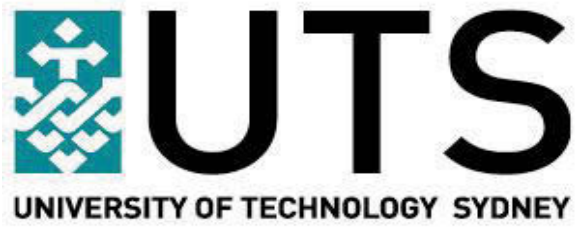
Effects of Maternal Cigarette Smoke Exposure on Renal and Other Health Outcomes in Mice Offspring

A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy

By

Ibrahim Al-Odat



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Dedication

To the memory of my late mother

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ACE	angiotensin-I-converting enzyme
AT	angiotensin type
ATGL	adipose triglyceride lipase
AUC	area under the curve
BMI	body mass index
BMP	bone morphogenetic protein
BW	body weight
CKD	Chronic kidney disease
CPT	carnitine palmitoyl transferase
DDti3	DNA damage-inducible transcript 3
E	embryonic day
ECM	extracellular matrix protein
eNOS	Endothelial nitric oxide synthase
ET	endothelin
Eya1	eyes absent 1
FASN	Fatty acid synthase
FGF	Fibroblast growth factor
FOXO	forkhead box protein O
GDNF	glial-cell line-derived neurotrophic factor
GFR	Glomerular Filtration Rate
GFRα 1	GDNF-family receptor α 1
Glut	Glucose transporter
h	hour
H&E	hematoxylin and eosin
HRP	horse radish peroxidase
HSL	hormone-sensitive lipase
IHC	immunohistochemistry
i.p	intraperitoneally
IPGTT	Intraperitoneal glucose tolerance test
IGF	Insulin-like growth factor
IL	interleukin
IM	intermediate mesoderm

IUGR	intrauterine growth retardation
LBW	low birth weight
LC	L-Carnitine
LIF	leukaemia inhibiting factor
mA	milliAmpere
MCP	monocyte chemoatractant protein
MM	metanephric mesenchyma
MSA	murine serum albumin
NC	nephrogenic cord
NEFA	non-esterified fatty acids
NHEBSA	normal horse serum with bovine serum albumin
NFκB	nuclear factor kappa B
NO	nitric oxide
NOS	NO synthase
Pax	paired box transcription factor
PGC	Peroxisome proliferator-activated receptor gamma coactivator
P	postnatal day
PAS	periodic acid Schiff
PPAR	peroxisome proliferator-activated receptor
RAS	renin-angiotensin system
RCF	relative centrifuge force
RET	receptor tyrosine kinase
ROS	reactive oxygen species
RV	renal vesicle
SE	smoke exposure
SEM	standard error of the mean
TG	triglyceride
TGF-β	transforming growth factor-β
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNFα	tumour necrosis factor α
T-TBS	tween twenty trise buffer saline
UB	ureteric bud

UV	ultra violet
W13	week 13
WNT	wingless–type MMTV integration site family member
Wt1	Wilms tumor 1
Xbp	X-box binding protein

List of peer reviewed Publications

- **Al-Odat I**, Chen H, Chan Y-L, Sawiris A. Wong MG, Gill A, Pollock C, Saad S, (2014). The impact of maternal cigarette smoke exposure in a rodent model on renal development in the offspring. *PLoS ONE* 9(7): e103443.

- Stangenberg, S., Nguyen, L.T., Chen, H., **Al-Odat, I.**, Killingsworth, M.C., Gosnell, M.E., Anwer, A.G., Goldys, E.M., Pollock, C.A. & Saad, S. 2015, 'Oxidative stress, mitochondrial perturbations and fetal programming of renal disease induced by maternal smoking', *The International Journal of Biochemistry & Cell Biology*, vol. 64, pp. 81-90.

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List of conference abstracts

Oral presentations

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Poster presentations

- **Ibrahim Al-Odat**, Hui Chen, Amgad Zaky, Carol Pollock and Sonia Saad, Maternal L-Carnitine Supplementation Reversed Kidney Underdevelopment and Adulthood Renal Dysfunction in Male Mice Offspring of Cigarette Smoke Exposed Dams. *New Horizons Conference* 2015, University of Technology Sydney, Sydney.

- **Ibrahim Al-Odat**, Hui Chen, Yik Lung Chan, Carol Pollock and Sonia Saad The prophylactic effect of l-carnitine against glucose intolerance and metabolic disorders induced by maternal smoke exposure in female offspring mice. *The 51th Annual Scientific Meeting of the Australian and New Zealand Society of Nephrology*, 2015. Canberra, ACT, Australia.

- **Ibrahim Al-Odat**, Hui Chen, Yik Lung Chan, Amgad Sawiris, Carol Pollock and Sonia Saad. The effect of maternal cigarette smoke exposure on offspring predisposition to kidney disease in male and female mice. *The American Society of Nephrology annual meeting*, 2014, Philadelphia, PA, USA.

- **Ibrahim Al-Odat**, Hui Chen, Amgad Sawiris, Carol Pollock and Sonia Saad. l-carnitine supplementation during gestation and lactation improve glucose intolerance induced by maternal smoking in the offspring. *The 50th Annual Scientific Meeting of the Australian and New Zealand Society of Nephrology*, 2014. Melbourne, Victoria, Australia

- **Al-Odat, Ibrahim**, Chen, Hui, Chan, Yik Lung, Sawaris, Amgad, Pollock, Carol and Saad, Sonia. Gender related differences in susceptibility to kidney disease in the mice offspring due to maternal cigarette smoke exposure**.** *New Horizons Conference 2014*, Kolling Institute of Medical Research, Sydney.

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Abstract

Background

The burden of kidney disease is significantly increased worldwide. Developmental programming of non-communicable diseases is an established paradigm. Therefore, there is increasing attention to the contribution of intrauterine and early post-natal environmental insults to the risk of adulthood kidney diseases. One of such insults is maternal cigarette smoke exposure (SE), which is associated with intrauterine growth retardation (IUGR) and some adulthood diseases in offspring. However, it is unknown whether maternal SE can increase the risk of developing chronic kidney disease (CKD) in offspring. Maternal SE was also shown to increase renal oxidative stress in offspring; while the anti-oxidants L-carnitine (LC) supplementation has been shown to be beneficial in many human diseases, which may benefit such offspring.

Objectives

This thesis aimed to study the effect of maternal SE on, 1) kidney development and renal function; 2) glucose and lipid metabolic markers in the liver, and; 3) the effect of maternal LC supplementation during gestation and lactation on health outcome of the SE offspring.

Methods

Female Balb/c breeder mice were exposed to cigarette smoke for 6 weeks prior to mating, during gestation and lactation; with sham exposure as control. A subgroup of the SE dams was treated with LC (SE+L-C) during gestation and lactation via drinking water. The offspring were sacrificed at postnatal day (P)1, P20 (weaning age) and 13 weeks (mature age). Blood, urine, kidneys and livers were collected. Renal Morphology and function, renal development factors, and metabolic markers in the liver were examined in the offspring.

Results

Reduced nephrons number, enlarged glomerular size and altered renal expression of developmental factors such as glial cell line-derived neurotrophic factor (GDNF) and paired box binding protein (Pax)2 were observed in the offspring. This was linked to increased mRNA expression of pro-inflammatory marker, monocyte chemoatractant

protein (MCP)-1, and urine albumin/creatinine ratio at adulthood in the male offspring. However female offspring were protected from such maternal effect. Both genders developed glucose intolerance. mRNA expression of IL-1β and TNF-α was upregulated in the liver in the female offspring, with hyperlipidemia. Maternal LC supplementation during gestation and lactation ameliorated these changes in the offspring by maternal SE.

Conclusion

Maternal SE led to kidney underdevelopment, adulthood renal dysfunction, lipid and glucose metabolic disorders, and increased renal and liver inflammation in the offspring in a gender-specific manner. Maternal LC supplementation has a beneficial role in ameliorating the detrimental impact of maternal SE on the offspring.

Chapter One

1

General Introduction

and

Literature Review

1.1. General introduction

It is now recognised that kidney disease is an important global public health burden associated with morbidity and premature mortality, decreased quality of life and increased health-care expenditure (Centers for Disease Control and Prevention 2007). Unfortunately, more attention is given to the end-stage kidney disease compared to the early-stage kidney disease (Eknoyan 2008). This is because early-stage kidney disease is normally asymptomatic, thus most patients with first-stage kidney disease remain undiagnosed until more than 50% of normal kidney function has been lost before when any symptom is manifested. By this stage, the health care cost is significantly increased compared with the early-stage (Australian Institute of Health and Welfare 2009; Eknoyan 2008).

Kidney disease has been defined as a heterogeneous group of disorders that affect kidney structure and function (Eckardt et al. 2013). CKD refers to all kinds of renal conditions that last at least for three months and are usually associated with kidney damage and/or kidney dysfunction, such as reduced glomerular filtration rate (GFR), protein in the urine (proteinuria or albuminuria), or blood in the urine (haematuria) (Australian Institute of Health and Welfare 2009; Eckardt et al. 2013). Acute kidney injury is a subgroup of acute kidney disease which causes sudden changes in renal function within one week (Eckardt et al. 2013). The pathological conditions in patients with acute kidney disease last for less than three months (Eckardt et al. 2013).

The prevalence of CKD is more than 10% globally and more than 50% in certain populations that are at high risk, such as African American and indigenous Australian populations (Cueto-Manzano et al. 2014; Eckardt et al. 2013). Its prevalence is also high in aging population with more than 20% in the ≥ 60 years old and 35% in the ≥ 70 years old (Eckardt et al. 2013). Around 1.5 million patients with CKD are undergoing renal replacement therapy worldwide (Uhlenhaut & Treier 2008). In Australia, 1 in 7 Australians who are 25 years old and above can have one symptom of CKD (Mallett et al. 2014). The incidence of end-stage kidney disease among the Aboriginal Australian and Torres Strait Islander populations are 30 times higher than the general Australian population (Australian Institute of Health and Welfare 2011). As such, CKD is considered as the 12th leading cause of death and top 17th cause of disability around the world (Nugent et al. 2011).

Several studies have reported the link between the intrauterine environment, fetal development and the development of adulthood diseases (Barker 1995; Bezek et al. 2008; Chen et al. 2013; Koleganova, Piecha & Ritz 2009). These kind of studies led to the hypothesis of 'a developmental origin of adult diseases', which highlights the importance of the intrauterine environment in programming permanent physiological changes in the growing foetus that can predispose it to various diseases in postnatal life, such as cardiovascular and kidney diseases (Barker 2013; Kim, Friso & Choi 2009).

Maternal cigarette SE during gestation and lactation is a significant insult that can disturb normal fetal and postnatal development in the offspring (Hackshaw, Rodeck & Boniface 2011; Koleganova, Piecha & Ritz 2009). The chemicals in the cigarette smoke can cross the placental barrier (Hackshaw, Rodeck & Boniface 2011) and be secreted into the breast milk which affect the nutritional and immunologic status in the offspring (Santos-Silva et al. 2011). Nicotine can restrict placental blood flow to reduce oxygen and nutrient supply to the growing foetus disrupting fetal development (Mantzoros et al. 1997). Importantly, SE during gestation can induce oxidative stress in both the mothers and the fetus which might play a role in the development of adulthood diseases in the offspring (Bruin et al. 2008).

This chapter will review the theory of the developmental origins of adulthood disease, early-life kidney development and the growth factors involved in this process, epidemic of smoking during gestation, and the short-term and long-term health consequences of maternal smoking in the offspring and its impact on the development of kidney diseases. The hypothesis and the aims of my PhD research project are included at the end of this chapter.

1.2. The kidney

1.2.1. Background

The kidney is a complex organ composed of blood vessels, nephron and branches of collecting system (Costantini 2010). Nephron is the basic excretory unit of the kidney which consists of many specialized cells forming the glomerulus (the filtration unit), proximal tubule, loop of Henle, distal tubule and connecting tubule (Figure 1.1). The

collecting system consists of cortical and medullary collecting ducts, calyces, papilla and ureter (Costantini 2010).

The main function of the kidney is to filter the blood in order to regulate the level of water and various chemicals in the body. The vital substances, such as water, glucose and other electrolytes, are reabsorbed back into the bloodstream and the waste products, such as urea are cleared into the urine. In addition, kidney produces some essential hormones in the body, such as erythropoietin to stimulate the synthesis of haemoglobin (Redmond & McClelland 2006).

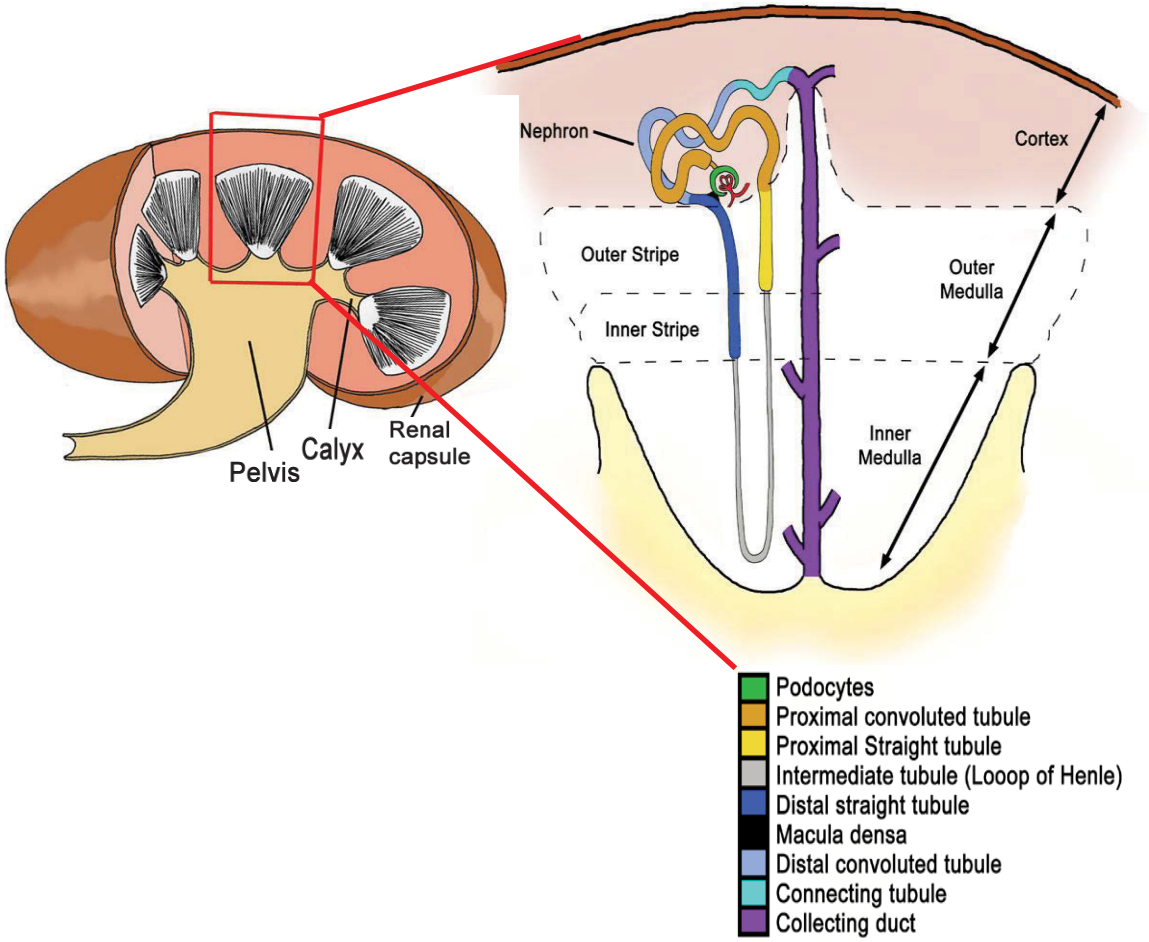


Figure 1.1: Kidney and nephron anatomy. Adapted from (Davidson 2009).

1.2.2. Kidney morphogenesis

In vertebrates, the kidney is derived from the intermediate mesoderm (IM) of the embryo located lateral to the somites (Costantini 2010) (Figure 1.2A). The nephric duct (also called the Wolffian duct) originates from the dorsal part of the embryonic IM layer. The tip of the nephric duct elongates caudally toward the region of cloaca between embryonic day (E) 8.0 and E9.5. A population of mesenchymal cells known as nephrogenic cord (NC) are remained in the ventral part of the IM. Both rostral Wolffian duct and NC form the early transient stages of the kidney, the pronephros, mesonephros and primitive renal tubules (Costantini 2010; Dressler 2002; Lechner & Dressler 1997; Michos 2009). The pronephros arises first. It is functional during development only in lower vertebrates and consists of simple tubules that empty into the primary nephric duct (Rumballe et al. 2010). The mesonephros develops caudally along the nephric duct when the pronephros degenerates. It consists of glomerulus and proximal tubule-like structures. It is functional in adult fish and amphibians and during embryogenesis in mammals (Rumballe et al. 2010). The metanephros is the final stage of the three embryonic organs in mammals which forms the permanent kidney (Rumballe et al. 2010).

Mammalian kidney morphogenesis is a complex process relying on the inductive interaction between two embryonic structures, the epithelial Wolffian duct-derived ureteric bud (UB) and metanephric mesenchyma (MM), which reciprocally regulates cell development and differentiation (Bard 1992; Lechner & Dressler 1997; Oliver & Al-Awqati 1998; Schedl & Hastie 2000; Weller et al. 1991). This process starts with metanephros formation (Dankers et al. 2011).

In mice, metanephros form at E10 (Merkel, Karner & Carroll 2007; Schedl & Hastie 2000). Metanephros formation involves UB outgrowth and branching, and the differentiation of MM cell into epithelial cells (Figure 1.2) (Abdel-Hakeem et al. 2008). At E10.5, a group of epithelial cells at the caudal end of the Wolffian duct, which originate from the dorsal part of the IM, swell dorsally by the inductive signals from the adjacent MM within the NC to form the UB, which subsequently invades the MM (Figure 1.2A) (Costantini et al. 2011; Merkel, Karner & Carroll 2007; Zohdi et al. 2012). At E11.0, the UB penetrates into the blastema of the MM. It bifurcates and forms a T-shaped structure (Figure 1.2B), and then branches repetitively during the next few

days (Figure 1.2C). The UB and its branches form the renal collecting ducts and the ureter respectively (Costantini 2010; Costantini et al. 2011; Dankers et al. 2011; Dressler 2009; Merkel, Karner & Carroll 2007; Michos 2009; Schedl & Hastie 2000; Zohdi et al. 2012). At E12.5, the MM cells get closer to each other, condense, and then are divided into two separate aggregates, forming two caps at the tip of the UB (Figure 1.2D) (Costantini et al. 2011; Glassberg 2002; Merkel, Karner & Carroll 2007; Zohdi et al. 2012). At E14.5, a group of cells within the MM convert to epithelial cells and rearrange themselves into a spherical shape which forms the renal vesicle (Figure 1.2D) (Zohdi et al. 2012). Then the renal vesicle is transformed into a comma-shape followed by an S-shape (Figure 1.2E). This structure is developed into different sections of nephron tubules, including the proximal tubule, loop of Henle and the distal tubule, in addition to the glomerulus (Costantini et al. 2011; Mantzoros et al. 1997; Zohdi et al. 2012).

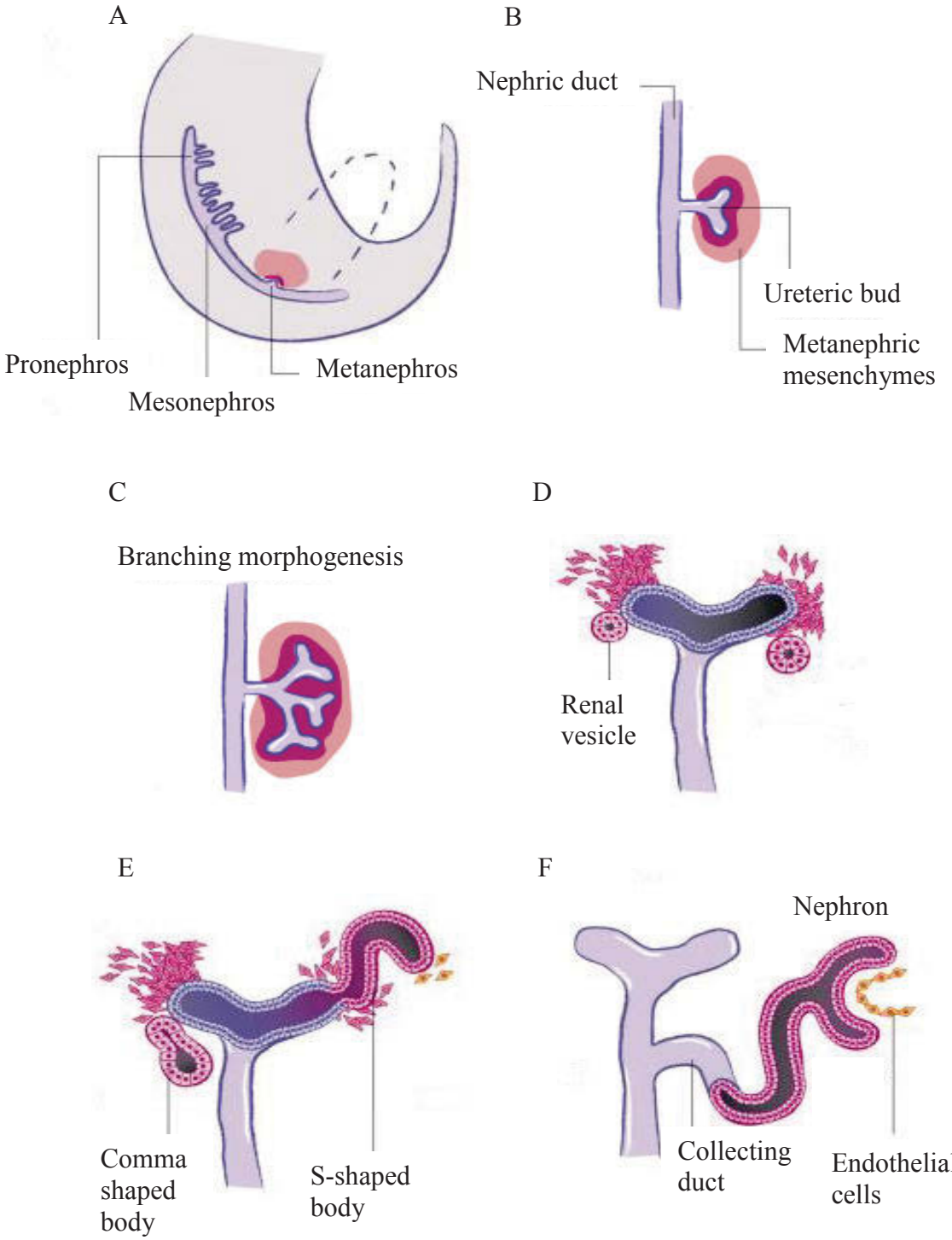


Figure 1.2: kidney development and morphogenesis in mammals. This process involves UB outgrowth (A), T-shape formation and mesenchymal cells aggregation, condensation and transformation into epithelial cells (B), UB branching within the MM (C), formation of renal vesicle (D), formation of comma-shape and S-shape (E) and glomerulogenesis (F). Adapted from (Uhlenhaut & Treier 2008).

During the glomerulogenesis (Figure 1.2F), the inner cells of the lower end of the S-shape differentiate into glomerular podocytes and the outer cells into parietal podocytes (Figure 1.2F) (Zohdi et al. 2012). At the same time, the endothelial precursor cells within the MM invade the lower cleft of the S-shape and differentiate to form the capillary loop in the glomerulus, which is the filtration system in the kidney (Zohdi et al. 2012). The podocyte epithelium and the developing endothelial cells participate in the synthesis of the glomerular basement membrane which later integrates with the podocytes and differentiate into foot processes, the fenestrated endothelium (Bard 1992; Dankers et al. 2011; Glassberg 2002; Lechner & Dressler 1997; Schedl & Hastie 2000; Zohdi et al. 2012). This process continues after birth in humans for a week (Bard 1992; Dankers et al. 2011; Dressler 2002; Lechner & Dressler 1997; Merkel, Karner & Carroll 2007; Michos 2009; Oliver & Al-Awqati 1998; Schedl & Hastie 2000). Thus, renal cells that are the lineage of UB and MM are derived from the IM; while the third renal cell lineage, the stromal cells which develops to interstitial cells, is derived from the somitic mesoderm (Costantini et al. 2011). In mice, glomerulogenesis and glomerular differentiation continues for one week after birth (Little & McMahon 2012; Loggie, Kleinman & Maanen 1975).

In humans, adult kidney morphogenesis starts by forming metanephros at E32 (Merkel, Karner & Carroll 2007; Schedl & Hastie 2000). Nephrogenesis starts at gestation week 5, and terminates at gestation week 36 (Walker & Bertram 2011). From this point onward, the nephrons undergo maturation until 12 years of age (Loggie, Kleinman & Maanen 1975). However, nephrogenesis process may continue for several weeks after birth following premature birth (Walker & Bertram 2011).

1.2.3. Growth and transcriptional factors in early kidney development

Nephrogenesis is regulated by multiple growth and transcriptional factors. Any internal or external teratogenic factors that alter these growth factors can interrupt the normal process of renal development and decrease nephron number in the kidney (Charlton, Springsteen & Carmody 2014), including the failure of UB to elongate and branch, as well as the failure of mesenchymal cells to proliferate and differentiate into epithelial

cells (Merlet-Benichou 1999). Both can lead to abnormal nephron structure, and predispose individuals to kidney diseases later in life (Merlet-Benichou 1999).

Moreover, nephrogenesis relies on a sequential activation of various proteins from several different families (Kuure, Vuolteenaho & Vainio 2000). These include bone morphogenetic proteins (BMPs), fibroblast growth factors (FGF) family, paired box binding proteins (Pax), wingless–type MMTV integration site (WNT) family, glial cell line-derived neurotrophic factor (GDNF), and Wilms tumour (Wt)1 inhibitory protein (Costantini 2010; Little & McMahon 2012).

GDNF signalling pathway is believed to be the most critical factor for the early-stage nephrogenesis during fetal life (Bard 1992; Dankers et al. 2011; Dressler 2002; Lechner & Dressler 1997; Merkel, Karner & Carroll 2007; Michos 2009; Oliver & Al-Awqati 1998; Schedl & Hastie 2000). GDNF is expressed by the MM, which can induce UB outgrowth and branching by binding to its receptor GDNF family receptor α (GFRα1). The latter is attached to the plasma membrane by glycosylphosphatidylinositol anchor, which is cleaved by unknown phospholipase and protease (Airaksinen & Saarma 2002). Then the GDNF-GFRα1 complex in turn binds to receptor tyrosine kinase (RET) to activate several intracellular cascades including the Mitogen-activated protein kinase pathway that is involved in ureteric branching during nephrogenesis (Sariola & Saarma 2003). As such, Both GFRα and RET are expressed in the mesonephric duct (Glassberg 2002; Walker & Bertram 2011).

BMPs play various roles in kidney development. Among the isoforms, the most important ones are BMP4 and BMP7 (Nishinakamura & Sakaguchi 2014). BMP4 is expressed in the mesenchyme surrounding the UB known as the cap mesenchyme and it regulates UB elongation by inhibiting the budding of the ureteric tips. However, the function of BMP4 is antagonized by Gremlin molecule so that only the ectopic budding is inhibited (Nishinakamura & Sakaguchi 2014). BMP7 enables the survival of MM (Little & McMahon 2012). Several members of FGFs such as FGF7 and FGF10 can also induce UB outgrowth (Costantini 2010). FGF2 is a proliferating and differentiating signalling factor that also promotes MM survival (Little & McMahon 2012). Wnt4 is expressed in the condensed mesenchyme and the comma- and S-shaped bodies, which is a signal that regulates the transformation of MM to epithelia and the formation of the renal vesicle (Krause et al. 2015; Little & McMahon 2012). Therefore, Wnt4 is essential

for proximal tubule development (Cizelsky et al. 2014). Wt1 is expressed in the MM and essential for MM survival through inducing FGF signalling pathway (Motamedi et al. 2014).

The nephrogenesis process is also accompanied by the expression of other molecules that can initiate the nephrogenesis including leukaemia inhibiting factor (LIF) and Eyes absent 1 (Eya1) (Bard 1992; Schedl & Hastie 2000; Walker & Bertram 2011). Eya1 can also upregulate Pax2 expression to regulate mesenchyme condensation around the tips of the UB and MM transdifferentiation into epithelial cells (Walker & Bertram 2011). Proteoglycans macromolecules, such as glycosaminoglycan, syndecan-1, glypican (glypican-5 and glypican-3), and integrin α8, can mediate the inductive interaction between the UB epithelial cells and mesenchymal cells that initiates kidney morphogenesis (Kuure, Vuolteenaho & Vainio 2000).

