeding in the first year was beneficial for telomeres in late adolescence. As a result of the known antioxidant properties of breast milk (749-751), this observation may suggest the involvement of ROS regulation in long term telomere maintenance. Breast milk antioxidants are thought to protect neonates against increasing oxidative stress in early postnatal life (733). The likelihood of the importance of this early postnatal window for enhancing metabolic outcomes as well as telomere maintenance in later life is demonstrated by the inverse relationship between BMI and the length of exclusive breastfeeding at age 17.

From these findings it is hypothesised that the mother's various antioxidant-rich milks (749-751) create a biochemical barrier that protects the telomeres in peripheral blood cells. In studies of pigs, prior to weaning, continued suckling

has been shown to improve intestinal function, increase scavenger superoxide dismutase activity, and decrease ROS concentration (806). Mechanistic studies might be needed to support this in the Raine cohort. Furthermore, extending the animal studies to adulthood could shed light on how the suckling period, a crucial window for developmental plasticity, influences the postnatal telomeric response to stress. The challenges in Chapter 3 and 4 regarding Dihydroethidium staining have already been outlined. And further work is necessary to confirm ROS involvement in nuclear DNA damage and hence contribution to the shorter telomeres observed. The absence of oxidative damage to proteins or lipids, however, suggests that ROS may not be present in sufficient quantities to cause irreversible macromolecular tissue damage.

These findings initially appear to suggest that telomeres play a direct role in the emergence of future metabolic disease. Poor prenatal and postnatal environments can increase the risk of many diseases. But not all exposed to such environments develop disease. Studies into the fundamental causes of disease programming have a tendency to focus on specific cell types and organs in relation to their functional impairment. Because proliferation continued as normal and *mTert* mRNA levels increased in both HF and DR fetuses, it appears that cells in fetal livers in this case were not compromised to the point where they were unable to perform their normal developmental functions. Telomerase modulates development-related signalling pathways and promotes proliferation in resting stem cells (807), which may be the case in these studies. However, from

these data, it is still unclear how *mTert* contributes to the preservation of tissue homoeostasis. The increased *mTert* gene expression in fetal livers may be due to telomerase maintenance of telomeres or other functions, such as Wnt signalling linked to the aforementioned proliferation, or associated with increased ROS. To provide an answer to these queries, additional analyses incorporating telomerase activity assays and pathway analysis would be needed. The implications of the telomere shortening for overall cellular functionality are thus brought up by these data.

In this thesis, it is posited that the developmental origins of health and disease may be influenced by interactions between the genome and environment. The genome is not only nuclear, but also encompasses the mitochondrial genome. If combined with an investigation into variations in mitochondrial DNA, TL might be a more significant indicator of future disease. In addition to demonstrating that TIN2 can localise and interact in both the nucleus and cytoplasm (373), Chen and colleagues also shed light on TIN2 function in the regulation of mitochondrial metabolism (528). They demonstrated that silencing TIN2 augmented mitochondrial ATP synthesis and therefore glucose metabolism, and lowered ROS concentration (528). The increase in cytoplasmic TIN2 localisation in DR livers may be associated with inverse outcomes in mitochondria, leading to increasing ROS. However further measures are required to verify this.

Alterations in metabolism can occur at both the cellular and organism level due to DNA damage (808). The study of mtDNA copy number and ROS damage to macromolecules was used as a proxy for mitochondrial dysfunction and aberrant metabolism. From the mouse studies, the changes in mtDNA copy number seemed to provide more precise information about the type of insult the fetuses were exposed to, despite the fact that there was a similar decrease in TL for both DR and HF fetuses. In the HF livers, there was an increase in mtDNA present, whereas in the DR group, there was no change. Further to that, there was proof that suggested mitochondria had been modified in HF fetuses to facilitate the uptake and metabolism of fatty acids. The processes responsible for the pathogenesis of metabolic diseases may once again be interrupted if an intervention can be implemented during this critical developmental stage.

Ultimately, the second hypothesis is not fully supported as there are no data directly tying the telomere shortening mechanisms to mitochondrial dysfunction. It was expected that animals with shorter telomeres would also have compromised mitochondria as a result of suboptimal diets (under- and overnutrition) during pregnancy. Previous research has highlighted a potential mechanism for shorter telomeres contributing to mitochondrial dysfunction and the generation of ROS (506,523) and trapping cells in a vicious cycle that advances senescence (809). While it is likely that this may be the case, more in-depth studies are required to verify this. This outcome in fetuses may help to shed light on the telomere-related mechanisms causing these effects in response to various diets.

What is clear is that mitochondria are affected differently depending on the diet in question. On the basis of these results, telomeres appear to signpost, but are not the mechanistic engine driving programming effects.

Also, important to consider are the effects on mothers. While maternal blood and bone marrow telomeres in the DR group were significantly shorter than in the CON group, there was an increase in the number of maternal blood mtDNA copies. The high energy demands following fertilisation and implantation as well as the requirement for ongoing metabolite delivery to the growing fetus (810,811) could be responsible for the rise in DR mtDNA copy number. It stands to reason that increased mitochondrial biogenesis and a more efficient respiratory apparatus would be required to maximise this process. It is also possible that increased maternal mtDNA synthesis may be related to increased oxidative stress in order to replace damaged mitochondria (621,622). However, no measures of oxidative stress in maternal blood were taken, which is a limitation.