1.2.4. Risk factors for kidney disease

The risk factors for kidney disease can be classified into three categories, biomedical, fixed, and behavioural factors including genetic, environmental and life-style factors (Figure 1.3). It has been proposed that the genetics factor cannot act without the input from the environment. Life-style factors (such as cigarette smoking, alcohol consumption and drugs and other substance usage) and the exposure to environmental insults (such as toxins and intrauterine environmental abnormalities) can affect fetal genome profile and thereafter affect the expression of different genes involved in the development and progression of CKD (Cass et al. 2004).

1.2.4.1. Biomedical factors

Individual's health status is a significant risk factor that can lead to CKD. In a ten years follow-up community-based population study, obesity, hyperlipidaemia, glomerulonephritis, metabolic syndrome, kidney and urinary stones, and urinary tract infections were shown as risk factors for developing CKD (Yamagata et al. 2007).

Obesity as a risk factor of CKD is related to diabetes and high blood pressure (Haroun et al. 2003; Stengel et al. 2003). Diabetes is a main cause of CKD as hyperglycaemia can increase the thickness of the basement membrane and proteins accumulation in the glomeruli, therefore affecting kidney function (Redmond & McClelland 2006).

Cardiovascular risk factors also can lead to the progression of CKD (Redmond & McClelland 2006). A recent report showed that hypertension is an independent risk factor for CKD (Gansevoort et al. 2013). In addition, coronary artery disease, atherosclerosis, stoke, left ventricular hypertrophy, and hyperlidemia are also important risk factors for CKD (Kasiske et al. 1990; Redmond & McClelland 2006; Schaeffner et al. 2003). The underlying mechanism is not fully clear, however, lipid nephrotoxicity has been suggested for decades (Moorhead et al. 1982). Circulating lipids can bind to and be trapped by extracellular matrix molecules within the wall of the atherosclerotic blood vessel in the kidney and then undergo oxidation (Trevisan, Dodesini & Lepore 2006). Lipids can stimulate the mesangial cells to recruit macrophages by producing chemokines such as monocytes chemoatractant protein (MCP)-1 (Rovin & Tan 1993). The activated mesangial cells and accumulated macrophages then produce reactive oxygen species (ROS, for more details, see section 1.3.1.1) that oxidize the low-density lipoprotein. This in turn stimulates the production of pro-inflammatory and pro-fibrotic cytokines (Keane 2000; Turner et al. 2012). Furthermore, the oxidised lipids are phagocytised by macrophages which are transformed into foam cells that release cytokines. This in turn recruit more macrophages to the site of the lesion which affects lipid deposition, endothelial cell function and vascular smooth muscle proliferation (Abrass 2004). Insulin resistance may also mediate the causative association between lipids and renal dysfunction (Trevisan, Dodesini & Lepore 2006).

Kidney stones obstructed uropathy can lead to renal vasoconstriction and inflammation in response to intratubular pressure (Klahr 1991; Rule et al. 2009; Rule, Krambeck & Lieske 2011). The latter in turn can lead to reduced renal perfusion, which can cause ischemia that leads to glomerulosclerosis, tubular atrophy and interstitial fibrosis eventually leading to acute renal injury (Tanner & Evan 1989).

1.2.4.2. Fixed factors

Genetic factors play a key role in the initiation and progression of kidney disease (Cass et al. 2004). More than 110 gene mutations have been found to be linked to the diseases with a renal phenotype (Eckardt et al. 2013). For example, the alterations in the fetal expression of osteopontin-CD44-integrin-receptor-system and renal insulin-like growth factor (IGF) I and II were shown to have a causal effect in reduced nephron number in the newborns which leads to hypertension at adulthood (Freese et al. 2013; Rothermund et al. 2006).

The genetic factors can be manifested by high incidence of kidney diseases within certain families, races or ethnic groups (Cass et al. 2004). For example, Australian Aboriginal and Torres Strait Islander populations are at higher risk of CKD compared to the reaming Australian population (Hazel & Hill 2006). In addition, studies have discovered some potential candidate genes among particular ethnic groups. For example, the deletion/deletion (DD) genotype of the angiotensin I-converting enzyme (ACE) is an independent risk factor for developing kidney disease in the Caucasians (Cass et al. 2004). The risk of developing kidney disease in the black people is four times higher than in the white individuals which has been linked to the polymorphism in the MYH9 and APOL1 gene regions (Eckardt et al. 2013; Hoy et al. 2005; Shoham, Vupputuri & Kshirsagar 2005). Moreover, mutations in genes involved in nephrogenesis, such as Pax2 can also result in oligomeganephoria, a rare congenital and sporadic anomaly characterised by bilateral renal hypoplasia, reduced nephron number and enlarged nephron size (Salomon et al. 2001).

Increased age is another independent risk factor for CKD, characterized by renal dysfunction and proteinuria due to glomerular hypertension and hyperfiltration (Eriksen & Ingebretsen 2006; O'Hare et al. 2007), as well as increased renal fibrosis due to age-related increase in ROS production (Iglesias-De La Cruz et al. 2000). Aging is normally accompanied by a reduction in functional nephrons in the kidney and hence declining in GFR which normally starts after the age of 30 in humans (Yamagata et al. 2007).

Moreover, it has been shown that reduced glomerular number in the kidney is closely correlated to low birth weight (LBW) and gender (men 17% higher than women) (Hoy et al. 2005). However, human population studies reported conflicting findings in respect

to male gender as a risk factor for developing CKD. Such bias in the study results is mainly due to the nature of subjects and the methodologies used.(Eriksen & Ingebretsen 2006; Jafar et al. 2003; Neugarten, Acharya & Silbiger 2000). The mechanism of how gender differences affects progression of kidney disease is discussed in more details in Section 1.2.5.

1.2.4.3. Behavioural factors

The socio-economic disadvantage can be a behavioural risk factor for CKD (Cass et al. 2004), which can influence the access to services of preventative medicine, examination, diagnosis and treatments (Patzer & McClellan 2012). Usually, a low socioeconomic status is also associated with other factors such as cigarette smoking and alcohol consumption where each forms an independent risk factor for CKD (Haroun et al. 2003; Yamagata et al. 2007).

Smoking has a direct effect on the kidney including renal hemodynamics, diuresis and electrolyte excretion and on the proximal tubules (Orth, Ritz & Schrier 1997). Smoking has a stimulatory effect on GFR, sodium and chloride excretion as well as urine flow which is mediated by catecholamines release from the adrenal medulla (Orth, Ritz & Schrier 1997). In addition, it has been reported that nicotine has an antidiuretic action in both animals and humans (Orth, Ritz & Schrier 1997). Moreover, smoking can cause damage to renal proximal tubules, by disrupting different cellular processes and functional integrity in the proximal tubules. The former includes the impairment of organic cation transport as well as the increase in urinary activities of lysosomal enzyme N-acetyl-β-D-glucosaminidase and cytoplasmic enzyme glutathione S-transferase which are established markers of proximal tubular damage (Brüning et al. 1999; El-Safty et al. 2004). Disruption of functional integrity of renal proximal tubules is also evident in the increase in urinary α_1-microglobulin, another marker of proximal tubular damage (Brüning et al. 1999; El-Safty et al. 2004; Wong, Smyth & Sitar 1992). However, only heavy alcohol consumption of more than three servings per day has been reported to be associated with the development of CKD (Shankar, Klein & Klein 2006), but not smoking itself.

Physical inactivity also contributes to the susceptibility to CKD as it doubles the risk of CKD due to the high risk of diabetes and hypertension, which further lead to the development of CKD (Stengel et al. 2003). A previous study showed that increased physical activity is associated with decreased albuminuria in the patients with CKD (Gansevoort et al. 2013).

1.2.4.4. Intrauterine environmental factors

1.2.4.4.1. Maternal malnutrition

It has been suggested that the intrauterine environment can contribute independently to the susceptibility to certain diseases in adulthood (Abdel-Hakeem et al. 2008; Shoham, Vupputuri & Kshirsagar 2005). For example, maternal undernutrition can lead to IUGR and Low Birth Weight (LBW) in offspring (da Silva Cunha et al. 2015; Mumbare et al. 2012). It has been reported that newborns with a birth weight of less than 2500 g have 30% fewer nephrons compared to those with a normal birth weight (Maria Pereira Pires, Barbosa Aguila & Alberto Mandarim-de-Lacerda 2006). Maternal malnutrition can affect the expression of several vital hormones and growth factors in the developing foetus, leading to underdeveloped kidneys (Luyckx & Brenner 2005). For example, FGF-2 is known to induce nephron formation and inhibit apoptosis of the mesenchymal cells (Dudley, Godin & Robertson 1999). mRNA expression of FGF-2 is upregulated by IUGR leading to renal malformation (Abdel-Hakeem et al. 2008). In addition, both mRNA and proteins levels of Pax-2 are decreased by IUGR; while mRNA and protein levels of WT1 are increased by IUGR (Abdel-Hakeem et al. 2008), leading to underdeveloped kidney at birth. These IUGR-induced alterations may occur through epigenetic mechanisms without changing the fetal genome structure, such as DNA methylation and histone protein methylation but can affect or program the fetal genomic expression (Koleganova, Piecha & Ritz 2009).

It has been reported that vitamin A deficiency in the growing fetus, due to maternal malnutrition, is also closely linked to smaller nephron numbers and abnormal vascular development in the newborn kidney (Merlet-Benichou 1999). This is because retinoic acid derived from vitamin A is a regulator of GDNF receptor, which is believed to play

a critical role in determining nephron numbers in the developing kidney (Gray et al. 2012; Merlet-Benichou 1999).

1.2.4.4.2. Gestational diabetes

Gestational diabetes is a strong risk factor for kidney disease in offspring (Mitanchez et al. 2015). Different studies have shown that foetal exposure to maternal high blood glucose concentration can cause reduced nephron numbers in the foetal kidney, leading to glomerular hypertension and proteinuria in offspring in later life (Burke et al. 2011; Simeoni & Barker 2009). Moreover, gestational diabetes has been associated with increased risks of renal agenesis (when one or both foetal kidneys fail to develop), the development of horseshoe kidney or hydronephrosis (dilation of renal pelvis calyces), and ureteric abnormalities (Chen 2005).

Increased oxidative stress and epigenetic changes are the suggested mechanisms underlying the effect of gestational diabetes on programming adulthood kidney disease in the offspring (Mitanchez et al. 2015). To this regards, a hyperglycemic environment can stimulates inflammatory response that leads to increased ROS production, decreased radical scavengers (such as superoxide dismutase), and thus increased oxidative stress in the placental microenvironment, which in turn leads to chromatin epigenetic modifications of genes involved in kidney development. As such, glomerular sclerosis, less nephron number, less GFR and microalbuminuria can occur at adulthood (Garcia-Vargas et al. 2012; Mitanchez et al. 2015).

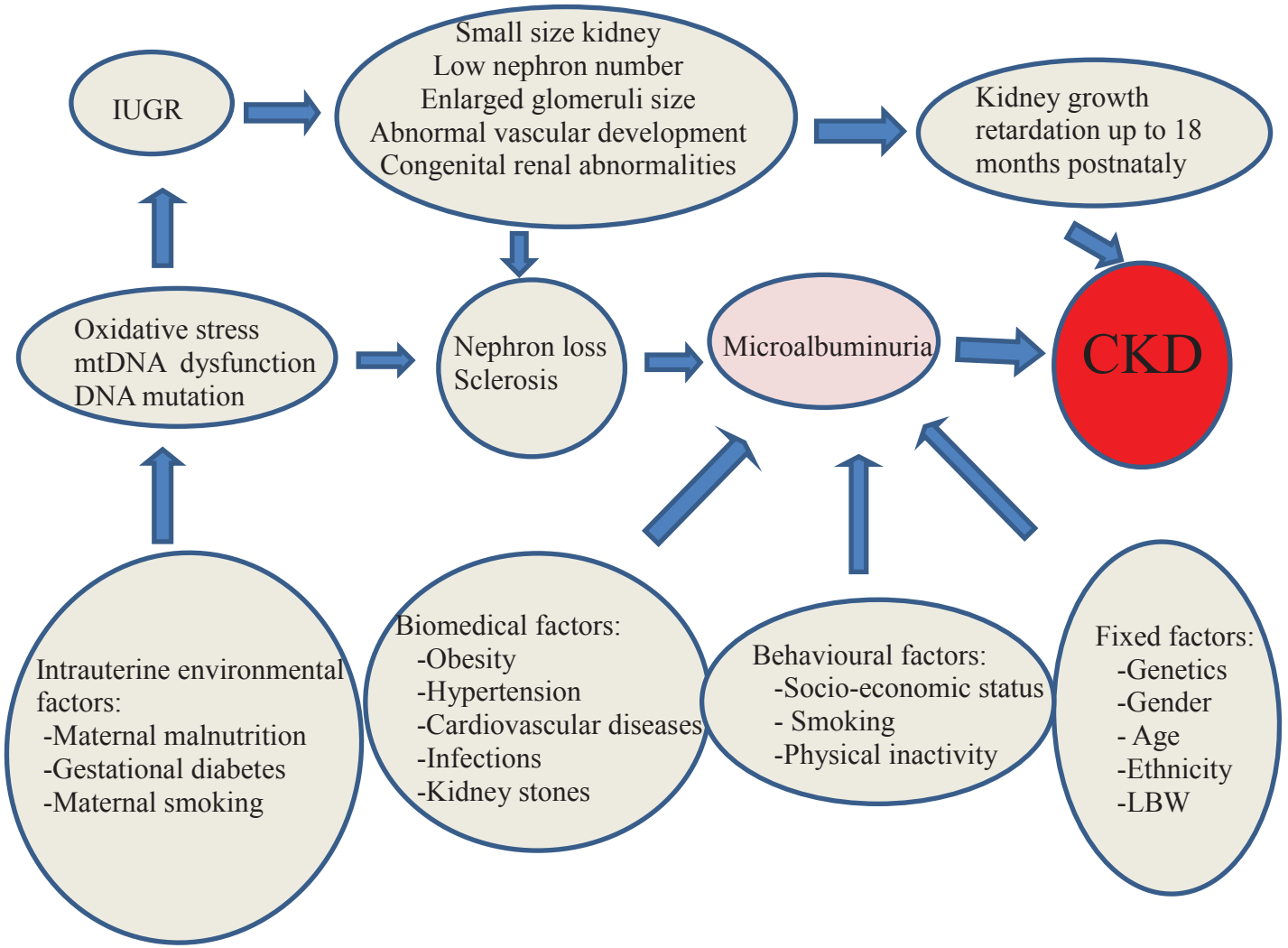


Figure 1.3: Risk factors for CKD. CKD: chronic kidney disease, IUGR: intrauterine growth retardation, LBW: low birth weight.

1.2.5. Gender differences in renal pathophysiology

Gender differences in different kidney diseases, including acute kidney injury and nephrotoxicity have been reported (Kwekel et al. 2013). Sex hormones can modulate the biosynthesis of different cytokines and growth molecules, such as different fibrogenic and inflammatory factors (including collagens, transforming growth factor (TGF)-β, tumour necrosis factor (TNF)-α, mesangial matrix), nitric oxide (NO), endothelin (ET)-1 and components of renin-angiotensin system (RAS) in the kidney, which significantly affect kidney response to inflammation and injury (Metcalfe & Meldrum 2006; Montezano et al. 2005; Neugarten & Golestaneh 2013; Silbiger & Neugarten 2008). Thus, females are more protected from renal injury than the males (Kang et al. 2004)

1.2.5.1. Fibrogenic and inflammatory factors

Renal fibrosis is characterized by the synthesis and accumulation of extracellular matrix (ECM) and increased expression of different fibrogenic factors such as TGF-β, fibronectin and collagen IV in the glomerulus and interstitium (Wang et al. 2012). Collagen IV is involved in the formation of the basement membranes underlying endothelial cells and it is essential for endothelial cell attachment and function (McLeod et al. 2014). TGF-β is an fibrogenic and inflammatory factor (Metcalfe & Meldrum 2006; Neugarten & Golestaneh 2013). It can promote the genes transcription and production of two renal fibrotic markers, mesangial collagen IV and fibronectin (Elmarakby & Sullivan 2012; Neugarten & Golestaneh 2013). It can also induce ECM deposition by inhibiting its degradation (Elmarakby & Sullivan 2012). TGF-β additionally upregulates the transcription of connective tissue growth factor which is a fibrogenic molecule that stimulates the proliferation of the connective tissue cells and ECM deposition (Elmarakby & Sullivan 2012). TGF-β along with IL-1 and TNF-α can stimulate the production of the pro-inflammatory marker, MCP-1, which recruits inflammatory cells, such as T cells, monocytes and dendritic cells to infiltrate the inflamed renal tissues (Elmarakby & Sullivan 2012). This in turn leads to renal damage and dysfunction (Elmarakby & Sullivan 2012). BMPs growth factors are a subfamily of the TGF-β superfamily. BMP 7 is expressed in the nephrogenic mesenchyme and collecting ducts. It is essential in kidney development for regulating cells proliferation and apoptosis (Adams et al. 2007).

In vitro study showed that androgens can enhance TGF-β transcription through androgen receptor binding to Smad-3 complex. This in turn activates fibroblasts to produce collagens hence promoting fibrosis (Kang et al. 2001; Metcalfe & Meldrum 2006; Zambrano et al. 2013). On the other hand, oestrogen decreases TGF-β availability and its effect on mesangial collagen IV gene transcription hence reducing renal injury (Guan 2006; Matsuda et al. 2001). Oestrogen can also indirectly decrease mesangial cells proliferation and collagen IV synthesis to inhibit fibrogenesis (Lee et al. 2013; Li et al. 2008). In addition, oestrogen has been shown to stimulate the activities of two enzymes involved in collagen degradation, metalloproteinase-2 and metalloproteinase-9 to reduce fibrosis in the mesangial cells isolated from the kidney of the mice (Guccione et al. 2002; Karl et al. 2006; Silbiger & Neugarten 2008). Oestradiol (an oestrogen hormone) has also been shown to inhibit collagen I gene transcription and protein synthesis in the mesangial cells which in turn decreases matrix accumulation and glomerular sclerosis (Kwan et al. 1996).

1.2.5.2. NO

The endothelium produces vasoactive mediators such as NO (Rudic et al. 1998). NO is a vasodilator synthesized by different NO synthases (NOS) from L-arginine which can provide protection against renal injury (Metcalfe & Meldrum 2006). Endothelial NOS (eNOS)-derived NO controls and maintains renal haemodynamics and function (Aydogdu et al. 2006). Human and animals studies showed gender differences in NO system activities, where female mice have higher level of NOS than the males (Reckelhoff et al. 1998; Vignon-Zellweger et al. 2014). Testosterone has been shown to affect the progress of renal injury via reducing both NO expression and NOS activity (Park et al. 2004). Therefore, renal disorder is more prominent in men than women.

Unlike testosterone, oestrogen can enhance NOS activity and induce the expression of NO molecule (Metcalfe & Meldrum 2006; Montezano et al. 2005). In addition, oestrogen increases the synthesis of prostaglandin E2 and prostacyclin that can also in turn increase–eNOS activity (Neugarten & Golestaneh 2013; Vignon-Zellweger et al. 2014). Thus females are protected from renal injury due to the beneficial effect of oestrogen.

1.2.5.3. ET-1

ET-1 is a vasoconstrictor that regulates kidney functions, including the constriction of renal vessels, mesangial cells contraction, the inhibition of sodium and water reabsorption by the nephron, promoting glomerular cells proliferation and stimulating ECM accumulation (Kittikulsuth, Sullivan & Pollock 2013; Kohan 1997; Rabelink et al. 1994). As such, ET-1 isoform is produced by most renal cell types (Kohan 1997). Overexpression, overactivation or dysfunction of ET-1 in podocytes and mesangial cells can cause glomerular dysfunction and proteinuria (Kittikulsuth, Sullivan & Pollock 2013). Increased ET-1 mRNA and protein levels mediated the progression of post ischemic renal injury to CKD (Zager et al. 2013). ET-1 has been shown to be increased in the serum of patients with acute renal failure, while the administration of ET-1 antibodies was shown to reduce ischemia-induced renal dysfunction (Wilhelm et al. 2001). Renal ET-1 synthesis is controlled by sex hormones (Polderman et al. 1993; Ylikorkala et al. 1995). Oestradiol and progesterone have been shown to decrease the expression of renal ET-1, which is beneficial for the females (Chen & Zhang 2011; Montezano et al. 2005; Neugarten & Golestaneh 2013; Tostes et al. 2008) . Men usually have higher level of ET-1 than the females (Montezano et al. 2005; Muller et al. 2002; Neugarten & Golestaneh 2013), therefore are more vulnerable to renal disorders.

1.2.5.4. RAS

RAS is the major regulator of blood pressure as well as fluid and electrolytes balance in the body. Renin is an enzyme that catalyses the conversion of angiotensenogin into angiotensin I, which is then further converted to angiotensin II by ACE. There are two types of angiotensin II receptor (AT), AT_1 and AT_2, and AT_1 is the functional receptor of angiotensin II. AT_2 counteracts the functions of AT_1 to maintain the balance (Baiardi et al. 2005). The function and response of RAS to renal pathology can vary between the males and females (Miller et al. 2006). For example, the activation of RAS can increase renal injury in men; however, the increase in angiotensin II activity does not always lead to renal injury in women (Sullivan 2008).

RAS inhibitors are the most commonly used medication in patients with CKD (Sullivan 2008). To this respect, epidemiological and experimental studies showed that testosterone activates RAS (Kang & Miller 2002; Reckelhoff, Zhang & Granger 1998), which can alter kidney hemodynamics and contribute to renal injury (Silbiger & Neugarten 2008). This was supported by a rat model of spontaneous hypertension where androgen was found to promote the blood pressure observed in the male rats (Reckelhoff, Zhang & Srivastava 2000).

On the other hand, epidemiological and *in vivo* and *in vitro* experimental studies showed that oestrogen decreases the expression of renin, ACE and angiotensin II as well as AT_1 in the female's kidney (Montezano et al. 2005; Nickenig et al. 1998; Silbiger & Neugarten 2008; Tanaka et al. 1997). This in turn can lead to improved renal hemodynamics thus reducing kidney disease progression (Silbiger & Neugarten 2008). Oestrogen can increase the levels of angiotensinogen and AT_2 receptor which in turn increase eNOS production in the cortical arteries and prostaglandin E2 in the renal medulla, thereafter providing the protection against renal injury (Baiardi et al. 2005).

1.2.6. Aetiology of kidney disease

The causes of kidney disease can be categorised into three groups; pre-renal, renal and post-renal causes (Haynes & Winearls 2010). The most common pre-renal cause of kidney disease is renal arterial disease (Haynes & Winearls 2010). There are many renal causes of kidney disease, including glomerular disease (such as diabetic nephropathy and glomerulosclerosis), and tubulointerstitial disease (such as reflux nephropathy and polycystic kidney disease) (Haynes & Winearls 2010; Levey & Coresh 2012). In addition, the other causes of CKD include diabetes mellitus, hypertension, ischemia, infection, obstruction, toxins, autoimmune diseases, and infiltrative diseases (Snively & Gutierrez 2004). The most common post-renal cause of kidney disease is prostatic obstruction due to benign enlargement, carcinoma or fibrosis (Haynes & Winearls 2010). However, it has been reported that CKD due to unknown reasons represents 10% of total patients with CKD in the world (Siddarth et al. 2014).

1.2.7. Signs and symptoms of kidney disease

The symptoms of kidney disease are developed over the time and may not be manifested until late-stage where irreversible damage has occurred in the kidney. The signs and symptoms include nausea, headaches, vomiting, loss of appetite, weight loss, increased frequency of urination with reduced volume of urine, fatigue and weakness, sleep problems, anxiety and depression, decreased mental sharpness, muscle twitches and cramps, hiccups, swelling of the feet and ankles, persistent itches, chest pain, shortness of breath and high blood pressure (Redmond & McClelland 2006).

1.2.8. Diagnosis of kidney disease

An early diagnosis of kidney disease can help in taking actions to slow disease progression. There are different ways to diagnose kidney disease.

Blood pressure can give an indirect indication of kidney disease. Urinalysis can assess the colour, appearance, quantity and chemical characteristics of the urine in addition to microscopic examination to identify any elements such as red blood cells and white blood cells, crystals, and bacteria in the urine (Levey & Coresh 2012; Redmond & McClelland 2006). Urine test using dipstick can detect the presences of blood cells, proteins (such as albumin and creatinine), leucocytes, nitrites, specific gravity, pH, ketones and glucose in the urine (Byrne & Cove-Smith 2015). At early stage, kidney disease can be diagnosed using biological markers in the blood and urine, including blood creatinine/creatinine clearance, urea nitrogen, C-reactive protein, and bicarbonates (Byrne & Cove-Smith 2015). However, the most reliable test for kidney functional deterioration is the measurement of GFR, which is an essential diagnostic tool in the kidney disease (Haynes & Winearls 2010). GFR is used to classify CKD into five stages based on millilitre of blood filtrated by the kidney per minute per 1.73 m^2 (Haynes & Winearls 2010). As GFR is difficult to measure, it can be estimated from serum creatinine using a mathematical formula that take in consideration age, sex, ethnicity and body size of the individual (Levey & Coresh 2012).

More advanced techniques are also used to diagnose kidney diseases including some invasive techniques such as kidney biopsy, and non-invasive techniques such as renal

imaging using B ultrasound and CT scan (Byrne & Cove-Smith 2015; Haynes & Winearls 2010; Redmond & McClelland 2006).

1.2.9. Complications and prognosis

CKD can affect many organs and systems in the body and leads to decreased kidney function and progression to kidney failure (Levey et al. 2007; Levey & Coresh 2012; Xie & Chen 2008). As kidney function deteriorates, patients normally develop complications related to fluid overload, electrolyte and acid-base imbalance and the accumulation of nitrogenous waste, which eventually require life-sustaining treatment for the patients to survive, such as dialysis and kidney transplant (Snively & Gutierrez 2004).

People with CKD are also at risk of developing different complications and comorbidities such as cardiovascular diseases (including hypertension), hyperglycemia, hyperlipidemia, anaemia, respiratory diseases, infection, bone and muscles problems, cognitive impairment and impaired physical functions (Haynes & Winearls 2010; Levey & Coresh 2012; Snively & Gutierrez 2004; Xie & Chen 2008). Other less common complications of CKD include low testosterone levels, sexual dysfunction, and reduced fertility (Haynes & Winearls 2010).

1.2.10. Treatment and management

The treatment and management of kidney disease depend on the underlying causes and the severity of kidney function impairment to slow the progress (Eckardt et al. 2013). The treatment for the patients with early-stage kidney disease is through the controlling and management of comorbidities that affect kidney function by taking medication and avoiding nephrotoxic substances. Currently, there is no curative medication for CKD; however, ACE inhibitors and AT blockers have been shown to be the most effective drugs in slowing down the progression of CKD (Levey & Coresh 2012; Okamoto 2003).

Patients with end-stage kidney disease require life-long renal replacement therapy of either dialysis or transplantation in order to prevent mortality (Levey & Coresh 2012; Snively & Gutierrez 2004). Dialysis can encounter the difficulties to access these health services; whereas kidney transplantation encounters the shortage of organ donors and possibility of tissue rejection. However, the high cost of these two therapies is striking in some part of the world, which increases the financial burden of CKD around the world (Eckardt et al. 2013; Levey & Coresh 2012). In Australia, the cost of renal replacement therapy has been increased by 18% between 2000 and 2010 (Tucker et al. 2014).

1.3. The developmental origins of adult health and disease

Developmental (or fetal) programming refers to the process by which the environmental insults during the critical periods of growth and development (fetal life and early postnatal life) can change the physiology, metabolism and genomic of the fetus. This in turn can predispose the fetus to certain diseases in adulthood (Zandi-Nejad, Luyckx & Brenner 2006). This programming is due to the plasticity of the developing fetus, which under different environmental conditions, a genotype can develop into different phenotypes (Barker 2004).

Based on epidemiological observations, Barker and colleagues first proposed "the developmental origins of adult health and disease" theory (or Barker Hypothesis) when they have addressed the influence of intrauterine and early postnatal environment on the development of diseases at adulthood (Barker 1995; Barker 2004). The "Thrifty Phenotype Hypothesis" was also proposed by Barker and Hales to explain the phenomenon of the fetal origins of disease (Hales & Barker 2001). This hypothesis suggests that under abnormal intrauterine conditions, such as under nutrition, the fetus makes reversible and/or irreversible adaptations to the environment in order to survive where the vital organs such as heart and brain receive more nutrition on the expense of the less vital organs, such as kidney and pancreas. This nutrient redistribution can have

a detrimental effect on the health later in life (De Boo & Harding 2006; Hales & Barker 2001; Koleganova, Piecha & Ritz 2009; Nuyt 2008; Warner & Ozanne 2010).

In addition, the "Postnatal Accelerated Growth Hypothesis" proposed by Singhal and Lucas explains the association between accelerated growth in the early postnatal life following IUGR, and increased risk of metabolic disorders in such offspring (Barker 2004; Singhal & Lucas 2004; Tomat & Salazar 2014). Decreased growth velocity in the fetus that alters organ development and maturation due to different intrauterine factors leads to LBW (Claris, Beltrand & Levy-Marchal 2010; Zohdi et al. 2014). This is accompanied by increased postnatal growth rate that exceeds the normal growth due to IUGR, which increases the risk of metabolic and cardiovascular disease in these offspring (Zohdi et al. 2014). A similar hypothesis of "catch-up or compensatory growth" was also proposed by Cianfaranri and colleagues (Cianfarani, Germani & Branca 1999).

1.3.1. Mechanisms for the developmental programming of adulthood diseases

1.3.1.1. Oxidative stress

ROS are oxygen-derived molecules (Nuyt 2008) including free radicals such as superoxide anion radicals ($O_2^{\cdot -}$) and hydroxyl radicals ($OH^{\cdot}$) or non-free radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Gulcin 2006). Normally ROS are produced in the body through different physiological reduction-oxidation reactions. They are involved in different cellular, metabolic, and transcriptional events such as proliferation, differentiation, apoptosis and morphogenesis (Vo & Hardy 2012). They are normally balanced by antioxidant mechanisms of ROS scavengers (eg. thiols and Vitamin C and E) and other detoxifying enzymes (eg. superoxide dismutase and glutathione reductase). When ROS production overwhelms the endogenous anti-oxidative capacity, oxidative stress causes cellular injury and tissue damage commonly seen in certain pathological conditions (Fernandez-Twinn & Ozanne 2006; Nuyt 2008; Sener et al. 2004; Vo & Hardy 2012). ROS production has been shown to be linked to vasoconstriction, vascular smooth muscle cell growth and migration, endothelial dysfunction, ECM protein modification, and kidney dysfunction through ROS induced-oxidative stress damage (Elmarakby & Sullivan 2012; Tomat & Salazar 2014).

During the critical period of development, the fetus is sensitive to the changes in ROS level as the fetal development occurs in a relatively low oxygen environment (Dennery 2010; Thompson & Al-Hasan 2012). Increased placental oxidative stress plays an important role in IUGR (Dennery 2010). Here, the anti-oxidative capacity of the developing fetus is unable to scavenge the excess of ROS (Thompson & Al-Hasan 2012), leading to adverse effect to the fetus (Ornoy 2007). Oxidative stress was recently suggested as a contributor to fetal programming of adulthood disease such as hypertension (Fernandez-Twinn & Ozanne 2006). This may act through the modulation of genes involved in development or lipids and proteins oxidation which is also important for fetal development (Luo et al. 2006).

Moreover, increased oxidative stress in the fetus can lead to endothelial cells necrosis and disrupt the angiogenesis process in the growing fetus leading to blood vessel structural abnormalities such as reduced density of arterioles and capillaries which can lead to hypertension later in life (Nuyt 2008). However, the link between oxidative stress on kidney development and health in adulthood is not fully elucidated.

1.3.1.2. Apoptosis

Apoptosis or programed cell death is a cell suicide process characterized by cellular and nuclear morphological changes, including the rearrangement of cell content, cell shrinkage, membrane blebbing, chromatin cleavage, nuclear condensation and the formation of apoptotic bodies of condensed chromatin (Doonan & Cotter 2008). In addition, the endoplasmic reticulum is transformed into vesicles and apoptotic bodies that are eventually eliminated by phagocytes (Doonan & Cotter 2008; Stewart & Bouchard 2011; Torban et al. 2000). It is an important cellular mechanism to eliminate unwanted cells during the development and during the exposure to toxins (Ashe & Berry 2003). The balance between cell proliferation and apoptosis is critical in determining the mass of an organ (Hershkovitz et al. 2007).

The intrauterine environment may drive the proliferating cells to undergo programmed cell death (Stewart & Bouchard 2011). For example, an animal study showed reduced number of neurons in some regions of the brain in the offspring following IUGR induced by unilateral uterine artery ligation (Mallard et al. 2000). This is due to the

alteration in genes expression of pro-apoptotic (P53) and/or anti-apoptotic (Pax2, FGFs, WT1) signals which can favour the apoptosis pathway leading to cell loss (Stewart & Bouchard 2011; Zandi-Nejad, Luyckx & Brenner 2006).

1.3.1.3. Epigenetic modifications

Epigenetic modification refers to the inheritable changes in the gene expression without changing the DNA sequence (Egger et al. 2004). There are three proposed epigenetic molecular mechanisms that can program embryos genome and predispose individuals to different diseases in adulthood life. These mechanisms include DNA methylation, histone modification and microRNA (Egger et al. 2004).

DNA methylation can occur by adding a methyl group to the cytosine residue at the cytidine-guanosine (CpG) nucleotides in the promoter region of some genes mediated by DNA methyltransferase (Egger et al. 2004). Histone modification can occur by adding acetyl or methyl group to the N-terminal end of the lysine amino acid in the histone protein which change the chromatin structure (Egger et al. 2004; Gluckman et al. 2008). These changes affect the ability of the transcription factors to access the target DNA which inhibits the translation and transcription processes (Gluckman et al. 2008). In addition, microRNAs which are small non-coding RNA can reduce protein transcription by binding to the complementary sequence of the mRNA (Egger et al. 2004; Gluckman et al. 2008). For example, hypomethylation of four specific CpG dinucleotides of PPAR-α promoter has been found in the liver of juvenile and adult offspring from the rat dams fed a protein restricted diet during pregnancy (Lillycrop et al. 2008). However, the contribution of epigenetic changes by maternal environmental change during pregnancy on kidney diseases in offspring has not been widely studied to date, which is a new area waiting for further investigation.

1.3.1.4. Gender specific programming

There is gender difference in the developmental programming of adulthood diseases (Chen & Zhang 2011). This means that the same intrauterine insult does not always have the same impact and/or same severity on the male and female offspring (Moritz et

al. 2010). For example, insulin resistance only occurs in the second generation female offspring, but not in the males, if the F0 dams were fed an isocaloric low-protein diet during lactation (Zambrano et al. 2005).

There are three main factors in gender differences during development; firstly, differences in the pattern of development, which include genetic, transcriptional and morphological differences; secondly, timing of the development, and; thirdly, the influence of sex hormones during fetal and postnatal life (Aiken & Ozanne 2013).

The embryo of different genders have different growth pattern and survival rate. It has been shown that the development of male and female bovine embryos can be influenced by early culture conditions. For example, the level of antiviral antibodies generated by the cultured female bovine embryos is twice higher compared to those by the male bovine embryo, which was suggested to be able to enhance the growth of the female embryos (Larson et al. 2001).

For the timing of the development, male and female undergo development at different rates starting from the fetal life until post-puberty age in humans. As such, male fast-growing fetus are more vulnerable to certain insults than female slow-growing fetus (Aiken & Ozanne 2013).

Different sex hormones has been shown to affect fetal and placental metabolism, nutrient delivery and endocrine function differently during fetal life (Tomat & Salazar 2014). During the fetal life, males are exposed to higher levels of androgen compared to the females (Barry et al. 2010) as the testes can synthesise androgen by gestation week 10 (Aiken & Ozanne 2013). Excess prenatal exposure to testosterone due to injection during pregnancy can lead to fetal growth retardation and postnatal catch-up growth in sheep offspring, which are both early determinants of future adulthood disease (Manikkam et al. 2004). In addition, the exposure to high level of androgen during childhood can also increase the risk of metabolic disorders, such as insulin resistance observed in pre-pubertal girls with premature adrenarche (Idkowiak et al. 2011; Oppenheimer, Linder & DiMartino-Nardi 1995). This suggests that males are at higher risk of developmental programming of adulthood diseases due to high level of male sex hormones.

1.4. The developmental programming of adulthood kidney disease

The most common paradigm in the developmental programming of kidney diseases is the alterations of nephron endowment (reduced nephron number) (Baum 2010; Dotsch, Plank & Amann 2012; Hoy et al. 2005; Ingelfinger & Woods 2002; Zandi-Nejad, Luyckx & Brenner 2006). Based on the developmental origins of adult health and disease theory and thrifty phenotype hypothesis, Brenner et al proposed a theory in regard to the kidney where growth restriction during critical time of fetal development can cause less nephron number in the kidney predisposing the individual to kidney dysfunction and diseases in adulthood (Brenner, Garcia & Anderson 1988; Koleganova, Piecha & Ritz 2009).

Human studies showed that intrauterine environmental insults (such as food restriction and toxins) can induce IUGR and LBW in the offspring (Barker & Clark 1997; Barker 1995; Bell & Ehrhardt 2002; Rosenberg 2008). IUGR is a decrease in growth velocity in the fetus as well as organ underdevelopment and delayed maturation. IUGR can usually lead to LBW (Claris, Beltrand & Levy-Marchal 2010; Zohdi et al. 2014). LBW is defined as birth weight of an infant less than 2500g at full term by the World Health Organization (Luyckx & Brenner 2005; Zohdi et al. 2012). IUGR and LBW in turn can lead to smaller kidney with low nephron number and its related compensatory enlargement of glomerular size, which results in the reduction of filtration surface area (Brenner & Anderson 1992). This will directly lead to low GFR and increased albumin in the urine (Luyckx & Brenner 2005).

The kidney normally overcomes the reduced nephron number by a compensatory increase in single-nephron GFR to maintain the body's demand (Tomat & Salazar 2014). The overload work of each individual glomerulus leads to an increase in glomerular capillary pressure and increase in the individual glomerular size (glomerular hypertrophy) in order to increase the filtration surface (Luyckx & Brenner 2005). This would increase the burden on the kidneys and lead to glomerular injury, scars (glomerulosclerosis), and nephron loss later in life (Moritz et al. 2009; Tomat & Salazar 2014; Warner & Ozanne 2010). This will eventually deteriorate renal function (Luyckx & Brenner 2005).

1.5. Maternal cigarette SE and its consequences

1.5.1. Smoking as a global public health problem

Cigarette smoking is currently considered to be the third leading preventable cause of death worldwide (Cnattingius & Lambe 2002; DeMarini 2004; Horta et al. 2011; World Health Organization 2011). It is the primary cause of cancer, cardiovascular disease and pulmonary disease (Bialous, Kaufman & Sarna 2003; IARC 2002).

There are more than one billion smokers around the world, with approximately 5 million deaths each year attributed to tobacco/cigarette smoking related diseases, costing billions of dollars per year (Warren et al. 2011; World Health Organization 2011). Of the adult population aged 15 years and over around the world, 22% are current tobacco smokers 8% of whom are women (World Health Organization 2011, 2013). It has also been estimated that by 2030, the mortality due to smoking-related diseases will rise to more than 8 million a year (Hussein et al. 2007; Warren et al. 2011).

Passive smoking also has a significant impact on the health of non-smokers (Janson 2004). It is believed that the concentration of harmful chemicals in second-hand smoke (from the side stream smoke which comes from the smouldering end of a cigarette which does not pass through the filter) is much higher than that in the original smoke inhaled from the cigarettes (Pieraccini et al. 2008) .

Maternal smoking during pregnancy is considered as a significant part of the global public health problem (Marufu et al. 2015; World Health Organization 2013). The prevalence of smoking during pregnancy varies worldwide although is declining in some countries (Al-Sahab et al. 2010; Mohsin, Bauman & Forero 2011). The World Health Organization reported that in the USA, Europe and Australia, the rates of smoking among women above 15-years-old are 24-27%; while in the rest of the world, the rates are 7-17% (Ng & Zelikoff 2007; World Health Organization 2011). In the USA, the prevalence of smoking during pregnancy is about 22.4% in 2006 (Mohsin, Bauman & Forero 2011). In Australia, 17.3% of pregnant mothers were smoking in 2006 with a variation of 13.5% in New South Wales to 29.3% in Northern Territory (Mohsin, Bauman & Forero 2011). Maternal smoking rates are three times higher

among the pregnant indigenous women relative to non-indigenous women in Australia (Passey et al. 2012). In addition, it has been estimated that 35% of non-smoking females around the world are exposed to passive smoking (Öberg et al. 2010; World Health Organization 2013).

1.5.2. Organ damage by chemicals in the cigarette smoke

Cigarette smoke has more than 4000 chemical substances, with 200 toxins and 50 carcinogens identified, such as ammonia, benzene, lead, chlorinated dioxins, and furans (Hussein et al. 2007; IARC 2002). Among them, hydrogen cyanide and arsenic are harmful to the cardiovascular system. In addition, thiocyanate can produce hypotensive effects (Raatikainen, Huurinainen & Heinonen 2007). Acrolein and acetaldehyde are harmful to the respiratory system. Arsenic and 1, 3-butadiene has detrimental effects on the reproductive system. Cyanide compounds and carbon monoxide can interrupt fetal oxidative metabolism through reducing oxygen supply to the fetus (Power, Atherton & Thomas 2010). Toluene and phenol can affect the normal function of nervous system (IARC 2002; Milner & Hill 1984; Ng & Zelikoff 2007). These toxic substances can also induce DNA damage (e.g. strand breaks), ROS production and oxidative stress, inflammatory responses and carcinogenic effects (Carnevali et al. 2003; Hergenhahn, Weninger & Bartsch 1999; Liu et al. 2005).

Nicotine within the cigarette smoke does not only induce addiction, but can also change energy homeostasis. Nicotine withdrawal contributes to smoking cessation-related hyperphagia and obesity. This is due to the suppressive effect of nicotine on appetite (Chen et al. 2008; Guan et al. 2004) and catecholamine-induced energy expenditure (Sen, Entezarkheir & Wilson 2010).

Studies have shown that smoking is one of the main causes of renal dysfunction in adults (Nogueira et al. 2010; Remuzzi 1999), possibly due to vascular mechanisms (Nogueira et al. 2010). The blood level of ET-1, a vasoconstrictive peptide produced by the endothelial cells, has been shown to be elevated in smokers compared with non-smokers individuals (Remuzzi 1999). The chemicals within cigarette smoke (such as carbon monoxide) may induce epithelial cells to synthesise and release large amount of ET-1, which then binds to its receptor in the vascular smooth muscle cells to cause

vasoconstriction and vascular damage, and subsequent renal dysfunction due to reduced filtration rate (Remuzzi 1999). This can also potentially lead to increased blood pressure.

1.5.3. Adverse impact of maternal smoking

In addition to the detrimental impact of cigarette smoke on the mothers, negative impacts upon foetal growth and development have also been reported with short and long-term health consequences (Andreski & Breslau 1995; Chen & Morris 2007; Horta et al. 2011; Hui et al. 2009; Lampl, Kuzawa & Jeanty 2005; Salihu & Wilson 2007).

1.5.3.1. Short term consequences

The short-term consequences of maternal smoking include complications related to pregnancy and birth, and LBW (Andreski & Breslau 1995; Browne, Colditz & Dunster 2000; Chen & Morris 2007; Marin et al. 2011). Gestational complications due to maternal smoking include placental detachment, miscarriage, preterm delivery, stillbirth, prenatal death, no lactation or short lactation period due to insufficient milk output (Andreski & Breslau 1995; Chen & Morris 2007; Hui et al. 2009; Marin et al. 2011; Oddsberg et al. 2008; Power, Atherton & Thomas 2010; Vio, Salazar & Infante 1991). It was reported that 14% of preterm deliveries and 10% of infant deaths were due to immature lungs caused by maternal smoking during pregnancy (Ng & Zelikoff 2007).

The link between placental abruption and maternal smoking could be due to an inflammatory response that increases oxidative stress and vascular hyperactivity, both of which lead to further placental damage, such as necrosis, apoptosis and extracellular matrix destruction (Salihu & Wilson 2007). As a result, the placenta can detach from the uterine wall, leading to miscarriage. Maternal smoking also can lead to DNA mutation, translocation and strand breaks in the fetus, which can lead to miscarriage or congenital defects (DeMarini 2004; Salihu & Wilson 2007).

LBW is defined by the World Health Organization as birth weight less than 2500g (Luyckx & Brenner 2005). Birth weight can be influenced by race, sex, socioeconomic conditions, and maternal or fetal disease (Black 1985). Approximately, 20-30% of the

occurrences of newborns with LBW are due to maternal smoking during pregnancy (Ng & Zelikoff 2007). The chemicals in cigarette smoke inhaled by pregnant mothers, such as nicotine and carbon monoxide, can cause blood vessel constriction that limits blood flow to the placenta and thereafter poor nutritional supply to the fetus (Fríguls et al. 2009; Lambers & Clark 1996). This will further affect fetal development, leading to IUGR and LBW (Chen & Morris 2007; Salihu & Wilson 2007). Furthermore, nicotine in the cigarette smoke is also an appetite suppressor that suppresses maternal food intake and increase energy expenditure which contribute to poor maternal nutrition that can decrease fetal growth (Bergen 2006).

1.5.3.2. Long term consequences

Maternal SE can predispose the newborn to various diseases later in life, such as cardiovascular disease, hypertension, kidney diseases, type 2 diabetes, cancers, cognitive abnormalities, behavioural problems, neurological disorders, respiratory diseases, obesity, and nicotine addiction (Andreski & Breslau 1995; Chen & Morris 2007; Heinonen et al. 2011; Marin et al. 2011; Ng & Zelikoff 2007; Oddsberg et al. 2008; Power, Atherton & Thomas 2010). Among them, obesity is a significant comorbidities for kidney disorders, due to its close link to hypertension and diabetes mellitus.

A catch-up growth in the first month of life commonly occurs in children from mother who smoked during pregnancy (Gravel, Potter & Dubois 2012). This has been confirmed by animal studies, where exposure of foetus or neonate to cigarette smoke led to increased adiposity at 26 weeks (Gao et al. 2005). The adverse impact of maternal smoking-induced LBW on health outcomes is worsened by rapid increases in body weight after two years of age (Simeoni & Barker 2009). Despite postnatal catch-up growth (Power & Jefferis 2002), the LBW may continue into infanthood and childhood (Taal et al. 2011). However, an increase in body mass index (BMI) in such offspring is also common in adulthood, especially with the preference for junk food in such offspring (Power & Jefferis 2002). Such increase in BMI mostly occurs in offspring from the mother who smoked during the first trimester of their pregnancy (Chen & Morris 2007). Additionally, there is high risk of developing hypertension (Jaddoe et al.

2008; Power, Atherton & Thomas 2010; Pringle 1998) and increase in total blood cholesterol levels, low density lipoproteins and triglyceride (TG), and a decrease in high density lipoprotein in the offspring of mothers who smoked during pregnancy (Horta et al. 2011; Jaddoe et al. 2008). Such disorders can further adversely affect the kidney health in such offspring.

Previous studies have demonstrated the detrimental impacts of cigarette smoking on kidney function in the smokers, such as abnormal renal haemodynamics, water diuresis and electrolyte excretion in humans (El-Safty et al. 2004). Smoking is also closely linked to proximal tubular damage, kidney cancer, and end-stage kidney disease in the smokers (El-Safty et al. 2004). The impact of maternal smoking during gestation itself on the risk of CKD in offspring is unknown. However, it is speculated that the mechanism is through increased ROS production and resulting oxidative stress (Noakes et al. 2007; Schwarz et al. 1997).

Other developmental abnormalities due to maternal smoking in humans include abnormal vascular development and small kidney size which can further lead to significant renal dysfunction, and the development of kidney-related diseases in adulthood such as hypertension (Figure 1.4) (Chou et al. 2008; Koleganova, Piecha & Ritz 2009; Mao et al. 2009; Merlet-Benichou 1999; Taal et al. 2011; Vielwerth et al. 2007).

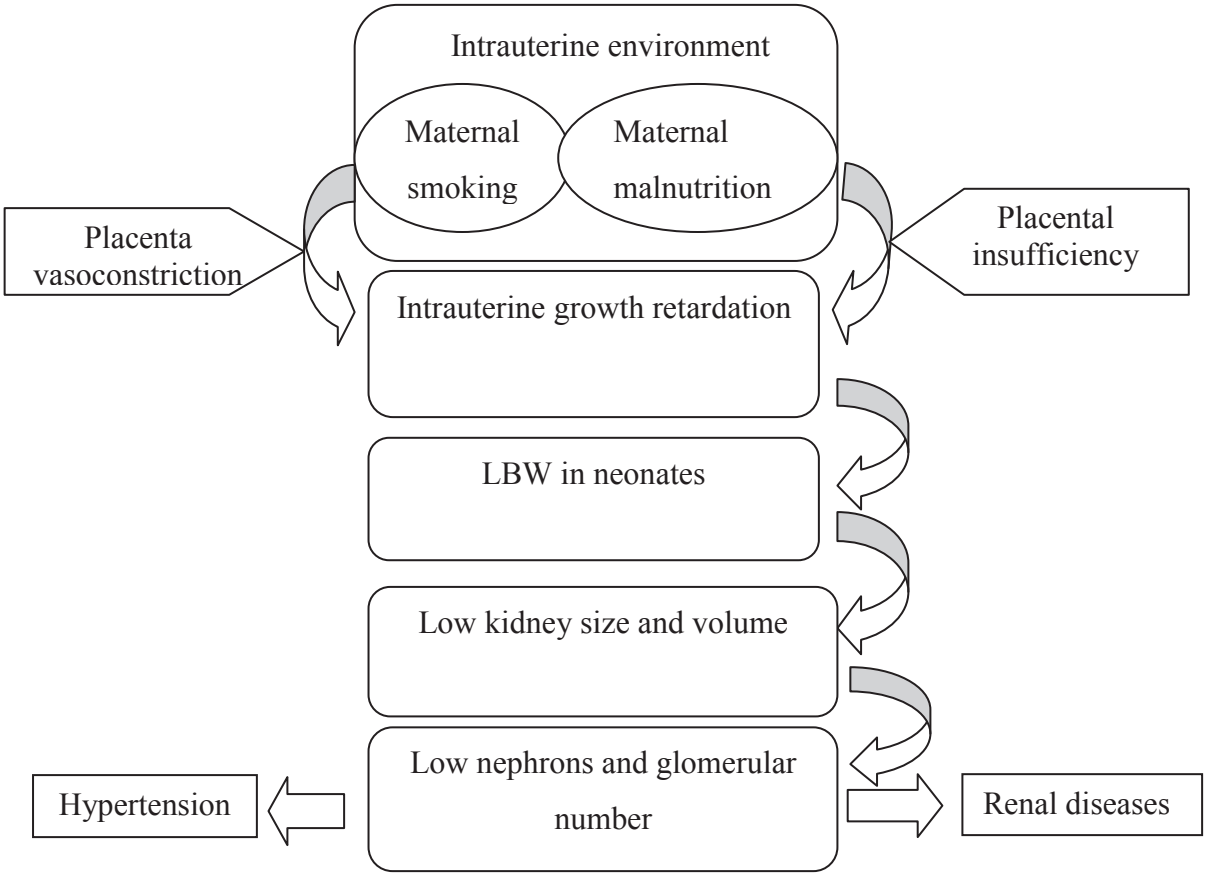


Figure 1.4: Flow chart of maternal cigarette smoking during pregnancy and adverse health consequences in offspring. Maternal cigarette smoking level is correlated to intrauterine growth retardation, leading to LBW, small kidney size and low nephron and glomerular numbers at birth, and renal dysfunction and hypertension in adulthood (Taal et al. 2011).

1.6. Carnitine

1.6.1. Background

Carnitine, also called β-hydroxy-γ-N-trimethyl aminobutyric acid, is a 162D water-soluble molecule. It is an essential nutrient that serves as a biological co-factor involving in transporting the activated long-chain fatty acid across the inner mitochondrial membrane for β-oxidation and ketones metabolism for energy production (Abdelrazik et al. 2009; Arenas et al. 1998; Gulcin 2006; Salama et al. 2012; Sener et al. 2004; Seong et al. 2010; Ye et al. 2010). Carnitine is also involved in the transportation of accumulated acetyl-CoA molecules from the outside of the mitochondria into the cytosol (Schmengler et al. 2013). It helps to excrete toxic exogenious and non-toxic endogenious organic acids via urine and participates in pulmonary surfactant production (Kępka et al. 2013). In addition, it has been shown to have anti-oxidant properties, including free radicals scavenging to protect the aorta, the heart, corpus and kidney from oxidative stress-induced injury in a rat model of partial nephrectomy-induced chronic renal failure (Sener et al. 2004). The anti-inflammatory effect of carnitine was also observed in the liver of mice fed a high-fat diet (Su et al. 2015). Moreover, carnitine was shown to have an anti-apoptotic effect on the human lymphoma cells that were exposed to apoptosis-inducing agents *in vitro* (Abdelrazik et al. 2009).

There are two isoforms of carnitine, L-carnitine (LC) and D-carnitine. LC is the biologically active isoform whereas D-carnitine is biologically inactive. In humans, 75% of the adult's requirement of LC is sourced from the diet, mainly from meat and to a less extent from the plant; while the infants source LC from milk (Arenas et al. 1998). About 25% of LC is synthesised by human body, particularly in the liver and kidney using amino acids lysine and methionine (Arenas et al. 1998; Gulcin 2006; Salama et al. 2012; Tein 2003). LC is mainly stored in the skeletal muscles and to a less extent in the heart, liver, kidney. Very little LC is stored in the blood (Arenas et al. 1998). LC is not degraded in the body but excreted via urine, bile and breast milk (Kępka et al. 2013). LC supplementation during lactation has been shown to increase milk production in sows (Ramanau, Kluge & Eder 2005). LC biosynthesis in infants is not as efficient as in adults, and thus LC supplementation is recommended in newborns with aciduria, childhood epilepsy associated with valproate-induced hepatotoxicity, and in those with kidney-associated syndromes (Kępka et al. 2013).

1.6.2. Carnitine deficiency

Normal plasma LC level is about 25 μmol/L during infancy and 54 μmol/L in aging population. A value of less than 20 μmol/L in all age groups is considered as LC deficiency (Lee Carter, Abney & Lapp 1995). Carnitine deficiency occurs in aberrations of carnitine regulation in disorders such as diabetes, sepsis, cardiomyopathy, hemodialysis, trauma, obesity and endocrine disorders (Flanagan et al. 2010). Furthermore, carnitine deficiency was recognize as a cause of myopathy in humans in 1973 (Matera et al. 2003). The pathological manifestations of carnitine chronic deficiency are the accumulation of neutral lipid within the skeletal muscle, cardiac muscle and liver, and the disruption of muscle fibres and accumulation of large aggregates of mitochondria within the skeletal and smooth muscle (Thorne Research Inc 2005).

Carnitine deficiency can be either inherited or acquired (Matera et al. 2003). There are two carnitine deficiency states, primary and secondary (Flanagan et al. 2010). Primary carnitine deficiency is a rare autosomal recessive disorder of fatty acid oxidation caused by deficiency of plasma membrane Organic cation/carnitine transporter 2 and is observed in some uncommon inherited disorders such as inborn errors of metabolism. It is characterized by low concentrations of carnitine in plasma, muscle and liver which is usually associated with muscle fatigue, cardiomyopathy, abnormal hepatic function, impaired ketogenesis and hypoglycaemia during fasting (Matera et al. 2003). LC was approved by the Food and Drug Administration to treat primary LC deficiency (Martinez et al. 2009).

Secondary carnitine deficiency normally correlates with nutritional inadequacies and can be observed in the LBW newborns, patients with renal tubular disorders and patients with chronic renal failure treated with haemodialysis (Matera et al. 2003; Thorne Research Inc 2005). Secondary carnitine deficiency is characterized by increased carnitine excretion in urine due to accumulation of organic acids. LC supplementation can improve the complications associated with secondary carnitine deficiency (Flanagan et al. 2010).

1.6.3. Carnitine and the kidney

CKD is associated with lipoprotein metabolic abnormalities (Tsuruya et al. 2014). Metabolic syndrome including dyslipidemia is also an independent risk factor for the progression of kidney disease (Cheng et al. 2012). It is suggested that the underlying mechanisms of lipid-induced renal damage could be through oxidative stress, as oxidative stress play a key role in the progression of chronic renal failure (Sener et al. 2004; Trevisan, Dodesini & Lepore 2006).

LC is secreted by the kidney as free carnitine or as acylcarnitine. The normal LC level in the kidney is 330-600 nmol/g (Lee Carter, Abney & Lapp 1995). The association of the endogenous LC level change in the kidney with renal injury, diabetes and obesity has not been reported in the literature. However, the protective effect of LC in the pathogenesis of oxidative stress-related diseases such as ischemia/perfusion kidney injury has been reported in animals (Liu, Yan, et al. 2012). LC treatment can enhance NO level in the endothelial cells (Aydogdu et al. 2006). LC has been used to treat haemodialysis caused dyslipidaemia in patients with CKD, as LC can increase fatty acids transportation into the mitochondria for oxidation to reduce blood TG level (Hurot et al. 2002).