One aspect of mtDNA analysis that hasn't been addressed in this thesis is the potential significance of circulating cell-free mitochondrial DNA (ccf-mtDNA), which is present during pregnancy under physiological homeostasis (812). When cellular stress occurs, mtDNA can degrade and mitochondrial autophagic responses can arise, causing a cytosolic release of mtDNA that may eventually enter the blood circulation and contribute to pregnancy pathology (813,814). In

contrast to typical term pregnancies, complications leading to intrauterine growth restriction (IUGR), and placental abruption can increase levels of whole blood mtDNA, according to research by Colleoni and associates (815) and more recently Williams *et al.*(816). In fact, clinical studies have also shown that a rise in ccf-mtDNA is a reliable indicator of pregnancy complications such as preeclampsia, particularly in later gestation (817,818).

It is possible that ccf-mtDNA may be a contributing factor to the measure of increased maternal mtDNA in DR fetuses, given the result from blood was sampled late in gestation in the DR mouse study. The concurrent telomere shortening in dam blood may be the indicator of a pregnancy in distress. As observed in this instance, the combination of telomere length and mtDNA content may potentially provide a predictive marker for future disease. However, additional research would be necessary to confirm the equilibrium of the maternal PGC-NRF-TFAM biogenesis pathway against mitochondrial autophagy and ccf-mtDNA in blood. Furthermore, if this dietary restriction model is used again, sampling of maternal circulatory ccf-mtDNA at different gestational stages could unveil a mechanism for programming of disease in the offspring.

Although an increase in mtDNA might be viewed favourably, any therapeutic action intended to promote mitochondrial biogenesis would need to be used with caution. Not all increases in mitochondrial DNA may be associated with favourable outcomes, as suggested in HF fetuses and DR mothers. It has been

shown in earlier studies using mouse models that an increase in mtDNA may result in nucleoid enlargement, which in turn can lead to a defect in the processes of mitochondrial transcription and replication (819). This opens up a new line of enquiry as to whether a comparable maladaptive nucleoid response might accompany the increased mtDNA copy detected in these studies. If so, this research could need to be expanded to look into the existence of deletions in the mitochondrial genome as a result of the different diets. Furthermore, studies on epigenetic modifications related to both telomeres and mitochondria crosstalk may further illuminate this work. The ability to analyse ccf-mtDNA in hypertensive mothers in the Raine cohort could provide a biomarker for not only identifying at-risk pregnancies but also highlighting a mechanistic pathway to metabolic disease in adolescents.

Combining TL and mtDNA may yield a useful indicator of cellular distress and future programming of disease. In the context of pregnancy, maternal weight gain, specifically daily weight gain in mice was shown to be a stronger measure than total weight gained. The DR study found that E10.5 predicted fetal TL and E13.5 predicted maternal TL at the end of pregnancy. These timepoints may be potential windows for intervention. According to the previously described Theiler stages of pregnancy (259,260), this corresponds to 28-3 and 48-50 days in human pregnancy respectively. Interestingly, maternal weight gain only predicts TL in maternal and fetal blood. To determine whether this timing extrapolates to a clinical setting, more research would be necessary. Because these studies would

involve using human subjects, many confounders would need to be taken into consideration, including socioeconomic, psychosocial, and other clinical variables. The DR study revealed that TL can be predicted by the amount of maternal weight gained each day compared to baseline, which is one of this thesis' most intriguing findings. Even though body weight gain is a rough indicator, if it is possible to link the predicted telomere length to impacts on mitochondria, it may prove to be a helpful diagnostic tool in the future.

Future studies

Further research would be required to clarify the mechanisms linking telomeres to programming outcomes based on this body of data. In addition to confirming whether telomerase activity is comparable to *mTert* gene expression, the examination of *mTert* signalling related to mitochondrial function would be a feasible extension of this work. Studies have shown that the trafficking of the TERT protein to mitochondria has a protective effect for cells against oxidative stress, mtDNA damage, senescence and apoptosis (575,576,662). In the brains of older animals, TERT has already been demonstrated to be diet-sensitive, with recovery of mitochondrial function, a decrease in ROS, and an increase in TERT protein being noted in response to rapamycin supplementation in chow (820). Whether this sensitivity extends to *mTert* in HF and DR fetal livers in a similar fashion will require further examination.

Although oxidative stress in macromolecules was not seen in these studies, given the effects on telomere length, it might still be present at lower levels. It would be beneficial to confirm increased ROS using alternative methods. It would then be worthwhile to investigate whether *mTert* localises to mitochondria in a manner similar to how TERT has previously been described to do (576). This research may shed light on *mTert* function in DR and HF fetuses even in the absence of the telomerase holoenzyme.

Following this initial cross-sectional study with TIN2 it would next make sense to study the other proteins in the shelterin complex to see how the differing diets might affect them. Seeing as a metabolic role for TIN2 has been established (528), it would be interesting to observe the effects of both under- and overnutrition on the shelterin complex as a whole.

This work was not able to be carried forward to postnatal time points. However, earlier work in our research group has already demonstrated that undernutrition programs metabolic disorders in this mouse model (201,211). It may be useful to study the shelterin response to interventions such as ω-3 fatty acid supplementation, which is shown to reduce or prevent insulin resistance and improve glucose tolerance in animal studies (821,822). As well as extending the research for both to include telomerase, it would be instructive to determine whether such dietary supplementation would have a postnatal effect on mouse

liver telomeres that was comparable to that observed in the Raine cohort from extended breastfeeding.

Overall, the observations in this thesis lay the groundwork for highlighting possible metabolic alterations that may increase susceptibility to disease in adulthood. Whether nutritional adversity from overnutrition or undernutrition, or exposure to hypertension, the eventual outcomes appear to be largely dependent on the environment in postnatal life. Further research will be required to determine effective therapeutic interventions that can translate from the laboratory into clinical settings.

CHAPTER 7

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