1.6.4. Carnitine and Liver fatty acids metabolism

Liver has a key role in lipid metabolism and body energy homeostasis (Nguyen et al. 2008). Fatty acids are derived from TG mediated by two major lipases, hormone-sensitive lipase and adipose triglyceride lipase (ATGL) (Gutgesell et al. 2009; Schweiger et al. 2006). The fatty acids are taken up by the hepatic cells through fatty acids transporter proteins, such as fatty acid transporter protein 2 and fatty acid translocase CD36 (Karagianni & Talianidis 2015; Reddy & Rao 2006). The mitochondria are the main organelle where the fatty acids are oxidised for energy production. Unlike the short-chain and medium-chain fatty acids that cross the mitochondrial membranes by diffusion, the long-chain fatty acids are unable to cross the mitochondrial membranes. Therefore, LC is required to bind to the long-chain fatty acids to facilitates its crossing the mitochondrial membranes (Gutgesell et al. 2009). This transfer process also requires carnitine palmitoyl transferase (CPT)1, CPT2, and

carnitine-acylcarnitine translocase, which are the key enzymes involved in the regulation of intracellular long-chain fatty acids transportation from the cytosol to the mitochondrial matrix (Matera et al. 2003).

The normal LC level in the liver is 500-1000 nmol/g (Lee Carter, Abney & Lapp 1995). High-fat diet has been shown to increase LC biosynthesis in the liver (Rigault et al. 2013); whereas LC supplementation was shown to ameliorate fatty liver in high fat diet-induced type 2 diabetic mice (Xia et al. 2011), as well as hepatotoxicity in different animal models and human studies (Romero-Falcón et al. 2003; Sugimoto et al. 1987; Yapar et al. 2007). However, the effect of LC on liver metabolic markers in offspring of the smoking mothers has not been studied to date.

1.7. Significance, hypotheses and aims

Significance:

Kidney disease is a significant health problem associated with increased morbidity and mortality rates which negatively affects the patients' life quality and leads to huge economic costs (Cueto-Manzano et al. 2014). This is because the kidney is not only a target organ of diseases such as glomerulosclerosis, but is also involved in the initiation of several pathophysiological processes such as hypertension, cardiovascular disease and diabetic nephropathy, due to its complex effects on body homoeostasis. Different animal studies have shown that changes in the intrauterine environment may predispose the offspring to kidney diseases (Figueroa et al. 2012; Ojeda, Grigore & Alexander 2008). Maternal smoking during pregnancy are associated with many adverse fetal outcomes including an increased risk of obesity, hypertension, and type 2 diabetes later in life, all of which are risk factors for CKD (Li, Sloboda & Vickers 2011; Robinson et al. 2000). It is a major cause of IUGR, leading to LBW and renal disorders in adulthood (Merlet-Benichou 1999; Taal et al. 2011). However, the underlying mechanisms of maternal cigarette SE on renal development and susceptibility to kidney diseases in offspring in adulthood is not fully understood and needs further investigation.

Hypothesis 1: Maternal cigarette SE before and during gestation and lactation can lead to kidney underdevelopment in the offspring, which in turn leads to renal dysfunction and CKD in adulthood.

Aim 1: To examine the impact of maternal cigarette SE prior to, during gestation and lactation on:

a) Nephron number and size at birth, weaning and adulthood as well as renal function at adulthood
b) Changes in different renal growth and transcription factors,
c) Renal expression of pro-inflammatory and injury markers.

Aim 2: To evaluate the gender differences on renal disorders in offspring by maternal SE.

Hypothesis 2: Maternal LC supplementation during gestation and lactation can ameliorate the adverse maternal SE effect on kidney and liver disorders in the offspring.

Aim 3: To examine the effects of maternal LC supplementation during gestation and lactation on:

a) Renal development and structure at early postnatal age and function in adulthood
b) The changes of renal growth and transcription factors,
c) The changes of renal pro-inflammatory and injury markers, in the male offspring.
d) Glucose intolerance as well as liver lipids metabolic and inflammatory markers in the female mice offspring at different time points.

Chapter Two

2

Materials and Methods

2.1. Animal experiment

2.1.1. Ethics approval

The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology, Sydney (ACEC#2011-313A). All protocols were performed according to the Australian NH&MRC Guide for the Care and Use of Laboratory Animals.

2.1.2. Animal model and tobacco cigarette smoke exposure

Female Balb/c mice (6 weeks old, n=20) were purchased from the Animal Resources Centre, Perth, Australia. Upon arrival, the mice were housed in the animal facility at 20 ± 2 ^{o}C and maintained on a 12:12 hour light/dark cycle (lights on 06:00h). They were given 1 week acclimatization with free access to standard rodent chow food (Specialty Feeds, Glen Forest, Western Australia) and water.

After 1 week, the mice were weighed and divided into 2 groups with equal body weight (BW), the sham exposure group (control) and the cigarette smoke exposure group (SE). BW of the mice was recorded once a week.

Breeder mice were exposed to cigarette smoke as per an established protocol (Chen et al. 2008). In details, SE mice were removed from their home cages and placed in a Perspex chamber (18L) in a biological hood. The chamber was filled with cigarette smoke produced by two cigarettes (Winfield Red, 16 mg or less of tar, 1.2 mg or less of nicotine and 15 mg or less of CO; Philip Morris, Melbourne, Australia) a time with a 5-minute interval between, twice (10:00 and 15:30) daily. This dosage is equivalent to human consumption of 1-2 cigarettes per day by human smokers. Control mice were exposed to air in an identical chamber at the same time. When smoke exposure was completed, the mice were returned to their home cages.

After 6 weeks of cigarette smoke exposure, the female mice were mated with 8 weeks old male mice and smoke exposure was continued; while the male mice were left in the

home cages during the exposure. When pregnancy was confirmed by significant increase in BW, the pregnant females were housed separately.

SE was not performed on the day the pregnant breeders gave birth and was resumed the following day as described above. SE was continued during lactation while the pups were left in the home cages during the exposure with no signs of distress on breeders and offspring. The pups were weighed every five days until weaning and then weekly until the end point at week 13. After weaning, offspring were housed under the same conditions with no further SE

2.1.3. Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed at 12 weeks in the offspring. After 5 hours fasting, a baseline glucose level was measured in the tail blood (Accu-Chek, Roche Diagnostics, Indianapolis, IN, USA). Then 50% Glucose was administered intraperitoneally (2 g/kg , n = 9-10) and blood glucose levels were measured at 15, 30, 60, and 90 min post-injection (Chen et al. 2011). The area under the curve (AUC) of the glucose levels was calculated for each mouse.

2.1.4. Tissues collection

Offspring mice were scarified at three different time points; Postnatal day (P) 1, P20 (normal weaning age) and at 13 weeks old (W13, mature age).

A day after birth, litter size was adjusted to 3-4 pups per breeder mouse. All the other pups were weighed and then scarified by decapitation. Trunk blood glucose was measured immediately (Accu-chek®, Roche Diagnostics, Nutley, USA). The kidneys and livers were harvested; one kidney was placed in 10% formalin solution natural buffered (Sigma, VIC, Australia). The other kidney and liver were weighed and snap frozen in liquid nitrogen, and then stored at -80 ^{o}C for later analysis.

At P20 and W13, pups were weighed and anaesthetized with sodium thiopental (0.1ml/g, i.p., Abbott Australasia PTY. LTD, NSW, Australia). Blood was collected

through direct cardiac puncture using a heparinzed syringe and 23 G needle. The blood glucose was measured as described above and then blood was centrifuged immediately at 2000 RCF for approximately 5 minutes at room temperature. The plasma was transferred into a new labelled tube and stored at -20 ^{o}C for analysis. Urine was collected from the bladder; then stored at -20 ^{o}C for later analysis. The offspring were decapitated and the kidneys and liver were harvested and processed as described above.

2.2. Real-time PCR

2.2.1. Total RNA extraction

Total RNA was extracted from chipped kidney and liver tissues (20-50mg) using TRIzol reagent (Life Technologies, CA, USA) or RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturers' instructions.

Using TRIzol method, kidney tissues were homogenized in 1 ml of cold TRIzol in 2ml Eppendorf tube containing 2 tungsten beads (Qiagen, Valencia, CA) using automatic Mixer Mills MM 301 (Retsch GmbH & Co., KG, Haan, Germany) for 5 minutes at 30/Second frequency. The homogenates were incubated for 5 minutes at room temperature for TRIzol reaction with the tissues. The RNA in the homogenates was separated from the DNA and proteins by adding 0.2 ml of chloroform (Sigma, VIC, Australia) into each samples and vortexed for 15 seconds and then incubated at room temperature for 2 minutes. The samples were then centrifuged at 12000 xg for 15 minutes at 4^{o}C. The upper transparent layer that contains the RNA was transferred into new clean tubes. The RNA was precipitated by adding 0.5ml isopropanol (Sigma, VIC, Australia), vortexed and incubated at room temperature for 10 minutes. Samples were centrifuged at 12000g for 10 minutes at 4^{o}C. The supernatant was discarded. The RNA (white pellet) was washed with 80% ethanol (absolute ethanol, (Sigma, VIC, Australia) + MilliQ water) and vortexed again, and then centrifuged. The supernatants were discarded. The RNAs were left to dry for 2 minutes then redissolved in 30µl RNase-free water. Then the RNA samples were quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Using RNeasy plus mini kit, the kidney tissues were homogenized in 350 µl of lysis buffer in 2ml Eppendorf tube using hand-operating homogenizer (Qiagen, Valencia, CA), the homogenate was centrifuged and transferred into clean tube. The total RNA purification was done using automated QIAcube robot system (Qiagen, Valencia, CA). The resulting volume was 50 µl of total RNA. RNA samples were quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Any DNA contamination in the total RNA samples was digested using DNase I, Amp Grade enzyme (Life Technologies CA, USA). For each total RNA sample, 10 µl volume was prepared by mixing 1 µl of 10x DNase I Reaction Buffer, 1 µl of DNase enzyme (DNase I amplification Grade), 1µg of total RNA (volume varies) and the volume was completed with RNase-free water. The mixture was kept on ice, flicked to mix, and then incubated at room temperature for 15 minutes. Then 1 µl of 25mM EDTA solution was added into the reaction in order to deactivate the enzyme. The reaction was heated at 65 ^{o}C for 10 minutes in the PCR machine. The RNA samples were quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and then stored at -80 ^{o}C.

2.2.2. First strand cDNA synthesis

The first strand of cDNA was synthesised using SuperScript® VILO cDNA synthesis kit (Life Technologies, CA, USA) or Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany).

Using SuperScript® VILO cDNA synthesis kit, each reaction was prepared in 20 µl volume by adding 4 µl of 5x VILO^{TM} Reaction Mix, 2 µl of 10x SuperScript® Enzyme Mix, 1 µg of total RNA sample and the volume was completed with RNase-free water. The mixture was kept on ice, flicked and then centrifuged. The reaction tubes were incubated in PCR machine at 25 ^{o}C for 10 minutes followed by 42 ^{o}C for 60 minutes and finally at 85 ^{o}C for 5 minutes.

First strand of cDNA templates were also created using Transcriptor First Strand cDNA synthesis Kit (Roche Diagnostics Gmbh, Mannheim, Germany). The reaction was prepared in 20 µl volume by mixing 1 µg of total RNA (volume varies), 2 µl of Random

Hexamer primer (600pmol/ µl), 4 µl of the 5x concentrate Transcriptor Reverse Transcriptase Reaction Buffer, 0.5 µl of 40U/ µl protector RNase Inhibitor, 2 µl of 10mM Deoxynucleotide Mix, 0.5 µl of 20U/ µl Transcriptor Reverse Transcriptase. The volume was completed with PCR-grade water (Roche, Germany). The reaction was run in a PCR machine at the following thermal cycle: 25 ^{o}C for 10 minutes, 55 ^{o}C for 30 minutes, 85 ^{o}C for 5 minutes. The cDNA samples were quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

cDNA templates were tested for integrity by amplifying 1 µl of the templates in traditional PCR machine using Taq DNA polymerase enzyme (Life technologies, CA, USA) or MangoMix^{TM} kit (BioLine, Toronto, Canada).

Using Taq DNA polymerase enzyme, the master mix was prepared by adding 17 µl of RNase-free water, 2.5 µl of 10xPCR buffer (-$MgCl_2$), 1.25 µl of 50nM of $MgCl_2$, 1.25 µl of dNTP, 0.25 µl of DMSO, 0.25 µl of Taq 0.25 µl of 10µM β actin forward primer and 0.25 µl of 10µM reverse primer. The mixture was flicked and centrifuged. Twenty three (23)µl of the master mix was transferred into PCR tubes and 2 µl of each cDNA template was added into each tube. The tubes were placed in PCR machine and incubated in a thermal cycle at 95 ^{o}C for 4 minutes for denaturing, followed by 35 cycles of amplification at 94 ^{o}C for 30 second, 60 ^{o}C for 30 second, and 72 ^{o}C for 1 minute, and then 72 ^{o}C for 10 minutes.

Using MangoMix^{TM} kit, 20 µl solution was prepared by mixing 10 µl of 2x Mango Mix, 7.4 µl of ultra-pure H_2O, 0.8 µl of each forward and reverse primer of 10µM β actin and 1 µl of cDNA template. The PCR thermal conditions were, initial denaturing at 95 ^{o}C for 4 minutes followed by 35 cycles at 95 ^{o}C for 30 seconds, 60 ^{o}C for 30 seconds, 72 ^{o}C for 1 minute with a final extension at 72 ^{o}C for 10 minutes.

Agarose gel (1.2%) was prepared by dissolving 1.2g agarose powder (Bio-Rad) in 100ml 1xTAE buffer, and then 10 µl Gel Green was added to the mixture. The mixture was heated in the microwave for 1 minute, mixed, let to cool down and then poured into the gel tray. When the gel was set, the tray was placed in the gel tank filled with 1xTAE buffer. The comb was removed and then 20 µl of the amplified cDNA templates that were created using Taq DNA polymerase enzyme were mixed with 2 µl of 10x

bluejuice and the mixtures were loaded into the gel. 50base pair and 1kb DNA ladder were loaded into the gel as well. No 10x bluejuice was mixed with the amplified cDNA templates that were created using MangoMix™ kit. The gel was run at 100V, 400mA for 30 minutes at constant voltage. cDNA bands in the gel were visualised in GelDoc (Bio-Rad) under UV light (Figure 2.1).

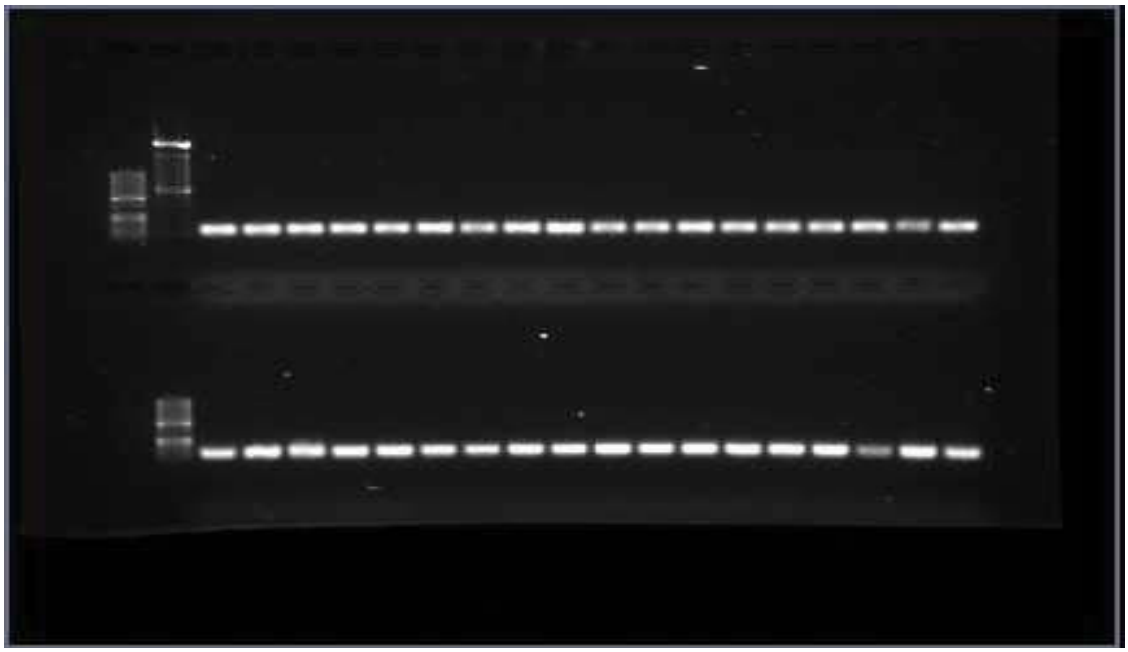


Figure 2.1: A sample of 1.2% agarose gel containing the amplified cDNA templates.

2.2.3. Quantitative real-time PCR (qPCR)

qPCR was performed using pre-optimized SYBR Green primers (Sigma-Aldrich) (Table 2.1) and either rt-PCR Master Mix kit (Life Technology, CA, USA) or SensiFAST™ SYBR Hi-ROX Kit (Bioline, Toronto Canada). The 10 µl final volume of each reaction contains 5 µl of SYBR Green Master Mix, 0.2µl of ROX, 0.2µl of 10µM forward target primer, 0.2µl of 10µM reverse target primer, 1.4µl of RNase-free water, and 3µl of 1:20 diluted cDNA samples. When SensiFAST™ SYBR Hi-ROX Kit was used, the 10 µl final volumes of each reaction contains 5µl of 2x SensiFAST SYBR Hi-ROX mix, 0.4µl of 10µM forward primer, 0.4µl of 10µM reverse primer, 1.2µl of RNase-free water and 3µl of 1:20 diluted cDNA samples.

The reaction was run in duplicate in a 384 well PCR plate (Life Technology, CA USA). The reagents and cDNA samples were loaded into wells using ep Motion 5070, (Eppendorf, Hamburg, Germany). The reaction was run in an ABI7900HT Sequence Detection System (SDS 2.4, Life Technologies, CA USA) using the following thermal condition (Saad et al. 2009): 2-step cycle at 95 ^{o}C for 2 minutes followed by 40 cycles at 95 ^{o}C for 5 seconds and 62 ^{o}C for 20 seconds (Figure 2.2). The melting curve was used for the experiment quality control (Figure 2.3).

Table 2.1: Forward and reverse sequences of the mice primers for rt-PCR

Primer	Forward 5′-3′	Reveres 5′-3′
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC
BMP7	CACTCCCTCCTCAACCCTCGGCA	TAGAGGCATCATAGGCCAGGTGCCC
BMP4	GGTCCAGGAAGAAGAATAA	GGTACAACATGGAAATGG
Collagen IV	TTAAAGGACTCCAGGGACCAC	CCCACTGAGCCTGTCACAC
FGF2	GACCCCAAGCGGCTCTACTGC	GTGCCACATACCAACTGGAGT
FGF7	GGCAATCAAAGGGGTGGA	CCTCCG CTG TGTGTCCATTTA
FGF10	TGAGACAATTTCCAGTGCCG	TATCTCCAGGACACTGTACG
Fibronectin	CACGGAGGCCACCATTACT	CTTCAGGGCAATGACGTAGAT
GDNF	ATTTTATTCAAGCCACCATTA	GATACATCCACACCGTTTAGC
MCP1	CATCCACGTGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT
Pax2	CGCCGTTTCTGTGACACACAATC	TGCTTGGGACCAAACACAAGGTG
WNT4	AGGAGTGCCAATACCAGTTCC	TGTGAGAAGGCTACGCCATA
WNT11	CAGGATCCCAAGCCAATAAA	GACAGGTAGCGGGTCTTGAG
WT1	ATCAGATGAACCTAGGAG	CTGGGTATGCACACATGA

18S: (Yu et al. 2013). BMP7: (De Petris et al. 2007). BMP4: (Talavera-Adame et al. 2013). Collagen IV: (Wong et al. 2014). FGF2: (Wake et al. 2009). FGF7: (Steiling et al. 2004). FGF10: (Itoi et al. 2007). Fibronectin: (Wong et al. 2014). GDNF: (Kuure et al. 2005). MCP1: (Kawasaki et al. 2013). Pax2: (Vigneau et al. 2007). WNT4: (Brisken et al. 2000). WNT11: (Ueno et al. 2007). WT1: (Wagner et al. 2004).

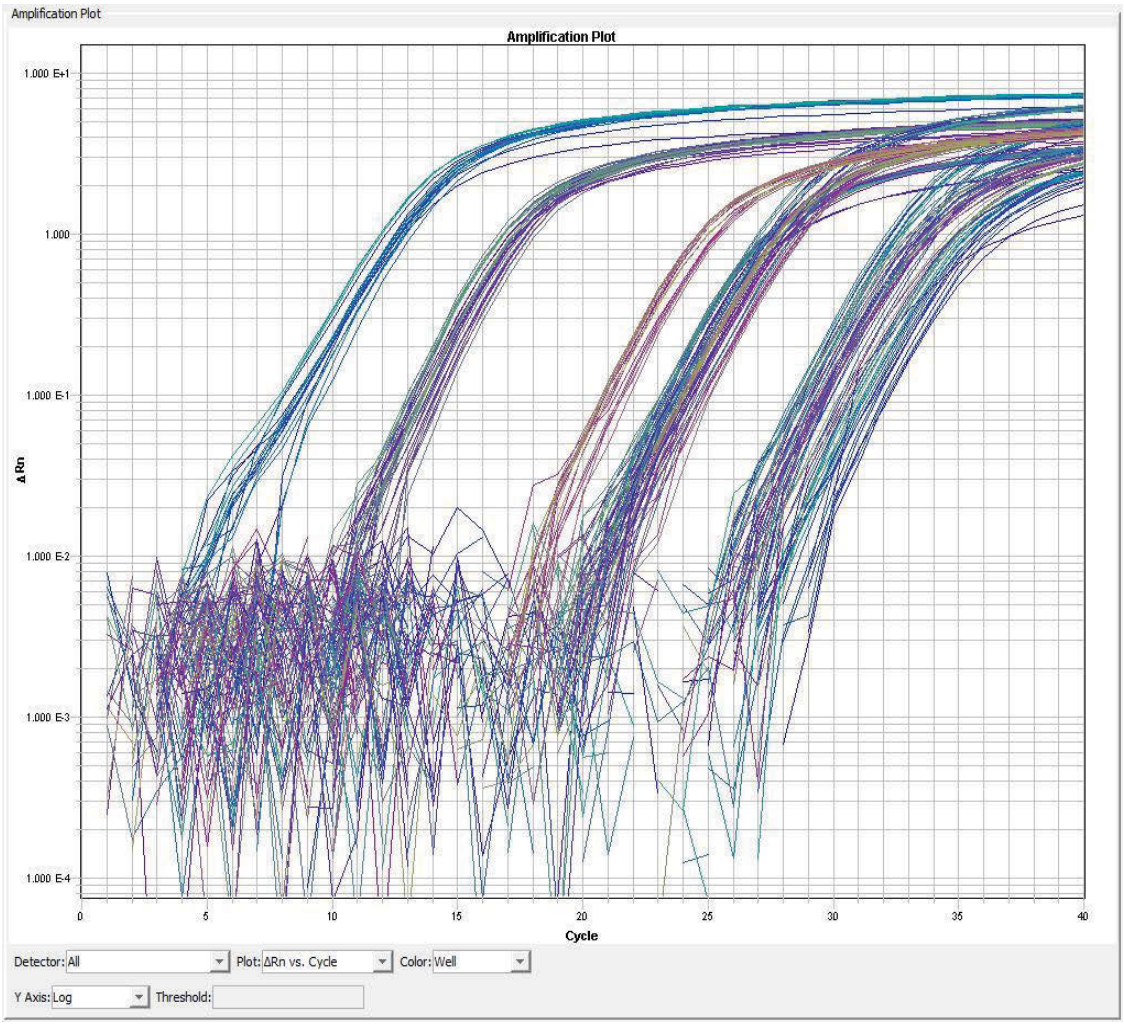


Figure 2.2: Sample of reaction curve using SYBR Green primers.

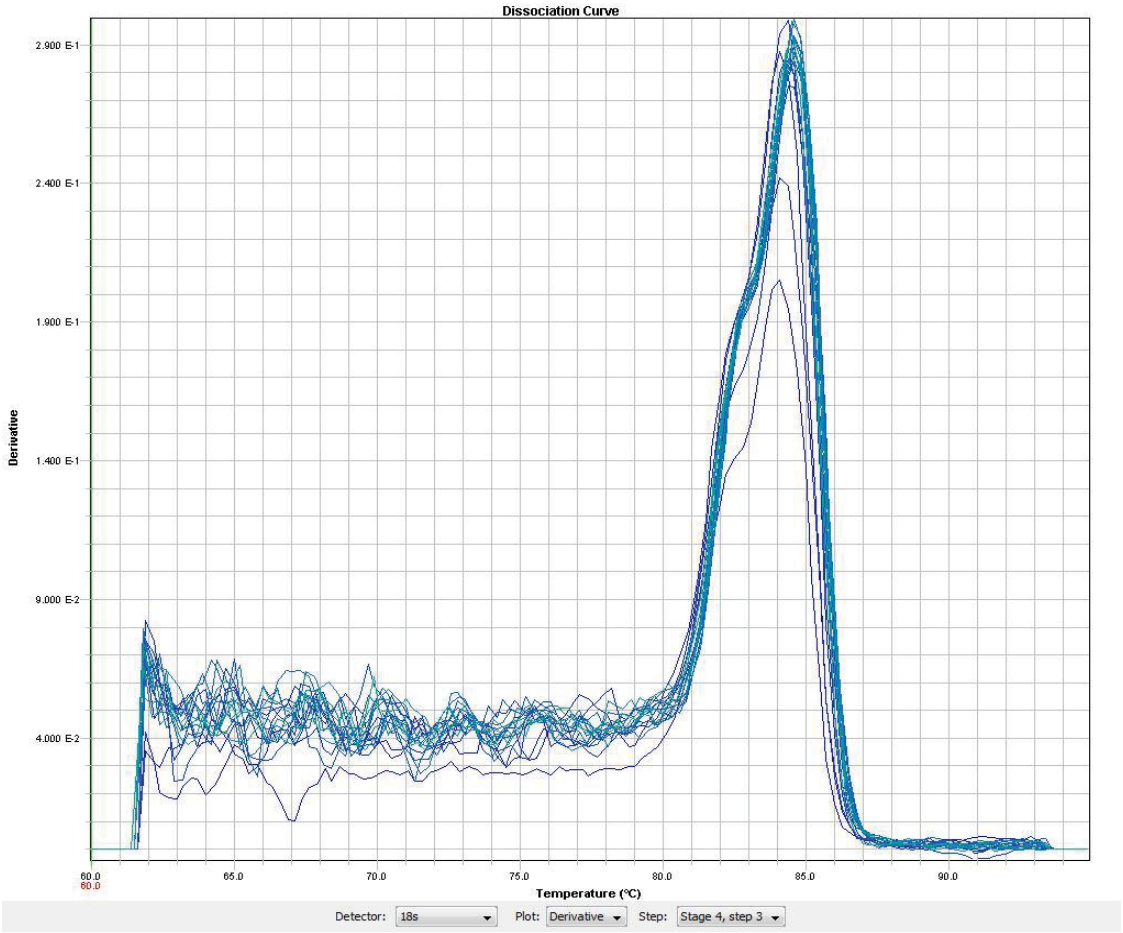


Figure 2.3: Sample of melting curve showing primers specificity.

qPCR was also performed using different Taqman probes (Life Technologies, CA USA) (Table 6.1) and Platinum® qPCR SuperMix-UDG kit (Life Technologies, CA, USA). The qPCR reaction was run in 10 µl final volume containing the following: 2.5µl of 0.2 µg/µl cDNA, 5µl of 25x Platinum® qPCR SuperMix-UDG, 0.5µl of the target probe (FAM), 0.5µl of 18S (VIC) and the volume was completed with UltraPure™ DNase/RNase-Free Distilled Water (Life Technologies, CA, USA).

The thermal cycle condition for using Platinum® qPCR SuperMix-UDG kit was 50 ^{o}C for 2 minutes, 95 ^{o}C for 2 minutes followed by 40 cycles of 95 ^{o}C for 15 seconds and 60 ^{o}C for 60 seconds (Figure 2.4).

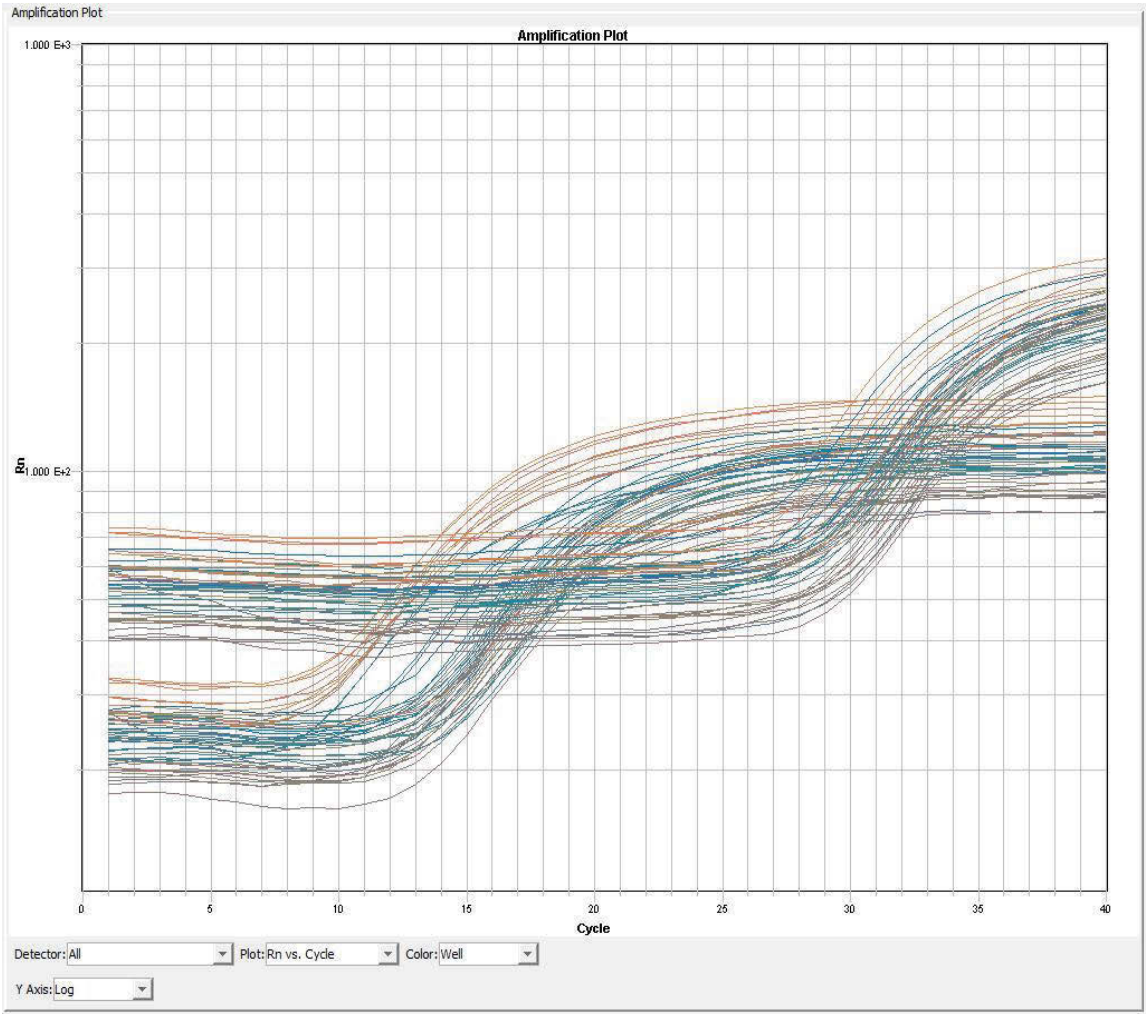


Figure 2.4: Reaction curve using Taqman probes

The data were analysed by relative quantitation using RQ Manager Software (RQ 1.2.1, Applied Biosystems), which is calculated as $2^{\Delta\Delta Ct}$, ΔCt value between the target primer and housekeeping gene, and ΔΔCt value between the experimental samples and the

calibrator. 18S rRNA was used as the housekeeping gene and the Sham group was used as the calibrator.

2.3. Kidney histology, glomerular number and size measurement

Fixed kidney samples in 10% formaldehyde were embedded in paraffin and sectioned in 2µm and 4µm. Kidney structure of the offspring was examined using hematoxylin and eosin (H&E), periodic acid Schiff (PAS) stain and Masson's trichrome stain of the 2µm kidney sections. Tubular structure was examined using both PAS and Masson's staining.

2.3.1. H&E staining

The kidney sections were de-waxed of paraffin by incubating the kidney section slides at 60 ^{o}C for 3 minutes followed by incubation in 100% xylene for 5 minutes twice. Then the sections were hydrated by incubation in 100% ethanol for 3 minutes twice, 95% ethanol for 3 minutes once, 70% ethanol for 3 minutes once, then washed in running water for 3 minutes. The sections then were incubated in haematoxylin for 3 minutes then washed under running water followed by decolourization in acid ethanol and then washed under running water. Then the sections were counterstained in eosin for 30 seconds then serially dehydrated in 100% ethanol 3 times and cleared in 100% xylene twice. Slides were coverslip mounted and left to dry in the fume hood.

Glomerular number for each animal was estimated by counting the developed glomeruli in 3-4 non-consecutive H&E stained kidney sections from the same animal. There were in average 6-8 different mice in each group. The results were expressed as the number of developed glomeruli /kidney section.

2.3.2. PAS staining

The kidney sections were de-waxed of paraffin by incubating the kidney section slides at 60 ^{o}C for 3 minutes followed by incubation in 100% xylene for 5 minutes twice. Then

the sections were hydrated by incubation in 100% ethanol for 3 minutes twice, 95% ethanol for 3 minutes once, 70% ethanol for 3 minutes once, and then washed in running water for 3 minutes. Then the sections were exposed to 0.5% periodic acid solution for 10 minutes, and then washed in running water followed by counterstaining with haematoxylin, then serially dehydrated in 100% ethanol 3 times and cleared in 100% xylene twice. Slides were coverslip mounted and left to dry in the fume hood.

Glomerular size was measured in the PAS stained kidney sections using Image J (Image J, NIH, USA) by drawing a circle around the outer border of the glomerulus in the kidney image and the perimeter was measured. In average of 6 different images for the same kidney section were used in the glomerular size estimation then they were averaged.

2.3.3. Masson's staining

The kidney sections were de-waxed of paraffin by incubating the kidney section slides at 60 ^{o}C for 3 minutes followed by incubation in 100% xylene for 5 minutes twice. Then the sections were hydrated by incubation in 100% ethanol for 3 minutes twice, 95% ethanol for 3 minutes once, 70% ethanol for 3 minutes once, and then washed in running water for 3 minutes. Then the sections were stained in Weigert's iron hematoxylin solution for 10 minutes, and then washed under running water. Then the sections were stained in Biebrich scarlet-acid fuchsin solution for 10 minutes, and then washed under running water for three minutes. And then the sections were were incubated in phosphotungistic acid solution for 10 minutes then transferred to aniline blue solution for 5 minutes. The sections were washed under running water and then transferred to 1% acetic acid solution for 2-5 minutes followed by washing under running water, then serially dehydrated in 100% ethanol 3 times and cleared in 100% xylene twice. Slides were coverslip mounted and left to dry in the fume hood.

The percentage area of positive stain in both PAS and Mason's staining was quantified using Image J (Image J, NIH, USA).

2.4. Immunohistochemistry (IHC) staining

For the IHC staining, 4µm kidney sections were de-waxed of paraffin by incubation in the oven at 60 ^{o}C for 3 minutes followed by incubation in 100% xylene for 5 minutes twice. Then the tissues were hydrated by incubation in 100% ethanol for 3 minutes twice, 95% ethanol for 3 minutes once, 70% ethanol for 3 minutes once then washed in running water for 3 minutes. The slides were boiled in pre-heated 10mM citrate acid (Sigma) buffer (PH 6) in a water bath at 99 ^{o}C for 20 minutes for heat-induced epitops retrieval. When they cooled down to room temperature, they were washed in running water and then incubated in 0.3% H_2O_2 for 5 minutes at room temperature to block the endogenous peroxidase. The slides were washed in running water for 3 minutes and then placed in sequenza tray filled with 1xT-TBS to check the leakage. The sections were washed in 1x T-TBS for 5 minutes, then 3 drops (100µl) protein block serum-free (Dako, CA, USA) was added to each kidney sections for 10 minutes at room temperature. Kidney sections were then incubated with rabbit anti-mouse primary antibodies against FGF2 (1:250), GDNF (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Pax 2 (1:1750), fibronectin (1:500) and collagen IV (1:500) (Abcam, Cambridge, UK) at 4^{o}C overnight. Negative controls were prepared by replacing the primary antibodies with rabbit IgG.

In the following day, the sections were washed in 1xTBS-T for 6 minutes and then exposed to Envision+system-HRP labelled secondary anti-rabbit antibodies (Dako, CA, USA) for 30 minutes at room temperature. Secondary antibody was detected by adding DAB + (liquid DAB + substrate chromogen system, Dako, CA, USA) to the kidney sections. The section were washed in running water and then counterstained with Mayer's Haematoxylin, washed in running water for 2 minutes followed by serially dehydration in 100% ethanol 3 times and then cleared in 100% xylene twice. Slides were coverslip mounted and left to dry.

The kidney tissues were examined and the images were captured by bright field microscopy using a Leica photomicroscope and a DFC 480 digital camera. In average, 6 different non-overlapping fields of the kidney tissues were captured for each section. Quantitation of the positive signals (the intensity of the brown colour) in the images was performed using Image J software and the percentage of the brown colour to the whole

field was determined and averaged in 6 different fields for each kidney section (Image J, NIH, USA). Three to four kidney sections were used from each animal and at least 6 animals were used from each group.

2.5. ELISA

Different commercial ELISA kits were used to measure urinary albumin and creatinine concentrations and serum insulin and cotinine levels.

2.5.1. Urinary albumin assay

Urine albumin and creatinine were measured using Murine Microalbuminuria ELISA kit (Albuwell M, Exocell Inc, PA, USA) and Creatinine Companion kit (Exocell Inc, PA, USA) respectively. Serum enzymatic creatinine levels were measured by an automated analyser (ARCHITECT, Abbott Australasia PTY. LTD, NSW, Australia).

For the albumin, 7 two-fold dilutions of the standard of murine serum albumin (MSA) were prepared from two-fold concentrated solution to the following concentration， 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0 μg/ml (diluent only which was used as a negative control). The urine samples were diluted （1:13） using normal horse serum with bovine serum albumin (NHEBSA). Controls, diluted standards and diluted samples were loaded into a 96 well plate in duplicate. The assay volume was 50μl.

Rabbit anti-murine albumin antibody (50μl) was added to the wells except for the negative control well. The plate was incubated at room temperature for 30 minutes and then washed using tap water for 10 times. One hundred (100) μl of anti-Rabbit HRP conjugate secondary antibody was added to the wells and then incubated for 30 minutes at room temperature. The plate was washed before 100μl of colour developer was added to each well and then incubated at room temperature for 5 minutes. Then 100μl of colour stopper was added to the wells. The absorbance was determined using microplate reader (LP400, Diagnostics Pasteur) at 450nm. The standard curve was generated by semi-logarithmic of the standard dilutions with the log[MSA] on the x-axis and mean absorbance on the y-axis. The data falling into the linear portion of the curve were used to calculate the albumin concentration and then multiplied by the dilution factor.

2.5.2. Urinary creatinine assay

To measure creatinine, the reagent was prepared by mixing 1N NaOH and Picrate reagent in 1:5 ratio. Urine samples were diluted (1:20) using distilled water. Creatinine standards 10, 3, and 1mg/dl and diluted urine samples were transferred into the plate. Picrate working solution (100µl) was added to each well and the plate was incubated at room temperature for 10 minutes. The absorbance was determined using microplate reader (LP400, Diagnostics Pasteur) at 492nm. Then 100µl of acid reagent was added to each well, and let to stand for 5 minutes. The absorbance was measured again at the same wavelength. Absorbance was calculated from the following formula:

$$A_{delta} = A_{alkaline\ picrate} - A_{alkaline\ picrate+acid}.$$

The square regression was determined using delta absorbance versus creatinine concentration for each standard. The concentrations of the diluted samples were determined from the standard curve (Figure 2.5) and then multiplied by the dilution factor for each sample.

The albumin/creatinine ratio was calculated from the albumin and creatinine assays results.

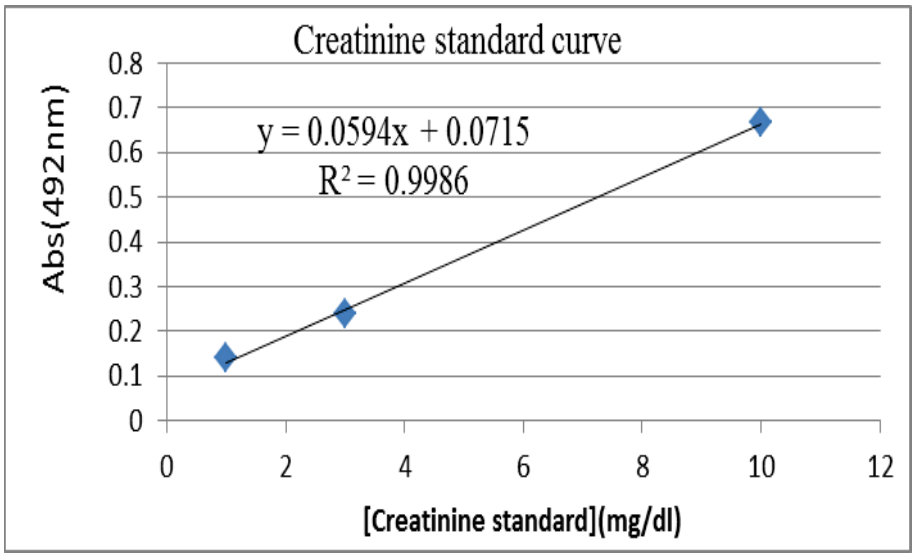


Figure 2.5: A sample standard curve used to calculate creatinine concentration in the urine.

2.5.3. Plasma insulin assay

The plasma insulin concentration was measured using a commercial ELISA kit (Insulin (Mouse) ELISA kit, Abnova, Taipei, Taiwan) according to the manufacturer's instructions.

In details, Plasma samples were coagulated at room temperature for two hours then centrifuged at 2000 xg for 20 minutes. Fifty (50) µl of the standards solutions in the following concentrations: 8µlU/ml, 16µlU/ml, 32µlU/ml, 80µlU/ml and 140µlU/ml in addition to plasma samples and control were loaded into the pre-coated plate in duplicate. Fifty (50) µl of the HRP conjugated anti-mouse insulin antibody into the wells except for the control well. The plate was sealed with a cover and incubated at 37 ^{o}C for I hour. Then the liquid was discarded from the wells and the plate was clapped on absorbent filter paper without washing. Then the plate was washed with wash buffer and then 50µl of Tetramethylbenzidine (TMB) substrate A was added into each well followed by 50µl of substrate B. The plate was shook for 30 seconds then incubated in dark at 37 ^{o}C for 15 minutes. Stop solution (50µl) was added to the wells. The absorbance was determined at 450nm (Bio-rad).

2.5.4. Plasma cotinine assay

The plasma cotinine concentration was measured using the commercial cotinine Direct ELISA kit (Abnova, Taipei, Taiwan) according to the manufacturer's instructions.

In details, all reagents were brought to room temperature, then 10µl of standards, controls and serum samples were plated into the plate in duplicate and them 100µl of enzyme conjugate was added to each well. The plate was shack for 30 seconds then incubated at room temperature for 1 hour. The wells then were washed 6 times with 300 µl distilled water then dried. And then 100 µl of the substrate reagent was added to each well followed by 30 minutes incubation at room temperature. Stop solution (100 µl) was added to each well, and then mixed. The plate was read at 450nm within 15 minutes from adding the stopping solution.

To analyze the data, the standards curve was constructed where the standards absorbances were plotted on the y-axis and the standards concentrations were plotted at x-axis. The controls and samples values were read from the standard curve.

2.6. Serum enzymatic creatinine

Serum enzymatic creatinine level was measured by an automated analyzer (ARCHITECT, Abbott Australasia PTY. LTD, NSW, Australia).

2.7. Plasma non-esterified fatty acids (NEFA) assay

NEFA in plasma samples were measured using HR Series NEFA-HR(2) kit (WAKO, Osaka, Japan).

For the standards solutions, 7 different standards solutions were prepared from the neat standard to the following concentrations, 15, 10, 5, 2.5, 1.25, 0.625, 0.3125 nmol. Five microliter of each plasma sample and the standards solutions were transferred into a 96 well plate in duplicate. And then 100 µl of reagent A was added and incubated at 37ºC for 10 minutes, followed by 200µl of reagent B and incubated at 37ºC for further 10 minutes. The absorbance was determined at 550nm using microplate reader (Bio-rad).

2.8. Plasma TG assay

Standards solutions (Roche Diagnostics, Mannheim, Germany) were first prepared from neat standard in the following concentrations: 0.025, 0.0125, 0.00625, 0.003125, 0.0015625 and 0 mg. Into a 96 well plate, 5µl of each standard and serum sample were transferred in duplicate. Then 150µl of TG reagent (Roche Diagnostics, Mannheim, Germany) were added into each well and incubated at 37ºC for 20 minutes. The absorbance was determined at 490nm (Bio-rad).

2.9. Statistical analysis

The differences between the Sham and SE groups were analysed using unpaired

Student's *t*-test ; one-way ANOVA followed by Fisher Least Significant Difference post hoc tests were used to compare the differences between more than 2 groups (Prism 6, Graphpad CA, USA). The results are expressed as mean ± SEM. $P<0.05$ is considered significant.

Chapter Three

3

The Impact of Maternal Cigarette Smoke Exposure on Renal Development and Function at Adulthood in the Male Mice Offspring

3.1. Introduction

Many studies have established the association between maternal smoking and long-term health consequences in the offspring, including obesity, respiratory and cardiovascular diseases (Chen & Morris 2007; Chen et al. 2012). Maternal smoking is associated with IUGR (Windham et al. 2000) while IUGR in turn, is associated with reduced nephron number in the offspring (Luyckx & Brenner 2005). Indeed, human research has shown that maternal smoking is closely linked to lower fetal kidney volumes during the second and third trimester, and lower birth weight (Anblagan et al. 2013). The reduction in fetal kidney volume correlates with the number of cigarettes smoked in a dose-dependent manner. Only maternal smoking greater than 10 cigarettes/day led to smaller fetal kidney volumes (Taal et al. 2011).

In humans, nephrogenesis starts at gestational week 6-8 (Merkel, Karner & Carroll 2007), and ends at gestational week 36 (Hinchliffe et al. 1991). Most nephrons are formed in the third trimester (Cass et al. 2004), and the final number of nephrons in each kidney is established at birth. However, in rodents, nephrogenesis continues after birth for a short period of time until weaning (Michos 2009). Kidney development is regulated by a highly coordinated activation of multiple growth factors and transcriptional regulators (Kuure, Vuolteenaho & Vainio 2000). Alteration of these growth factors at any stage of kidney development can lead to renal underdevelopment and potential renal dysfunction in the long term (Cunha, Aguila & Mandarim-de-Lacerda 2008; Merlet-Benichou 1999).

Few studies have directly investigated the mechanisms underlying renal developmental abnormalities induced by maternal smoking, and the results from available literature are contradictory. Animal studies have shown both increased (Zarzecki et al. 2012), and unchanged apoptosis (Karaoglu et al. 2012) in the offspring kidney of smoke exposed dams. One study using proteomic analyses in mice did not observe changes in protein expression of factors involved in renal growth, albeit small birth weight and kidney mass (Jagadapillai et al. 2012). However, it could be argued that the cigarette smoke dose in this model was greater than expected in human correlates (Jagadapillai et al.

2012). The cigarette smoke in the mentioned study was generated from Philip Morris Marlboro Red brand cigarettes ™ (Philip Morris, Richmond, VA; 15 mg of tar/cigarette; 1.1 mg nicotine/cigarette) for 6 hours a day, seven days a week (Canales et al. 2012). Maternal smoking may impair renal function in the offspring once adulthood is reached (Koleganova, Piecha & Ritz 2009; Taal et al. 2011; Uhlenhaut & Treier 2008), due to abnormal early development, including low numbers of nephrons and secondary hyperfiltration (Taal et al. 2011). However, it is still unknown how maternal smoking is linked to an increased risk of developing chronic kidney disease in offspring (Chong & Yosypiv 2012).

This study aimed to investigate the changes of kidney structure and factors that regulate renal development at different postnatal ages, including P1, P20 and W13, as well as renal function in adulthood, in mice offspring of dams exposed to cigarette smoke before and during gestation and lactation.

3.2. Materials and methods

3.2.1. Modelling cigarette smoke exposure in mice

The animal experiment was approved by the Animal Care and Ethics Committee at the University of Technology, Sydney (ACEC#2011-313A). All protocols were performed according to the Australian NH&MRC Guide for the Care and Use of Laboratory Animals.

Female Balb/c mice (6 weeks old, n=20, Animal Resources Centre, Perth, Australia) were weighed and divided into 2 groups with equal BW, the Sham exposure group (control, n=10) and the cigarette SE group (SE, n=10). Body weight of the mice was recorded once a week.

Breeder mice were exposed to tobacco cigarette smoke as per protocol described in Chapter 2, section 2.2. In brief, SE mice were exposed to cigarette smoke produced by two cigarettes (Winfield Red), twice daily. Control mice were exposed to air in an

identical chamber at the same time. After 6 weeks of cigarette smoke exposure, the female mice were mated with 8 weeks old male mice and smoke exposure was continued during gestation and lactation. The pups were weighed every five days.

3.2.2. Tissues collection

Offspring mice were scarified at three different time points. At the end point, pups were weighed and anaesthetized with sodium thiopental (0.1ml/g, i.p., Abbott Australasia PTY. LTD, NSW, Australia). Blood was collected through direct cardiac puncture using a heparinzed syringe and 23 G needle and glucose was measured (Accu-chek®, Roche Diagnostics, Nutley, USA). Urine was collected from the bladder. The plasma and urine samples were stored at -20 ^{o}C for later analysis. Mice were killed by decapitation. The kidneys were harvested, one was placed in 10% formalin solution natural buffered (Sigma, VIC, Australia). The other kidneys were weighed and snap frozen in liquid nitrogen then stored in -80 ^{o}C.

3.2.3. IPGTT

IPGTT was performed at 12 weeks of age in the offspring. After 5 hours fasting, a baseline glucose level was taken from tail blood (Accu-Chek, Roche Diagnostics, Indianapolis, IN, USA). Then 50% Glucose was administered intraperitoneally (2 g/kg , n = 9-10) and blood glucose levels were measured at 15, 30, 60, and 90 min post-injection (Chen et al. 2011). The AUC of the glucose levels was calculated for each mouse.

3.2.4. Real-time PCR

Total RNA was extracted from kidney tissues using TRIzol reagent (Life Technologies, CA, USA) according to the manufacturers' instructions as described in Chapter 2, section 2.5. Briefly, kidney tissues were homogenized in 1 ml of cold TRIzol (Life Technologies, CA, USA). The RNA, DNA and proteins in the homogenate were separated into layers by adding 0.2 ml of chloroform (Sigma, VIC, Australia) then

centrifugation. The upper layer that contains the RNA was transferred into clean DNase and RNase free tubes. The RNA was precipitated by adding 0.5ml isopropanol (Sigma, VIC, Australia), then centrifuged at 12000g for 10 minutes at 4^{o}C. The RNA (white pellet) was washed with 80% ethanol (absolute ethanol, (Sigma, VIC, Australia) + MilliQ water) then dissolved in 30µl RNase free-water. The DNA in the total RNA samples was digested using DNase I, Amp Grade enzyme (Life Technologies CA, USA). The first strand of cDNA was synthesized using SuperScript® VILO cDNA synthesis kit (Life technologies, CA, USA) or Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany).

The cDNA samples were quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and then tested for integrity by amplifying 1 µl of the templates in traditional PCR machine using MangoMix^{TM} kit (BioLine, Toronto, Canada).

The PCR products were run in 1.2% agarose gel stained with Gel Green on constant voltage, 100 V, 400mA for 30 minutes. The cDNA bands in the gel were visualized in GelDoc (Bio-Rad).

qPCR was performed using pre-optimized SYBR Green primers (Sigma-Aldrich) (primers sequence in Table 2.1) with either rt-PCR Master Mix kit (Life Technology, CA, USA) or SensiFAST^{TM} SYBR Hi-ROX Kit (Bioline, Toronto canada). The reaction was run in duplicate in a 384 well PCR plate (Life Technology, CA USA) in ABI7900HT Sequence Detection System (SDS 2.4, Life Technologies, CA USA) as described in chapter 2, section 2.5.

The results files were analyzed by relative quantitation using RQ Manager Software (RQ 1.2.1, Applied Biosystems) using ΔΔCt. At all the time, the average mRNA expression of the Sham (control) group was used as the calibrator and 18S rRNA was used as the housekeeping gene.

3.2.5. Kidney histology and IHC staining

Fixed kidney samples in 10% formaldehyde were embedded in paraffin and sectioned in 4μm. Kidney structure of the offspring was examined using H&E, PAS stain and Masson's stain.

For IHC staining, Kidney sections were de-waxed then dehydrated. Heat induced epitops retrieval was performed then endogenous peroxidase was blocked by incubated the sections in 0.3% H_2O_2 for 5 minutes at room temperature. The kidney sections were blocked using protein block serum-free (Dako, CA, USA) for 10 minutes at room temperature, and then incubated with rabbit anti-mouse primary antibodies against FGF2 (1:250 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), GDNF (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Pax 2 (1:1750 dilution, Abcam, Cambridge, UK) at 4^{o}C overnight. Negative controls were prepared by replacing the primary antibodies with rabbit IgG. In the following day, the sections were exposed to Envision+system-HRP labelled polymer secondary anti-rabbit antibodies (Dako, CA, USA) for 30 minutes at room temperature. Secondary antibody was detected by reaction of HRP enzyme and DAB+(liquid DAB + substrate chromogen system, Dako, CA, USA). The section counterstained in Mayer's Haematoxylin.

The kidney tissues were examined and the images were captured by bright field microscopy using a Leica photomicroscope and a DFC 480 digital camera. Four to twelve different non-overlapping fields of the kidney tissues were captured. Quantitation of the positive signals (the intensity of the brown colour) in the images was performed using Image J software and the percentage of the brown colour to the whole field was determined and averaged in four to twelve fields for each kidney section (Image J, NIH, USA). Three to four kidney sections were used from each biological repeat and at least 6 animals were used from each group.

3.2.6. Glomerular number and size measurement

The developed glomerular number in the kidney was estimated by counting glomeruli in 3-4 non-consecutive kidney sections and averaged. Six to eight different biological repeats from each group were used. The results were expressed as number of developed glomeruli /kidney section.

Glomerular size was measured using Image J (Image J, NIH, USA) by measuring the perimeter of the glomerulus in the kidney. Four to ten different images for the same kidney section and 3-4 kidney section from each group were used in the glomerular size calculation.

3.2.7. Albumin and creatinine assays

Urine albumin and creatinine were measured using Murine Microalbuminuria ELISA kit (Albuwell M, Exocell Inc, PA, USA) and Creatinine Companion kit (Exocell Inc, PA, USA) respectively according to the manufacturer instructions as described in chapter 2, section 2.8. Serum enzymatic creatinine levels were measured by an automated analyzer (ARCHITECT, Abbott Australasia PTY. LTD, NSW, Australia).

3.2.8. Statistical analysis

The differences between the Sham and SE groups were analyzed using two-tailed, unpaired Student's *t*-test assuming equal variances between the groups (Prism 6, Graphpad CA, USA). The results are expressed as mean ± SEM. $P<0.05$ is considered significant.

3.3. Results

3.3.1. Female breeders

Before the commencement of smoke exposure, the BW was similar between the Sham and SE groups (Table 3.1). Six weeks later, the SE dams had significantly smaller BW compared with the Sham exposed dams ($p<0.05$, Table 3.1). At the endpoint, BW, kidney weight and liver weight were significantly lower in the SE

dams ($p<0.01$). Blood glucose levels were similar between groups (Table 3.1).

Table 3.1: The effects of smoking on female breeders

Breeders	Sham	SE
Body weight before smoke exposure (g)	17.6 ± 0.2	17.4 ± 0.2
Body weight at mating (g)	19.2 ± 0.2	17.6 ± 0.2**
Body weight at cull (g)	24.6 ± 0.4	21.9 ± 0.2**
Kidney (g)	0.17 ± 0.003	0.15 ± 0.003**
Kidney%	0.69 ± 0.01	0.66 ± 0.04
Liver (g)	1.78 ± 0.05	1.44 ± 0.04**
Liver%	7.22 ± 0.12	6.53 ± 0.15**
Blood glucose (mM)	12.4 ± 1.1	10.6 ± 0.8

Results are expressed as mean ± SEM, n = 20. * $p < 0.05$, ** $p < 0.01$. SE: smoke exposed.

3.3.2. Parameters in the offspring

At P1 and P20, the BW and organ weights of the SE offspring were similar to the Sham (Table 3.2). At W13, BW and kidney weight were still significantly lower in the SE offspring ($p<0.05$, Table 3.2). There was no difference in blood glucose levels at all ages (Table 3.2).

Table 3.2: The effects of maternal SE on growth and development in offspring.

Male offspring	P1		P20		W13	
	Sham	SE	Sham	SE	Sham	SE
Body weight (g)	1.48 ± 0.05	1.51 ± 0.07	9.90 ± 0.22	9.70 ± 0.22	26.1 ± 0.4	24.6 ± 0.4*
Kidney (g)	0.01±0.001	0.01±0.001	0.07±0.002	0.07±0.002	0.22 ± 0.003	0.19 ± 0.005*
Kidney%	0.67 ± 0.11	0.60 ± 0.09	0.70 ± 0.01	0.71± 0.01	0.85 ± 0.01	0.78 ± 0.01*
Liver (g)	0.04 ± 0.01	0.04 ± 0.01	0.43 ± 0.01	0.42 ± 0.02	1.30 ± 0.02	1.27 ± 0.03
Liver%	2.92 ± 0.3	2.83 ± 0.5	4.38 ± 0.06	4.32 ± 0.09	4.99 ± 0.05	5.15 ± 0.07
Glucose (mM)	4.70 ± 0.2	4.23 ± 0.1	10.8 ± 0.5	11.4 ± 0.5	10.8 ± 0.4	10.1 ± 0.3

Results are expressed as mean ± SEM, n=11-23. * $p < 0.05$. SE: smoke exposed.

However, IPGTT showed a significantly increased blood glucose level at 15 and 30 min post-injection ($p<0.05$, Figure 3.1A). AUC also showed significant glucose intolerance ($p < 0.05$, Figure 3.1B) in the SE offspring compare to the Sham ($p<0.05$).

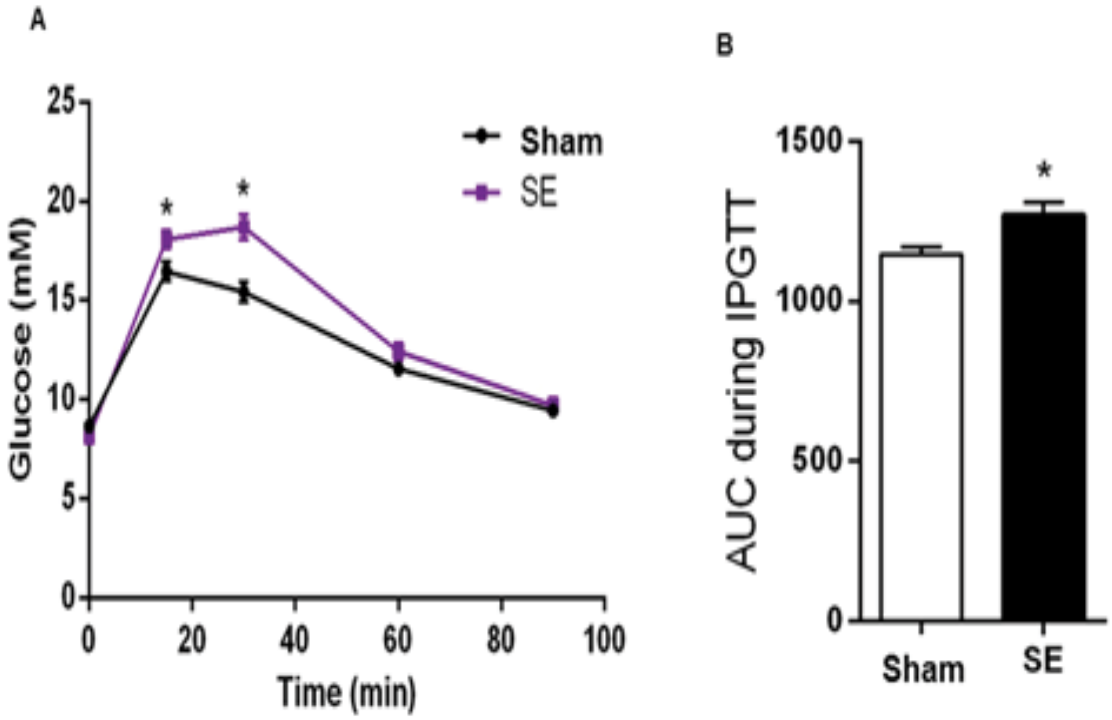


Figure 3.1. Glucose tolerance test in offspring of SE dams at Week 12. (A) Blood glucose changes during IPGTT over time. (B) AUC (A) showing that offspring from SE dams are glucose intolerant at adulthood (W13). n = 9-10. * $p < 0.05$, maternal SE effect.

3.3.2.1 Kidney histological changes and glomerular numbers

At P1, the SE offspring had fewer glomeruli and more immature (non-vascularized) glomeruli compared with the sham offspring ($p<0.01$, Figure 3.2A,B). At P20, fewer developed and more underdeveloped glomeruli were still evident in the SE offspring compared to the Sham offspring ($p<0.01$, Figure 2C,D). At W13, SE offspring still got smaller glomerular number than the sham offspring ($P<0.05$), although the glomeruli were structurally developed in the adult SE offspring (Figure 3.2E,F).

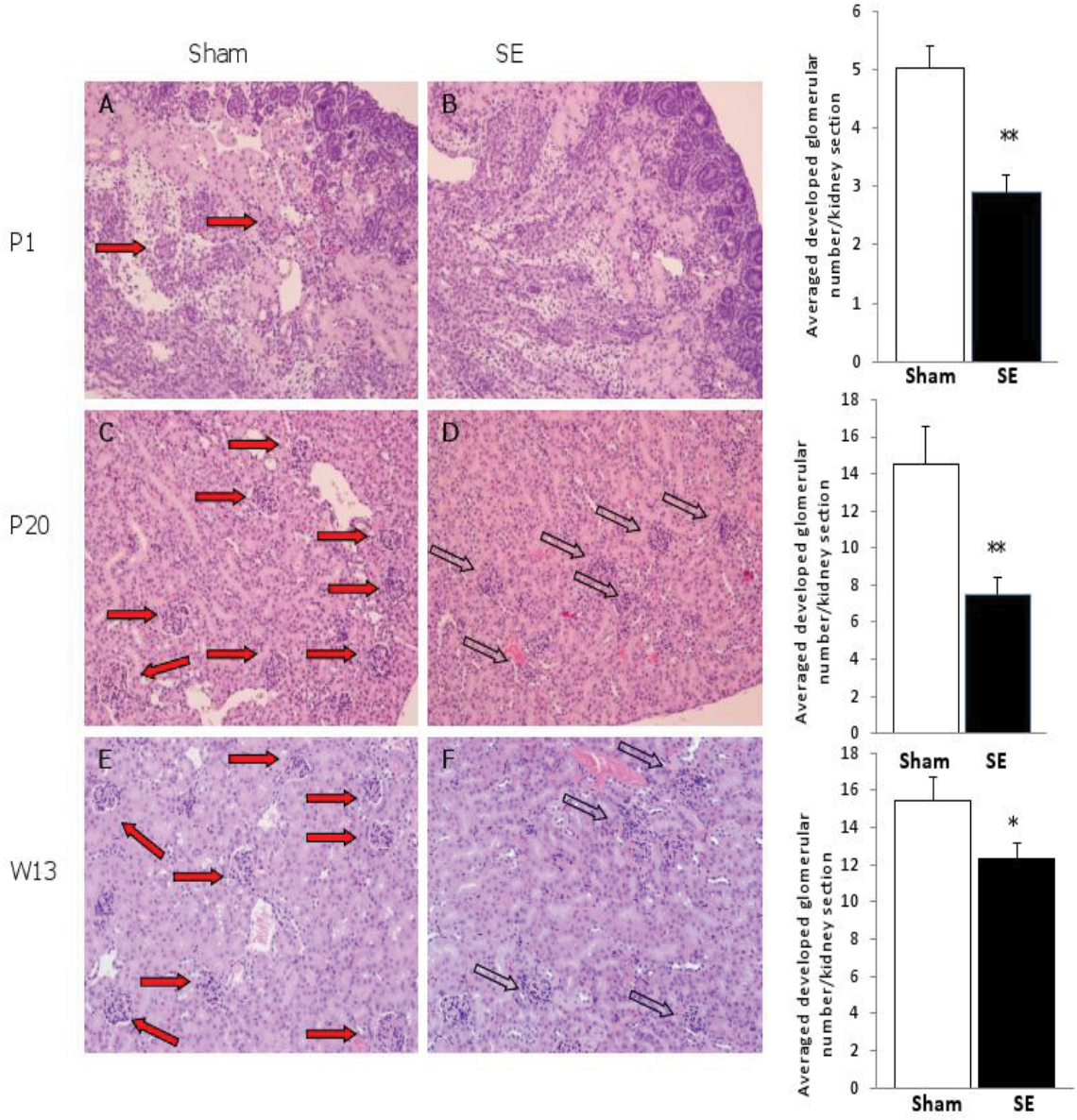


Figure 3.2. Glomerular number in offspring of SE dams. Kidney H&E stained sections from the offspring of Control (1st column) and smoke exposed dams (SE, 2nd column right panel) at postnatal (P)1 (A,B), P20 (C,D), and week (W)13 (E,F). Reduced glomerular numbers was shown in offspring from SE dams at birth (P1), early postnatal life (P20) and adulthood (W13). Mag. 20X. Closed arrows show mature and fully vascularized glomeruli and open arrows show underdeveloped glomeruli. Numbers of developed glomeruli are shown in the 3rd column. n = 6-8. * $p< 0.05$ and ** $p<0.01$, maternal smoke exposure effect.

The glomerular size in the SE offspring was similar to the Sham offspring at P1; at P20, the mature glomerular size in the SE offspring was significantly larger than the sham offspring ($p<0.05$, Figure 3.3). However, by W13 glomerular size was significantly decreased in the SE offspring compared with the sham ($p<0.05$, Figure 3.3).

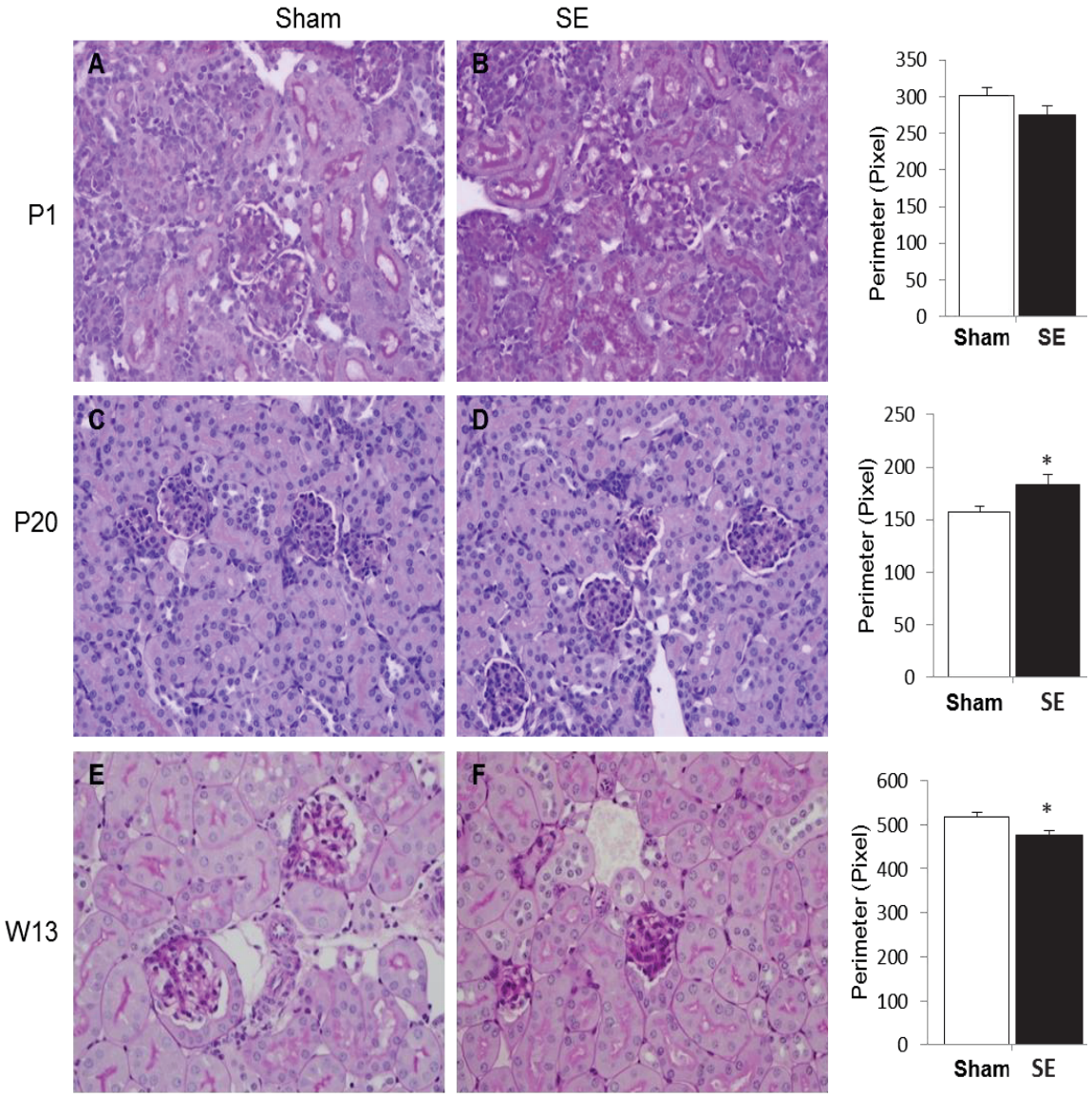


Figure 3.3. Glomeruli size in offspring of SE dams. Kidney PAS staining showing glomerular size in the offspring of Control (1st column) and smoke exposed dams (SE, 2nd column) at postnatal (P)1 (A,B), P20 (C,D) and week (W)13 (E,F). Enlarged glomeruli are shown in early postnatal life (P20) and reduced glomeruli size was shown at adulthood (W13) in offspring from SE dams. n = 3-4. * $p < 0.05$ maternal SE effect.

In the offspring of SE dams, PAS stain and Masson's stain showed some glomeruli had minor degrees (< 25%) of sclerosis, minor tubular dilatation and some tubules were elongated with proteinacious material. However, there was no statistical significant could be observed in the PAS and Mason's staining when images were quantified (Figure3.4).

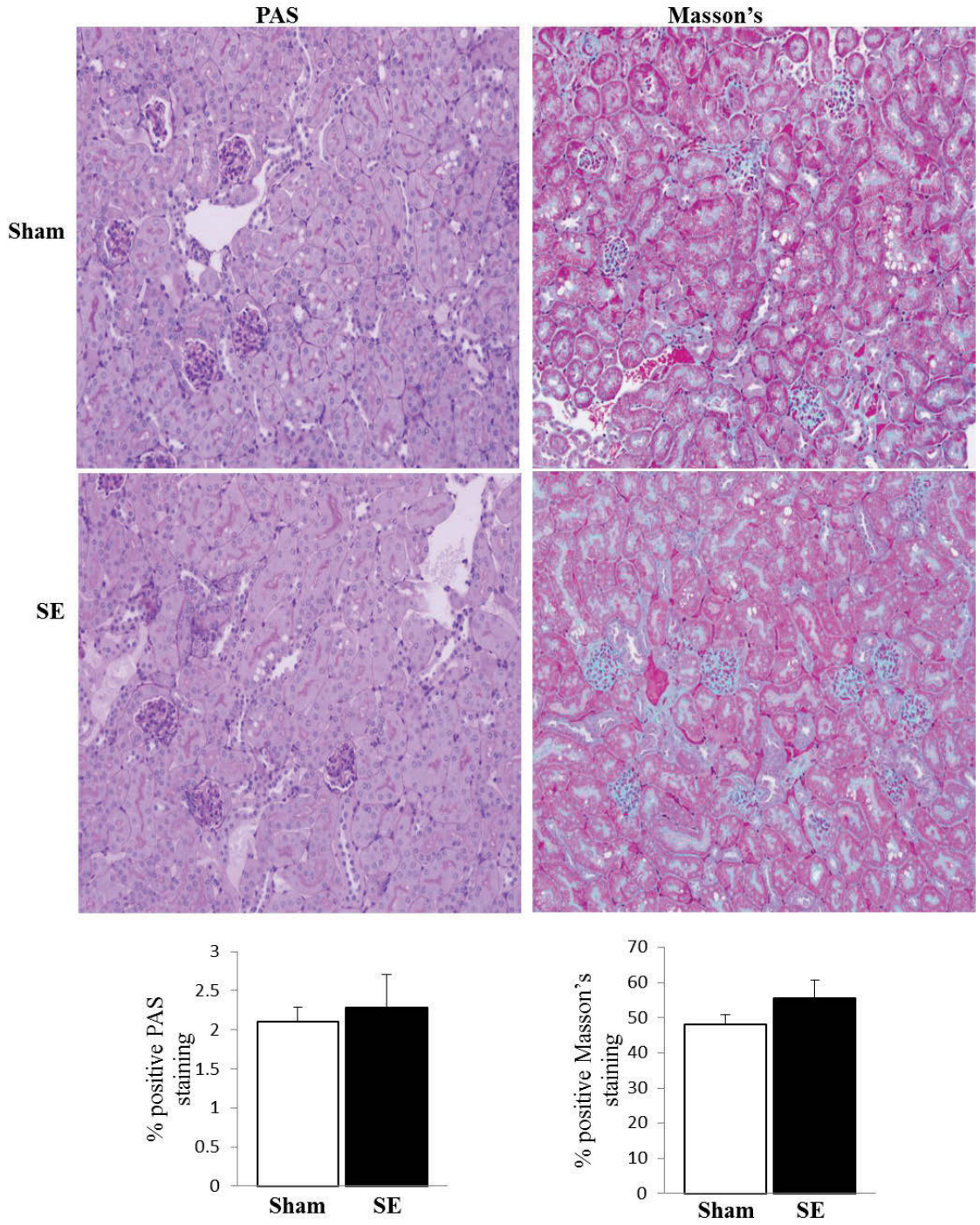


Figure 3.4: PAS stain (Left panel) and Masson's stain (Right panel) in the kidney from the offspring of the Sham exposed dams (Top panel) and SE dams (bottom panel) at W13. Mag. 20X. n = 3-4.

3.3.2.2 Kidney mRNA and protein levels

Maternal SE differentially affected mRNA expression of the growth and transcription factors involved in the renal development. Renal mRNA expression of FGF2, GDNF, Pax2, WNT11 and WT1 was significantly upregulated, whilst FGF7 and FGF10 were downregulated by maternal SE at P1 ($p<0.05$, Figure 3.5A). Renal BMP4, BMP7, WNT4 mRNA expression were not changed at this age. At P20, renal mRNA expression of FGF2, FGF10 and WNT4 was significantly upregulated by maternal SE ($p<0.01$, FGF2; $p<0.05$, FGF10, WNT4, Figure 3.5B). However, mRNA levels of renal developmental genes were not different between groups in adulthood when renal development has completed (Figure 3.5C).

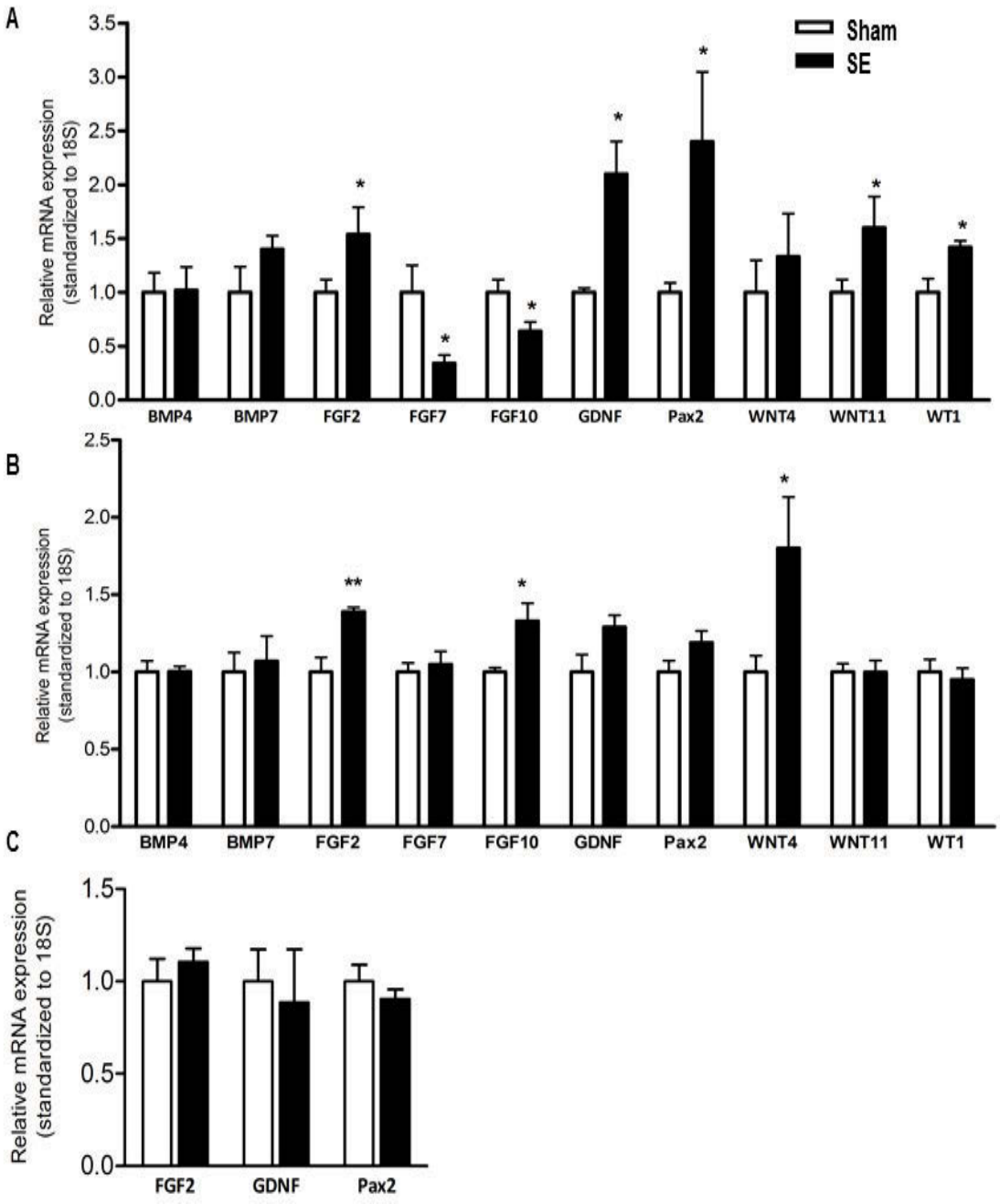


Figure 3.5. Renal growth and transcription factors mRNA expression in offspring mice. Renal mRNA expression of growth factors in the offspring at postnatal (P)1 (A), P20 (B) and week (W)13 (C) showing increased expression of FGF2, GDNF and Pax2 at birth and early postnatal life (P1 and P20 respectively). n = 3-6. * $p < 0.05$, ** $P < 0.01$ vs maternal SE effect.

Renal protein levels of FGF2 (Figure 3.6), GDNF (Figure 3.7), and Pax2 (Figure 3.8) were higher in the offspring of SE dams at P1 ($p<0.05$). FGF2 protein level was still higher at P20 in the SE offspring, but not at W13; while GDNF and Pax2 protein levels were not different between groups at P20 and W13.

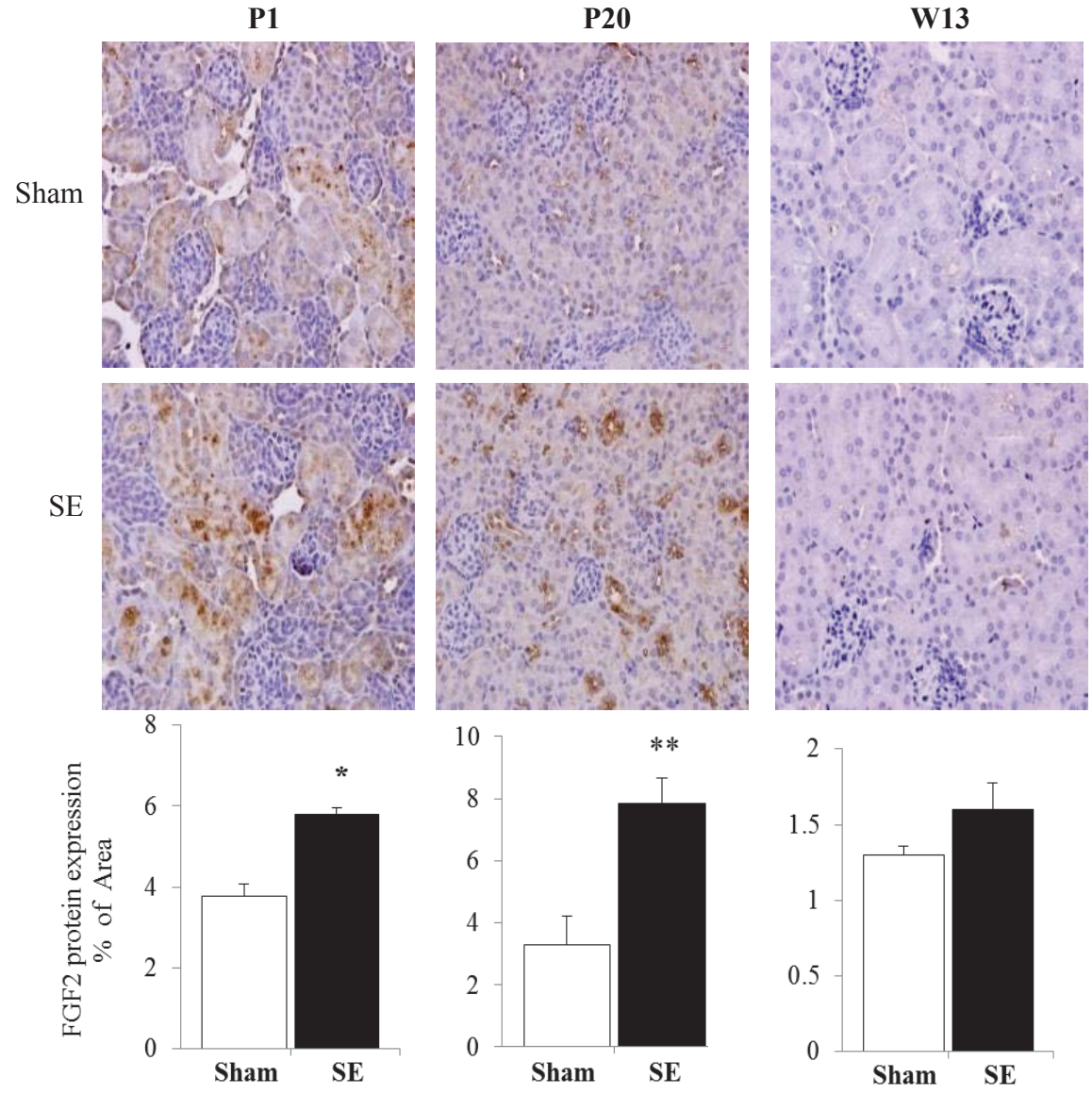


Figure 3.6. Renal FGF2 growth and transcription factor protein expression in offspring mice. Immunostaining showing increased protein expression of fibroblast growth factors (FGF2) at birth (P1) and early postnatal life (P20) but not at W13. 40X. n = 3-4. * $p < 0.05$, maternal SE effect.

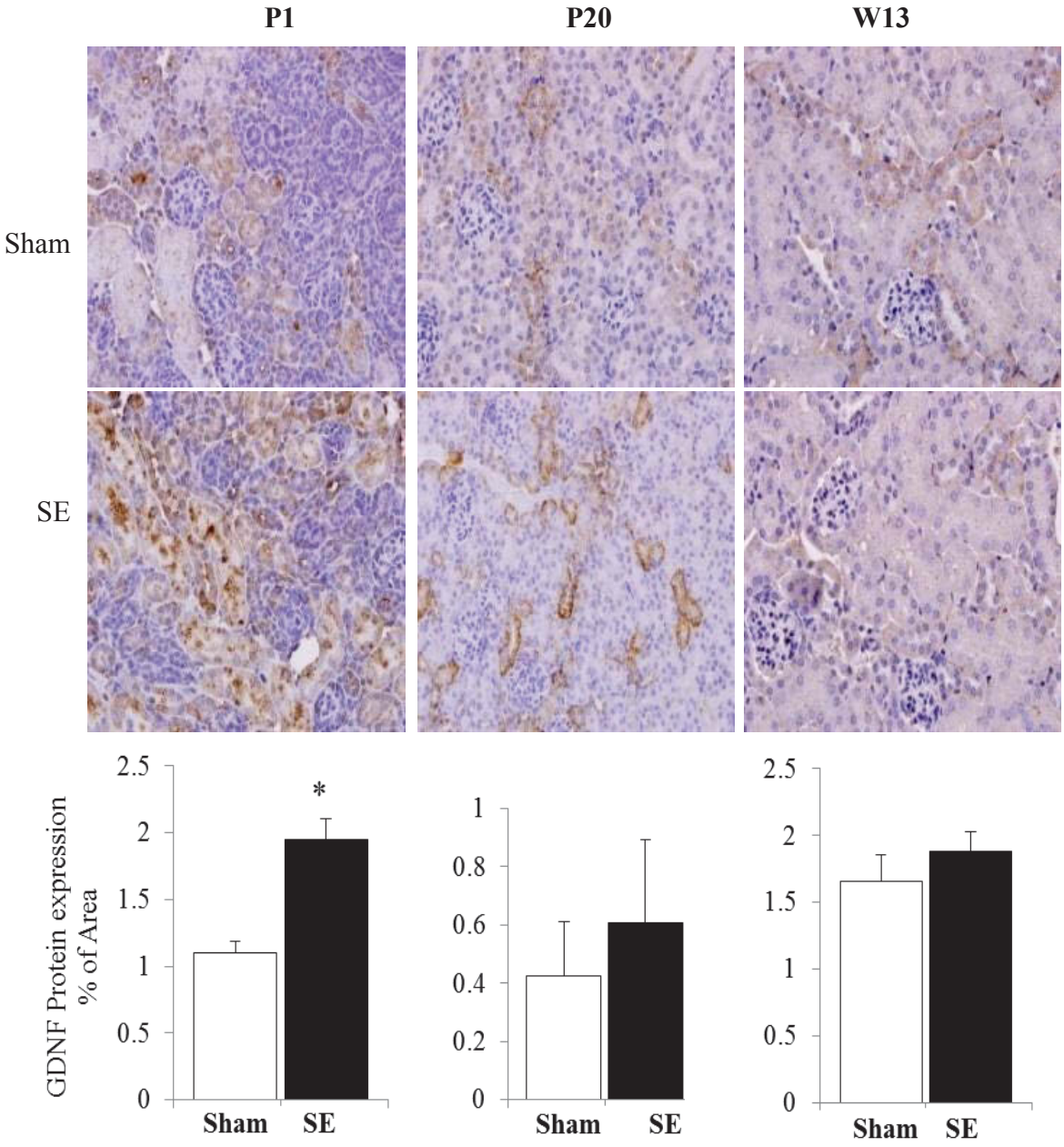


Figure 3.7. Renal GDNF growth and transcription factor protein expression in offspring mice. Increased protein expression of GDNF at birth. Mag. 40X. n = 3-4. * $p < 0.05$, maternal SE effect.

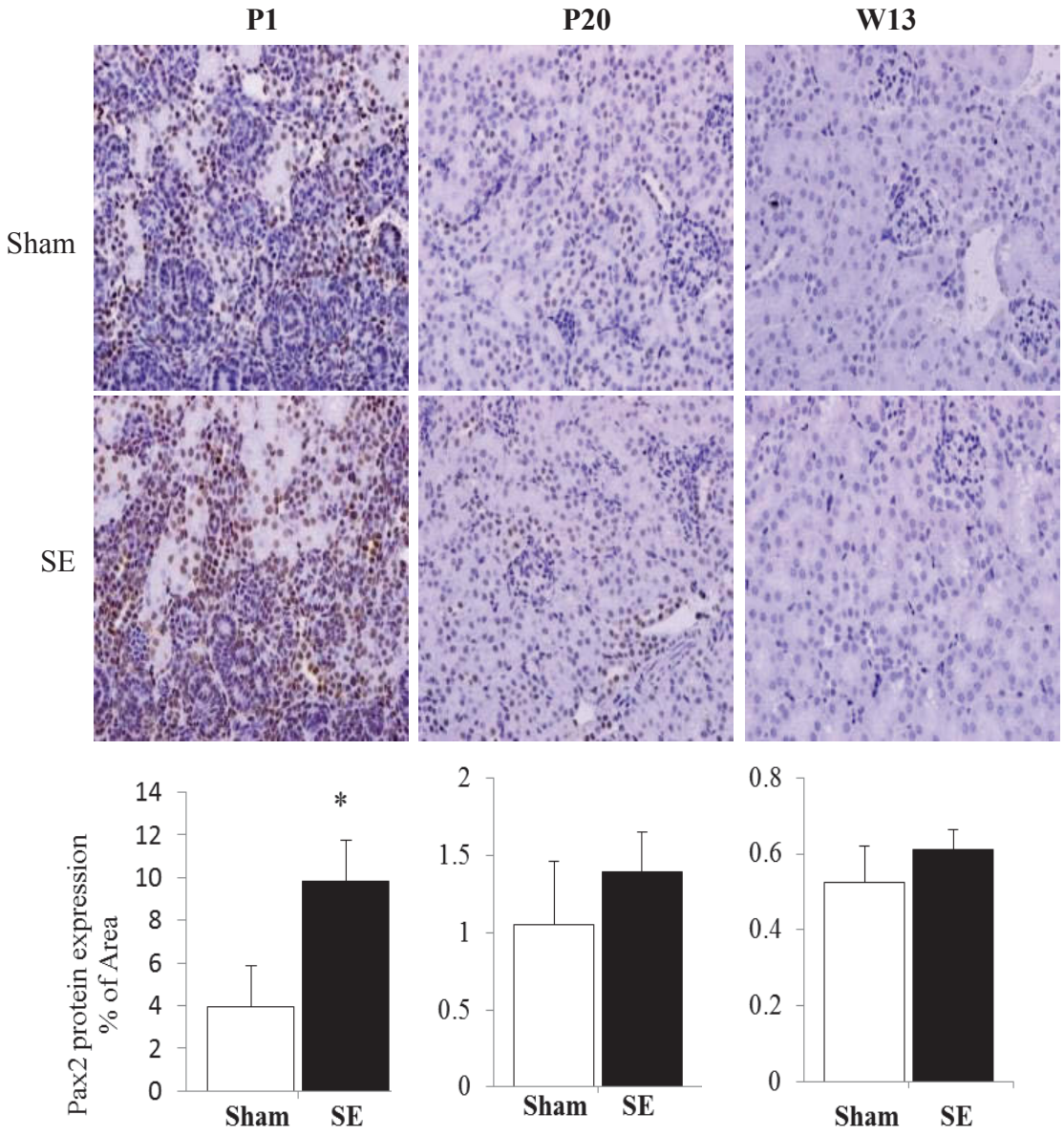


Figure 3.8. Renal growth and transcription factors protein expression in offspring mice. Increased protein expression of Pax2 in offspring from SE dams compare to offspring from control dams. Mag. 40X. n = 3-4. * $p < 0.05$, maternal SE effect.

3.3.2.3 Renal markers of inflammation, injury and function

mRNA levels of pro-fibrotic markers (fibronectin and collagen IV) and pro-inflammatory marker (MCP-1) were measured in the offspring kidney at adulthood. There was no difference between fibronectin and collagen IV mRNA levels at W13 (Figure 3.9 A,B). However, MCP-1 mRNA expression was significantly increased in the SE offspring ($p<0.05$, Figure 3.9 C). This was accompanied by increased urinary albumin/creatinine ratio at W13 ($p<0.05$, Table 3.3). There were also no differences in the serum enzymatic creatinine levels at all ages (Table 3.3).

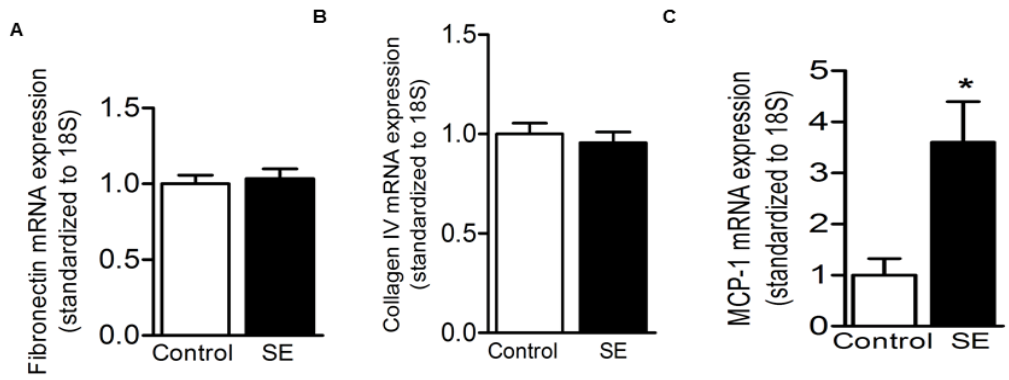


Figure 3.9. Expression of kidney injury markers in offspring mice at W13. Renal mRNA expression of pro-fibrotic marker fibronectin (A) and collagen IV (B) did not change between the offspring from SE dams and Sham dams due to maternal SE at week (W)13. Pro-inflammatory marker MCP-1 (C) mRNA expression in offspring from SE dams was significantly higher compare to offspring from sham dams at W13. n = 4-6. * $p < 0.05$, maternal SE effect.

Table 3.3: The effects of maternal SE on renal function in offspring

Male offspring	P20		W13	
	Sham	SE	Sham	SE
Urinary albumin/creatinine ratio (µg/mg)	8.7 ± 2.0	6.1 ± 1.5	7.00 ± 2.3	38.0 ± 6.3*
Serum enzymatic creatinine (µmol/l)	10.4 ± 0.7	12.1 ± 1.2	15.2 ± 1.3	14.2 ± 0.5

Results are expressed as mean ± SEM, n=3-11. * $p < 0.05$. SE: smoke exposed.

3.4. Discussion

The major finding of the current study is that maternal cigarette smoke exposure from pre-gestation to lactation period is clearly associated with abnormal early kidney development in offspring and resulting renal dysfunction and increased inflammatory markers in adulthood.

After 6 weeks of cigarette smoke exposure, a smaller body weight was observed in the SE dams, which is consistent with our previous study (Chen et al. 2008). Maternal smoking is known to cause intrauterine growth retardation and resultant small fetal kidneys in humans (Chen et al. 2013). We have demonstrated that offspring from SE dams have reduced nephron numbers at birth and weaning but no difference in kidney weight, which is consistent with previous animal studies (Gao et al. 2008; Zarzecki et al. 2012). This is not surprising especially since body weight and kidney weight don't correlate with nephron number (Murawski, Maina & Gupta 2010). In animal studies, only suprapharmacologically high dose of cigarette smoke have been shown to cause significantly smaller kidney size in the offspring (Jagadapillai et al. 2012). In this study, male SE offspring displayed smaller body weight, and kidney mass in mature age (13 weeks), which was opposite to our previous observation (Chen et al. 2011). This may be due to gender difference as only females were reported in our previous study. However, glucose intolerance developed at 12 weeks in the SE offspring was consistent with our previous study in the female offspring (Chen et al. 2011). We have shown that TNFα mRNA expression was upregulated in the fat tissue, which may play an important role in systemic glucose intolerance in the glucose deposit organs, such as fat itself and skeletal muscle (Chen et al. 2011). In the liver, insulin sensitivity did not seem to be affected by maternal cigarette smoke exposure in the females (Chen et al. 2011). Whether the same occurs in the male offspring requires further investigation, which is beyond the scope of the current study. In addition, the female offspring in our previous study were heavier with more adiposity than the control offspring. This may be due to the difference in the fat concentration in the diet, which was 14% in the previous study (Chen et al. 2011) versus 11% in the current study. It has been suggested that the offspring of the smokers are more likely to develop obesity due to their preference for

junk diet (Chen & Morris 2007). In addition, gender difference may also need to be taken into consideration. Nevertheless, it also suggests that the metabolic disorder induced by maternal SE can be independent of postnatal body weight. Diabetes is one of the risk factors to develop kidney functional disorders and smokers' offspring are predisposed to diabetes in human studies (Chen & Morris 2007). In addition, in this study, renal underdevelopment at early postnatal life and renal dysfunction in adulthood in the SE offspring is consistent with previous human studies (Taal et al. 2011), suggesting the close association between our mouse model and human conditions. As such, it cannot be excluded that glucose intolerance in the SE offspring may also contribute their already developed kidney dysfunction at 13 weeks, rendering them to possible chronic kidney disease later in life.

During kidney development, environmental factors can affect the nephron numbers during nephrogenesis. Maternal smoking, particularly in the first trimester, has been shown to impose a significant adverse impact on fetal renal development and the future risk of chronic kidney disease (Puddu et al. 2009; Solhaug, Bolger & Jose 2004). However, it has been reported that the correlations between maternal smoking and abnormal renal mass disappeared by age (Taal et al. 2011), potentially due to 'catch up' growth commonly seen in such offspring (Chen et al. 2012). In this study, the delayed renal development observed immediately after birth and at weaning led to fewer and smaller mature glomeruli in the adulthood in the SE offspring, regardless of the kidney mass, suggesting the importance of early renal development in determining future renal function.

There are several proposed mechanisms that may contribute to fetal renal underdevelopment (Dotsch, Plank & Amann 2012). Changes in the growth factors in the uterus can clearly play an important role. FGF7 and FGF10 are essential to stimulate the proliferation, migration and differentiation of epithelial cells at the growing tips of the ureteric bud (Abdel-Hakeem et al. 2008; Costantini 2010). Therefore, FGF7 and FGF10 may also determine the number of mature nephron (Qiao et al. 2001). In this study,

mRNA expression of FGF7 and FGF10 were lower at P1 in the SE offspring, which may directly lead to reduced ureteric bud growth and branching resulting in reduced numbers of glomeruli after birth. However at P20, FGF7 and FGF10 mRNA expression was either normalized or upregulated, which may promote the catch-up growth of kidney development and maturation after weaning. Indeed, at P20 although glomeruli number was still reduced by maternal smoking, the glomeruli size was significantly increased in the SE offspring suggesting a compensatory effect of the reduced numbers of glomeruli in order to maintain necessary renal function (Puddu et al. 2009).

In this study, adaptations of other growth factors have also been observed in the SE offspring. The GDNF signaling pathway is critical for the initial stage of nephrogenesis (Bard 1992; Dankers et al. 2011; Dressler 2002; Dressler 2006; Lechner & Dressler 1997; Merkel, Karner & Carroll 2007; Michos 2009; Oliver & Al-Awqati 1998; Schedl & Hastie 2000). GDNF is expressed by metanephric mesenchyme, which induces the ureteric bud outgrowth and branching. Disruption of the GDNF pathway has been shown to cause low nephron number due to its critical involvement in the initiation of nephrogenesis (Zandi-Nejad, Luyckx & Brenner 2006). Fibroblast growth factor, FGF2 promotes ureteric bud endothelial cell proliferation to form collecting ducts (Dressler 2006; Qiao et al. 2001). Here, offspring from SE dams have increased GDNF and FGF2 mRNA and protein levels at P1 and P20, suggesting early adaptation to intrauterine renal underdevelopment. Pax2 is expressed at the ureteric bud and metanephric mesenchyme in the developing kidneys, which is essential for developing renal epithelium and generating tubules from the mesenchyme (Dressler & Woolf 1999). WT1 is involved in metanephric cell differentiation into epithelial cells (Dressler 2009). Pax2 induces WT1 expression in the metanephric mesenchyme which acts as a negative feedback for Pax2 expression when the metanehpric mesenchyme has been differentiated into epithelial cells (Chi & Epstein 2002; Koleganova et al. 2011). Interestingly Pax2 mRNA and protein expression at P1 and P20 are positively correlated with WT1 expression. During glomerular formation, WNT 4 is a autocrine signal to promote the condensation and the aggregation of the metanephric mesenchyme around the tip of the T-shape ureteric bud to form glomeruli (Dressler 2006). Interestingly, WNT4 is increased at P20 but its level was not changed at P1, suggesting a possible role

of WNT4 to increase in glomerular size at P20. As expected, the basal levels of all the growth factors were low or undetectable at W13, most likely due to the completion of nephrogenesis.

WT1, BMP7, BMP4 and Pax2 are all anti-apoptotic factors (Dressler 2009) which function at different stages of renal development. During fetal development, apoptosis regulates ureteric budding, which is essential to determine nephron numbers (Zandi-Nejad, Luyckx & Brenner 2006). Placental insufficiency can promote cellular apoptosis resulting in reduced nephron number (Zandi-Nejad, Luyckx & Brenner 2006). Indeed, increased renal apoptosis has been reported in offspring of SE dams (Zarzecki et al. 2012). In the current study, the upregulation of WT1 at P1 by maternal SE may be an adaptation to reduce apoptosis and to increase nephrogenesis. BMP7 is expressed in the ureteric bud and cap mesenchyme to induce ureteric budding (Dressler 2009), while BMP4 can prevent ectopic budding (Walker & Bertram 2011). Therefore, they are more likely to change during intrauterine fetal development, but not after birth (Torban et al. 2000).

A previous study (Jagadapillai et al. 2012) in the offspring of cigarette smoke exposed mice proteomics showed subsequent changes in the expression of renal proteins that regulate inflammation, cell to cell signaling/interactions, lipid metabolism, small molecule biochemistry, cell cycle, nucleic acid and carbohydrate metabolism network. However, it did not address any of the growth factors involved in renal development. Short term high dose cigarette smoke exposure has been shown to increase oxidative stress in the mice kidney, which is one of the suggestive mechanisms leading to both chemical induced underdevelopment of fetal kidney and the onset of chronic kidney disease in adults (Kabuto, Amakawa & Shishibori 2004; Massy, Stenvinkel & Drueke 2009; Raza, John & Nemmar 2013). However, whether this is involved in maternal smoking related renal underdevelopment in offspring requires further investigation. Regardless, chemicals in the cigarette smoke are known to cause direct damage to the smokers' kidney, leading to proximal tubular damage, kidney cancer, and end-stage kidney disease (El-Safty et al. 2004). The chemicals, such as nicotine, inhaled by the

pregnant mothers, pass rapidly across the placenta and accumulate in the kidney, which has been shown to lead to smaller kidneys in offspring from rat dams with nicotine infusion (Chen et al. 2013). The exposure of other chemicals in the cigarette smoke, such as cadmium and polycyclic aromatic hydrocarbons, have been shown to be correlated with the risk of fetal growth restriction, however with the direct impact on kidney development unknown (Chen et al. 2013). As tobacco smoke is contains more than 4000 chemical substances (International Agency for Research on Cancer (IARC) 2002), using direct cigarette smoke expose will re-produce a better effect of such intrauterine factor in animal studies.

Here we show that underdevelopment of the kidney in the offspring of SE dams is closely associated with abnormal renal function, reflected by increased urinary albumin/creatinine excretion. Proteinuria is a biomarker reflecting progressive renal dysfunction (Murali et al. 2007). It has been suggested that reduced nephron number at birth can lead to the adaptation of glomerular hypertrophy and hyperfiltration in order to maintain sufficient renal function (Dotsch, Plank & Amann 2012; Hoy et al. 2006; Puddu et al. 2009; Zandi-Nejad, Luyckx & Brenner 2006). Prolonged hyperfiltration can in turn lead to hyperfiltration injury in the long term, resulting in structural injury and functional deterioration in adulthood (Puddu et al. 2009).

Structurally, subtle changes were observed in the tubules and glomeruli in the kidneys of the SE offspring at W13. However, the expression of the pro-fibrotic markers, fibronectin and collagen IV were not altered, maybe due to the sensitivity of the methodology or relatively young age of the mice. Inflammation correlates with renal damage and contributes to the development of chronic kidney disease (Agarwal 2006). MCP-1 is a pro-inflammatory and pro-fibrotic protein (Agarwal 2006). In recent studies, it has been demonstrated that MCP-1 is involved in the initiation and progression of glomerular and tubulointerstitial damage (Murali et al. 2007; Viedt & Orth 2002). Moreover, MCP-1 expression is also positively correlated with albuminuria (Viedt & Orth 2002), which was well presented in this study. As a result, the offspring

of SE dams may be prone to further renal damage as adulthood progresses.

Chapter Four

4

Impact of Maternal Cigarette Smoke Exposure on Renal Pathophysiology in the Female Offspring

4.1. Introduction

There is an increasing attention to the gender difference in the physiological process of diseases (Arnold 2014). The impact of gender disparities on the developmental programming of adulthood diseases has also been well documented. For example, prenatal insults such as infection and inflammation can change the development and physiology process of the fetus and newborn leading to decrease in hippocampal volume and cortical monocytes filtration resulting in neurological disorders in a gender-dependent manner (Dada et al. 2014). In such condition, the microglial colonization and activation induced neuronal loss was more severe in the male offspring compared with the female littermates (Dada et al. 2014). Chemical toxicity is suggested to be gender-dependent as well. For instance, the risk due to SE in humans is less severe in the males than the females because of the gastrointestinal absorption to cadmium in women is higher compared to the men (Vahter, Åkesson, et al. 2007).

Furthermore, there are growing evidences supporting the theory of gender differences in response to renal injury (Metcalfe & Meldrum 2006). For example, renal failure rate is higher in the male than the female patients independent of other health conditions (Metcalfe & Meldrum 2006). To this respect, etiology studies showed that the association between low birth weight and adulthood kidney disease is more evident in the males than the females (Li et al. 2008). The underlying mechanisms of such gender effect on the development and progression of kidney disease is not fully known (Ji et al. 2007) and therefore requires further investigation.

To date, most of the animal research studies have only been performed on males (Arnold 2014; Vahter, Gochfeld, et al. 2007) including our previous work (Chapter 3) reporting the impact of maternal SE on kidney underdevelopment and renal dysfunction in male offspring at different postnatal ages (Al-Odat et al. 2014). However, using a rat model of hypertension, Sandberg and colleagues suggested that females may be protected from the progression of kidney disease and decline in renal function

(Sandberg 2008). Different mechanisms underlying the gender disparity of kidney disease have been proposed, including sex hormones, kidney structure differences between the males and females, kidney hemodynamics, postnatal diet and lifestyle (Neugarten & Golestaneh 2013; Silbiger & Neugarten 2008).

Previously, we showed that male mice offspring from SE mothers who were continuously exposed to cigarette smoke prior to and during gestation and lactation are susceptible to renal underdevelopment and resulting kidney disorders and dysfunction at adulthood evidenced by reduced nephron number, reduced glomerular size, as well as changes in mRNA and protein expression of the renal growth and transcription factors at birth and increased albuminuria at adulthood (Al-Odat et al. 2014).

Therefore, the aim of this study is to determine whether maternal SE prior to and during gestation and lactation can affect the developing kidney in the female mice offspring and induce renal dysfunction at adulthood. This chapter aimed to determine, 1) the changes of kidney structure and growth factors that regulate renal development at different postnatal ages, including birth, weaning and adulthood; and 2) the effect of maternal SE on markers of renal function in adulthood.

4.2. Materials and methods

4.2.1. Animal model and cigarette smoke exposure protocol

The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology, Sydney (ACEC#2011-313A).

Female Balb/c mice (8 weeks old, n=20, Animal Resources Centre, Perth, Australia) were exposed to tobacco cigarette smoke as described in chapter 2, section 2.2. In brief, SE mice were exposed to cigarette smoke produced by two cigarettes (Winfield Red, 16 mg or less of tar, 1.2 mg or less of nicotine and 15 mg or less of CO; Philip Morris, Melbourne, Australia), twice (10:00 and 15:30) daily. Control mice were exposed to the air in an identical chamber at the same time. After 6 weeks of cigarette smoke exposure, the female mice were mated with 8 weeks old male mice. Smoke exposure was continued as usual throughout gestation and lactation. When pregnancy was confirmed by significant increase in body weight, the pregnant females were housed individually. SE was not performed on the day of delivery. Neither the male breeder nor the offspring were directly exposed to cigarette smoke at any time during the study.

4.2.2. IPGTT

IPGTT was performed at 12 weeks of age in the pups. After 5 hours fasting, a baseline glucose level was taken from the tail blood (Accu-Chek, Roche Diagnostics, Indianapolis, IN, USA). Glucose was administered (2 g/kg i.p., n = 9-10) and blood glucose levels were measured at 15, 30, 60, and 90 min post-injection (Chen et al. 2011). The AUC of the glucose levels was calculated for each mouse.

4.2.3. Tissues collection

Female offspring were scarified at three different time points; P1, P20 and W13. At the endpoint, pups were weighed and anaesthetized with sodium thiopental (0.1ml/g, i.p., Abbott Australasia PTY. LTD, NSW, Australia). Blood was collected via cardiac puncture and glucose was measured (Accu-chek®, Roche Diagnostics, Nutley, USA).

The plasma and urine samples were collected and stored at -20 ^{o}C for later analysis. Mice were killed by decapitation. The kidneys were harvested, one was placed in 10% formalin solution natural buffered (Sigma, VIC, Australia). The other kidneys were weighed and snap frozen in liquid nitrogen then stored in -80 ^{o}C.

4.2.4. Real-time PCR

Total RNA was extracted from the kidney using TRIzol reagent (Life Technologies, CA, USA) or RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturers' instructions as described in chapter 2, section 2.5. Briefly, kidney tissues were homogenized in 1 ml TRIzol (Life Technologies, CA, USA). The first strand of cDNA was synthesized from 1 µg of total RNA using SuperScript® VILO cDNA synthesis kit (Life Technologies, CA, USA) or Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany).

qPCR was performed in a 10 µl volume using pre-optimized SYBR Green primers (Sigma-Aldrich) (Table 2.1, Chapter 2) and SensiFAST^{TM} SYBR Hi-ROX Kit (Bioline, Toronto Canada). The reaction plate was run in ABI7900HT Sequence Detection System (SDS 2.4, Life Technologies, CA, USA) as described in Chapter 2.

The results were analyzed by relative quantitation using RQ Manager Software (RQ 1.2.1, Applied Biosystems) using ΔΔCt. The average mRNA expression of the Sham (Control) group was used as the calibrator and 18S rRNA was used as the housekeeping gene.

4.2.5. Kidney histology and IHC staining

Fixed kidney samples in 10% formaldehyde were embedded in paraffin and sectioned in 2µm and 4µm. Kidney structure in the offspring was examined using H&E stain and PAS stain of the 2µm kidney sections.

For H&E staining, the kidney sections were de-waxed and hydrated. The sections then were incubated in haematoxylin for 3 minutes followed by de-colourization in acid ethanol. The sections then were counterstained in eosin then dehydrated, cleared and mounted. Glomerular number was estimated by counting the developed glomeruli in 3-4 non-consecutive H&E stained kidney sections from the same animal. For this purpose, 6-8 different animals were used from each group.

For PAS staining, the kidney sections were de-waxed and hydrated. Then the sections were incubated with 0.5% periodic acid solution for 5 minutes, and then washed in running water followed by incubation in Schiff's reagent. The tissues were counterstained with haematoxylin, dehydrated, cleared and mounted. Glomerular size for each animal was measured in the PAS stained kidney sections using Image J (Image J, NIH, USA) in average of 6 different images for the same kidney section then averaged (Al-Odat et al. 2014).

For IHC staining, 4µm kidney sections were de-waxed then hydrated. Heat-induced epitops retrieval was performed then endogenous peroxidase was blocked by incubated the sections in 0.3% H_2O_2 for 5 minutes at room temperature. The kidney sections were blocked using protein block serum-free (Dako, CA, USA) for 10 minutes at room temperature, and then incubated with rabbit anti-mouse primary antibodies against FGF2 (1:250 dilution), GDNF (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Pax 2 (1:1750 dilution), fibronectin (1:500) and collagen IV (1:500) (Abcam, Cambridge, UK) at 4^{o}C overnight. Negative controls were prepared by replacing the primary antibodies with rabbit IgG.

In the following day, the sections were exposed to Envision & system-HRP labeled polymer secondary anti-rabbit antibodies (Dako, CA, USA) for 30 minutes at room temperature. Secondary antibody was detected by reaction of HRP enzyme and DAB + (liquid DAB + substrate chromogen system, Dako, CA, USA). Then the sections were counterstained with haematoxylin, dehydrated, cleared and mounted.

On average, 6 different non-overlapping fields of the same kidney section were captured. Quantitation of the positive signals (the intensity of the brown colour) in the captured images was performed using Image J software (Image J, NIH, USA) and the percentage of the brown colour to the whole field was determined and averaged.

4.2.6. Albumin and creatinine assays

Urine albumin and creatinine were measured using Murine Microalbuminuria ELISA kit (Albuwell M, Exocell Inc, PA, USA) and Creatinine Companion kit (Exocell Inc, PA, USA) respectively according to the manufacturer instructions as described in Chapter 2, section 2.5.1 and 2.5.2. Serum enzymatic creatinine level was measured using an automated analyzer (ARCHITECT, Abbott Australasia PTY. LTD, NSW, Australia).

4.2.7. Cotinine assay

Commercial cotinine Direct ELISA kit (Abnova, Taipei, Taiwan) was used to measure serum cotinine level according to the manufacturer instructions.

4.2.8. Statistical analysis

The differences between the Sham and SE groups were analyzed using two-tailed, unpaired Student's *t*-test assuming unequal variances between the groups (Prism 6, Graphpad CA, USA). The results are expressed as mean ± SEM. $P<0.05$ is considered significant.

4.3. Results

4.3.1. Body parameters of the offspring

In the female offspring, body weight, kidney weight and kidney as percentage of body weight were similar between the groups at P1 and P20 (Table 4.1). At W13, the offspring from the SE dams had significantly smaller body weight and kidney weight than the Sham offspring ($p<0.01$); however, when the kidney weight was standardized by the body weight, there was no difference between the groups. Blood glucose level was significantly lower in the SE offspring at P1 compared to the Sham offspring ($P < 0.05$, Table 4.1). Blood glucose level was similar between the groups at P20 and W13.

Table 4.1: Effects of maternal SE on growth and development in female offspring.

P1	**Sham**	**SE**
Body weight (g)	1.45 ± 0.05	1.34 ± 0.04
Kidney weight (g)	0.007 ± 0.0004	0.008 ±0.0004
Kidney/Body (%)	0.49 ± 0.02	0.56 ± 0.03
Glucose (mM)	4.2 ± 0.2	3.3 ± 0.3*
P20	**Sham**	**SE**
Body weight (g)	9.7 ± 0.2	9.1 ± 0.2
Kidney weight (g)	0.07 ± 0.01	0.06 ± 0.01
Kidney/Body (%)	0.66 ± 0.06	0.72 ± 0.02
Glucose (mM)	10.1 ± 0.4	9.7 ± 0.4
W13	**Sham**	**SE**
Body weight (g)	22.4 ± 0.2	20.8 ± 0.4**
Kidney weight (g)	0.13 ± 0.002	0.12 ± 0.002**
Kidney/Body (%)	0.57 ± 0.01	0.57 ± 0.01
Glucose (mM)	10.5 ± 0.4	10.8 ± 0.4

Results are expressed as mean ± SEM. n=11-23. Unpaired student's t test was used to analyse the data between Sham and SE groups at the same age. * $p < 0.05$, ** $p < 0.01$.

4.3.2. Kidney histological changes in the offspring

In the female offspring, there were no changes in developed glomerular number between the groups at P1 and P20 (Figure 4.1). At W13, fully developed glomeruli were presented in the kidneys of both groups and the glomerular numbers were still similar between the groups (Figure 4.1). No differences in glomerular size were observed at P1, P20 and W13 either (Figure 4.2).

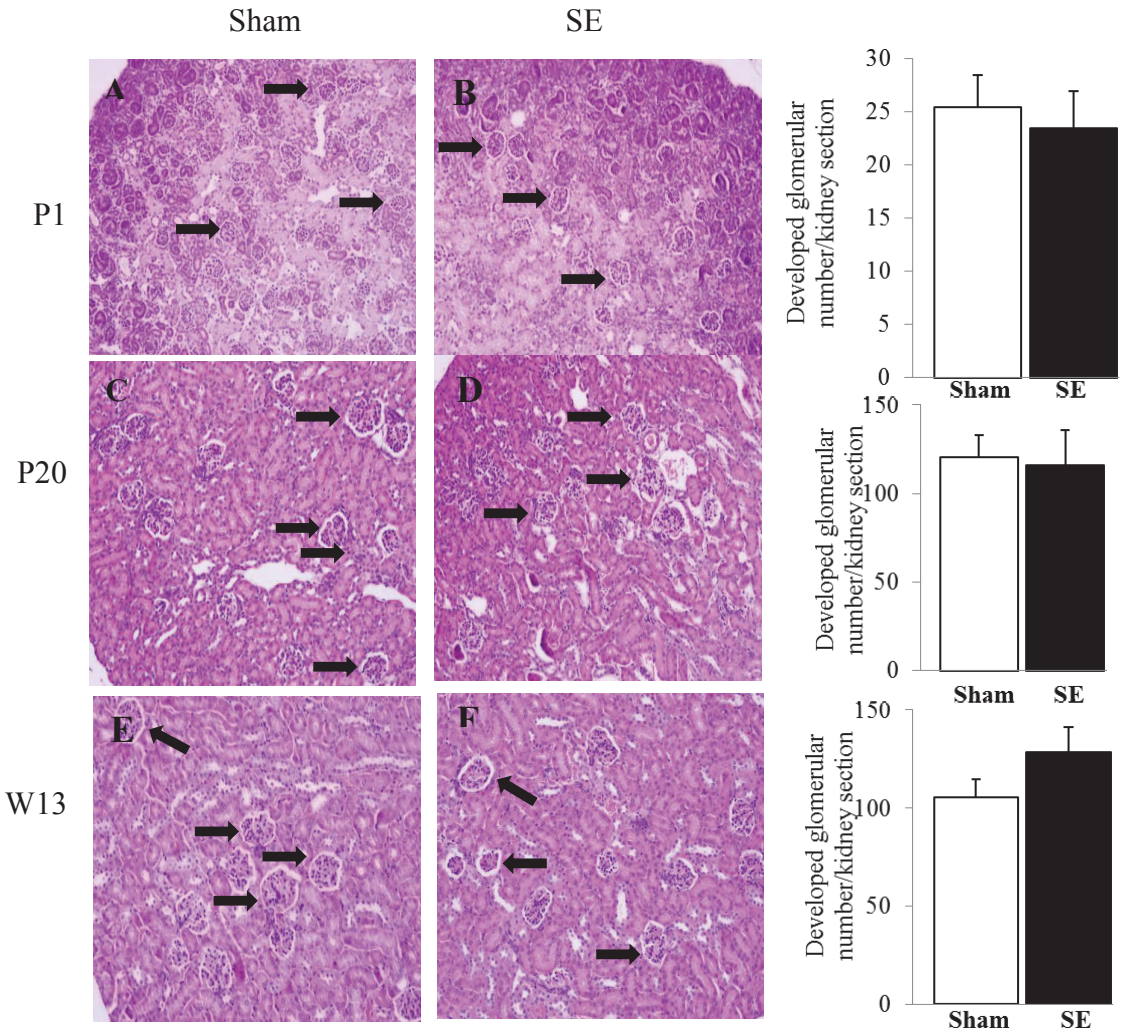


Figure 4.1: H&E stained Kidney sections from the Sham offspring (Left panel) and SE offspring (Right panel) at P1 (A and B), P20 (C and D) and W13 (E and F) showing glomeruli. Closed arrows indicate fully developed glomeruli. Results are expressed as mean ± SEM, n=6-8. Mag. 20X. Unpaired student's t test was used to analyse the data.

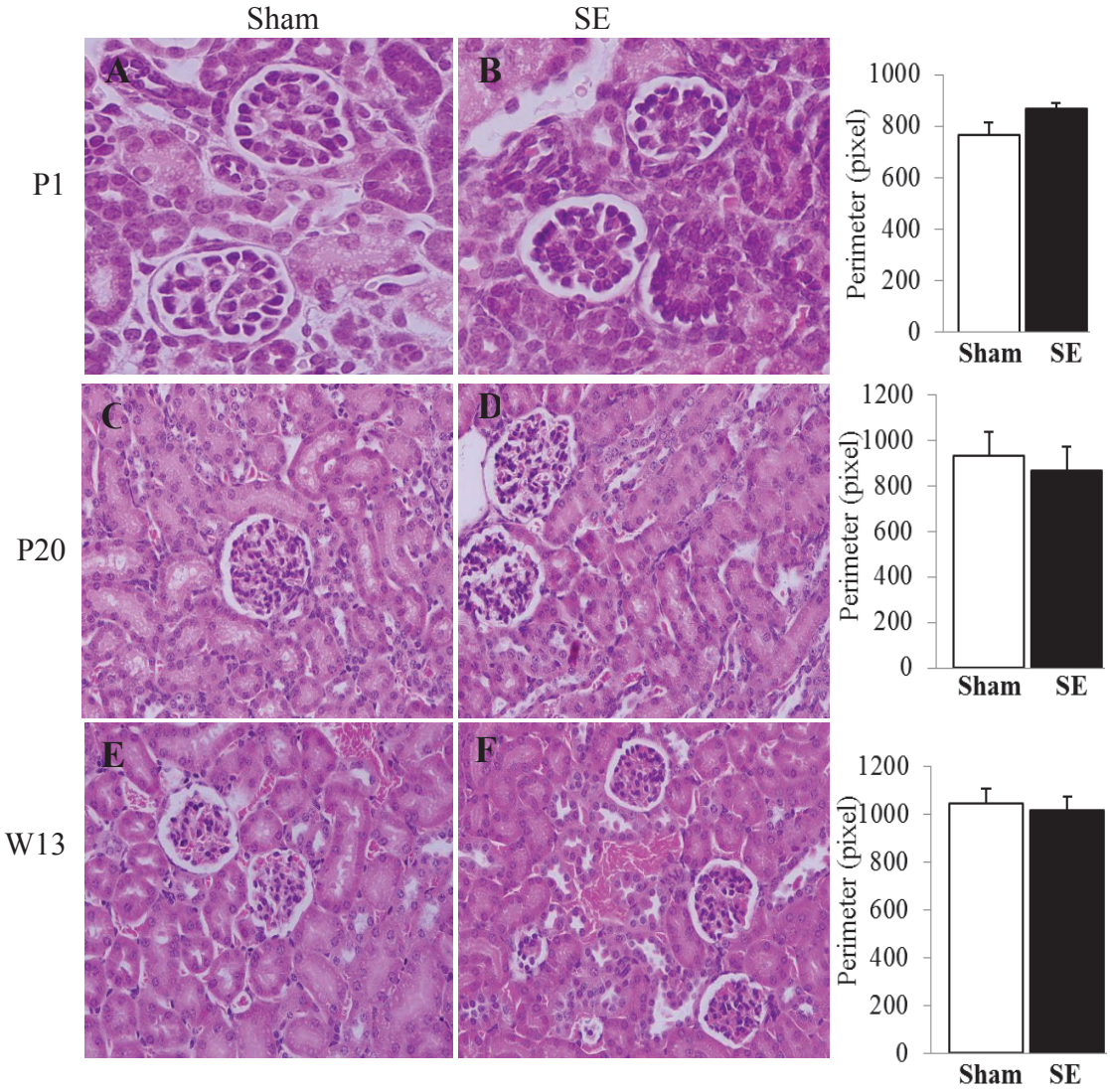


Figure 4.2: Kidney PAS stained sections from the Sham (Left panel) and SE offspring (Right panel) at P1 (A and B), P20 (C and D) and W13 (E and F) showing glomerular size. Results are expressed as mean ± SEM, n=6-8. Mag. 40X. Unpaired student's t test was used to analyse the data.

4.3.3. Kidney mRNA and protein levels of growth and transcription factors

Maternal SE differentially did not affect mRNA expression of the growth and transcription factors involved in renal development in the females at P1 (Figure 4. 3A). No changes were also observed in the mRNA expression of the growth and transcription factors at P20 (Figure 4.3B) and W13 (Figure 4.3C) between the groups.

While we have previously demonstrated that FGF2, GDNF and Pax2 protein levels were increased in males offspring due to maternal SE, the IHC analysis in female offspring showed no changes in renal protein levels of FGF2 (Figure 4.4), GDNF (Figure 4.5) and pax2 (Figure 4.6), which was consistent with the mRNA expression of these developmental markers at P1, P20 and W13. Both male and female protein expression showed that FGF2 and GDNF are cytoplasmic proteins while pax 2 is a nuclear protein.

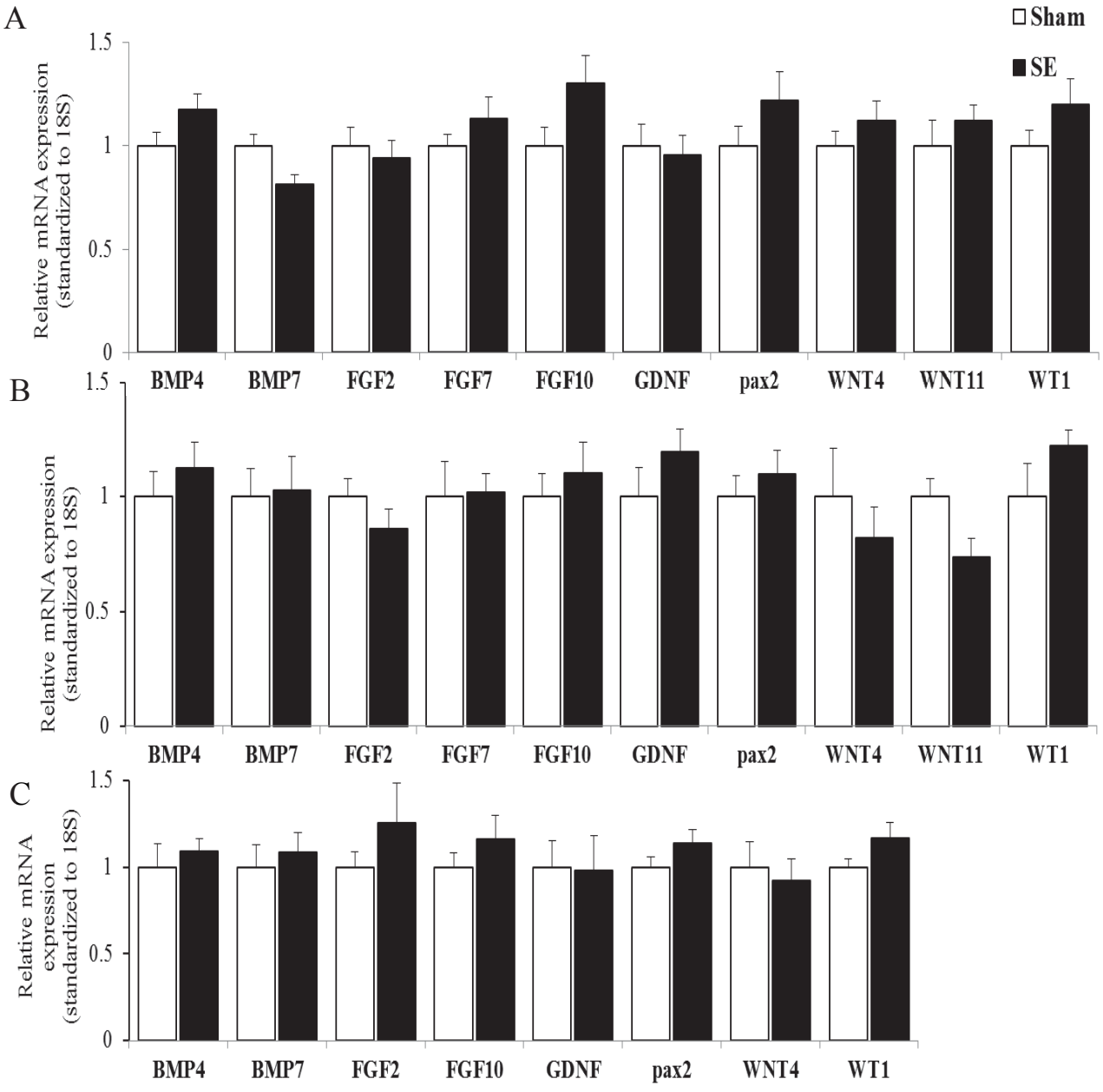


Figure 4.3: Renal mRNA expression of growth and transcription factors in the female offspring mice at P1 (A), P20 (B) and W13 (C). Results are expressed as mean ± SEM, n=6. Unpaired student's t test was used to analyse the data. * $p < 0.05$.

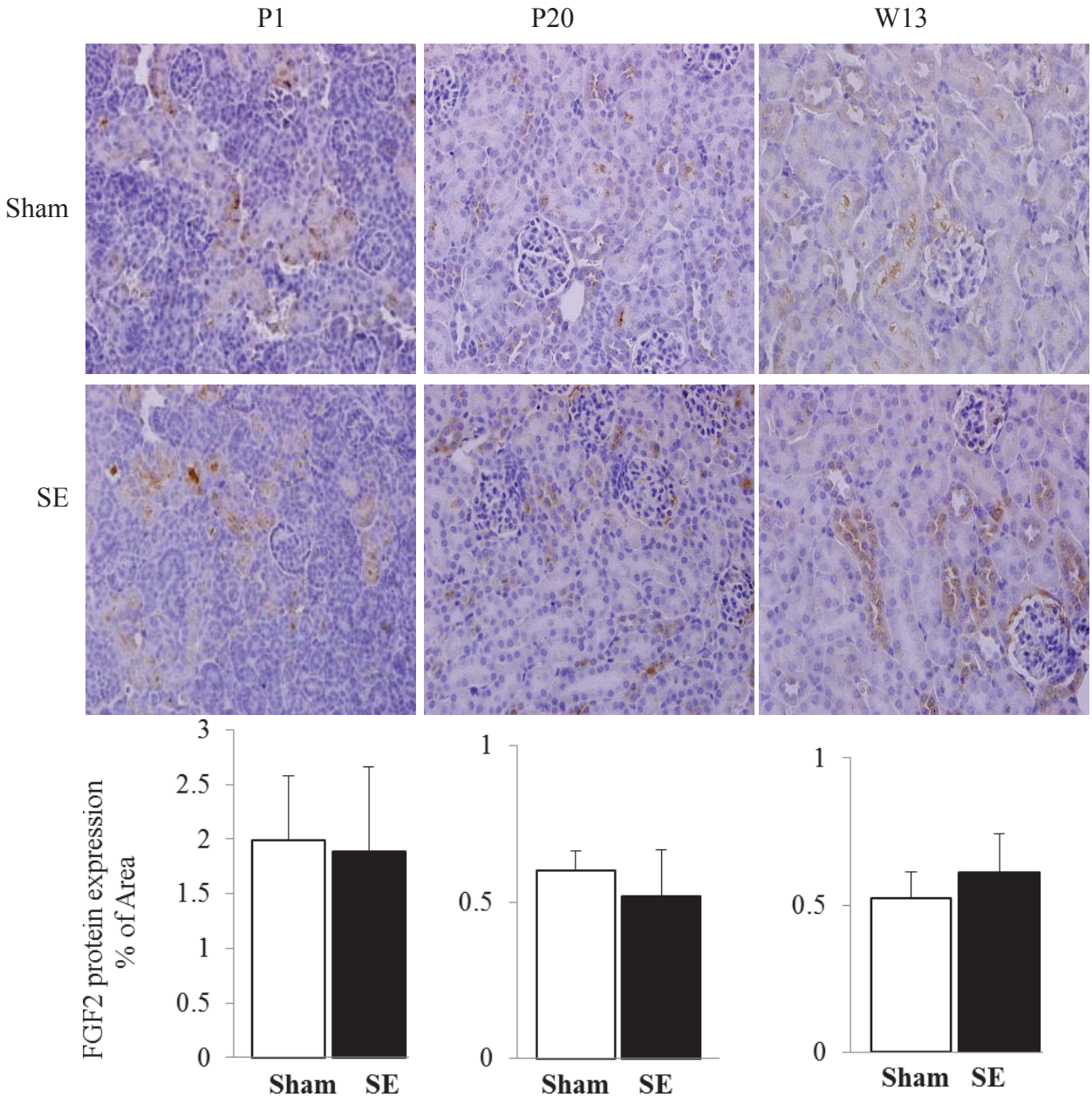


Figure 4.4: Renal FGF2 protein expression in the Sham offspring (Top panel) and SE offspring (Lower panel) at P1 (Left column), P20 (Middle column) and W13 (Right column). Results are expressed as mean ± SEM, n=6-7. Unpaired student's t test was used to analyse the data. Mag. 40X.

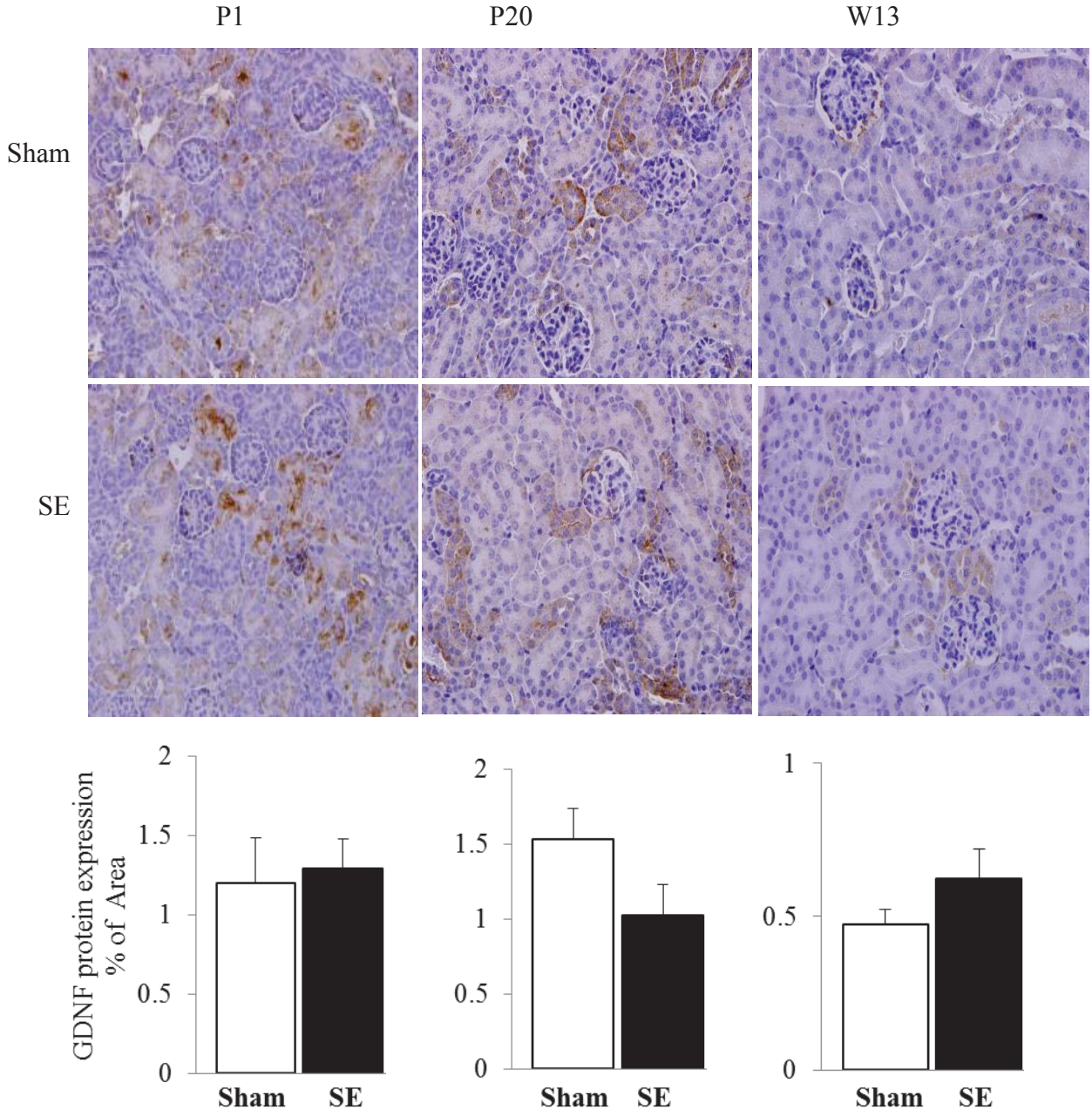


Figure 4.5: Renal GDNF protein expression in Sham offspring (Top panel) and SE offspring (Lower panel) at P1 (Left column), P20 (Middle column) and W13 (Right column). Results are expressed as mean ± SEM, n=6-7. Unpaired student's t test was used to analyse the data. Mag. 40X.

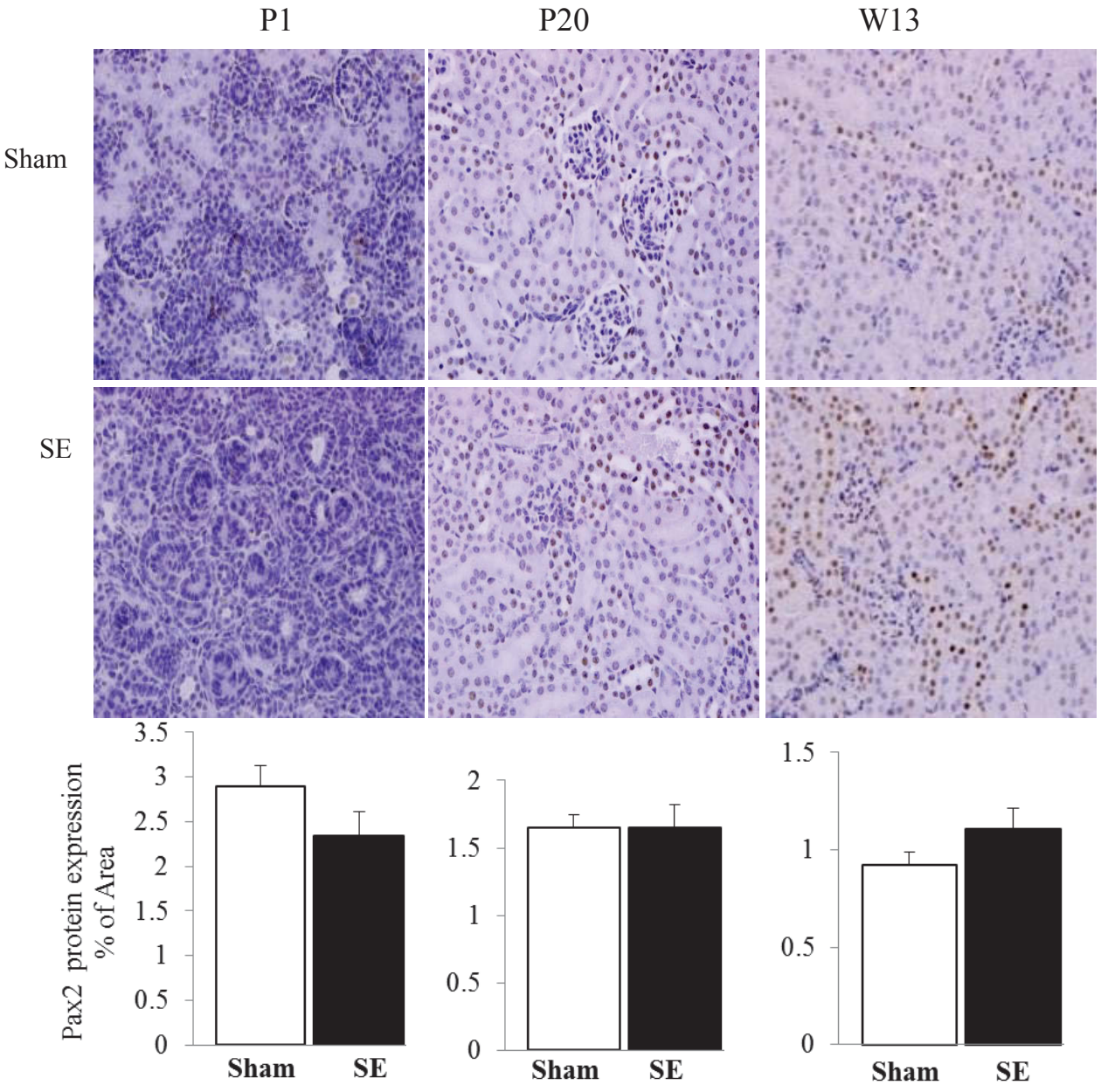


Figure 4.6: Renal Pax2 protein expression in the female offspring mice of the Sham (Top panel) and SE offspring (Lower panel) at P1 (Left column), P20 (Middle column) and W13 (Right column). Results are expressed as mean ± SEM, n=6-7. Unpaired student's t test was used to analyse the data. Mag. 40X.

4.3.4. Renal markers of injury, inflammation and function in the offspring

Renal mRNA expression of the fibrotic markers, fibronectin and collagen IV were not different between the two groups (Figure 4.7A and B). However, the pro-inflammatory marker, MCP-1 mRNA expression, had a trend of increase in the kidney from the SE offspring compared to sham offspring without statistical significance at W13 (Figure 4.7C). Proteins level of fibronectin and collagen IV were not different between the two groups either (Figure 4.8). Although cotinine level was higher in the SE offspring compared to the Sham offspring (Table 4.2, $p < 0.05$), there was no significant difference in urinary albumin/creatinine ratio and in serum enzymatic creatinine level at P20 and W13 between the two groups (Table 4.2).

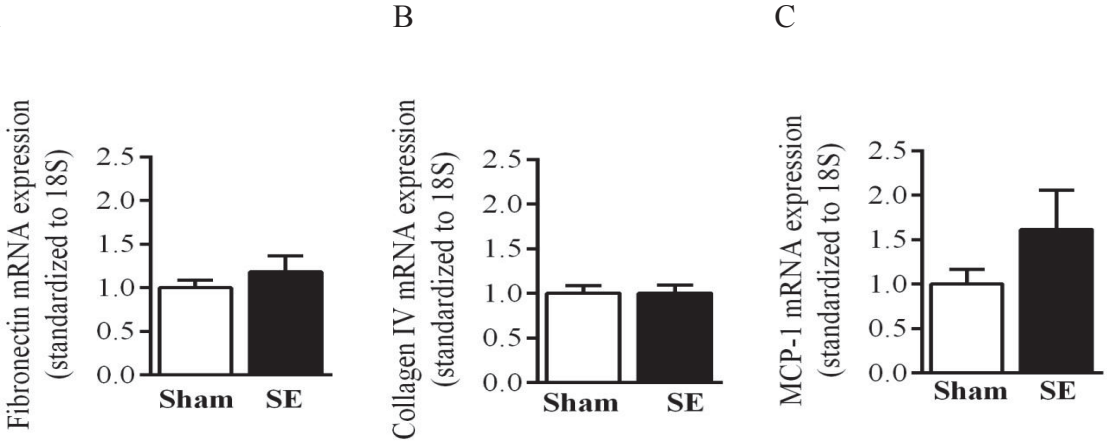


Figure 4.7: Renal mRNA expression of injury markers fibronectin (A), colagen IV (B) and MCP-1 (C) in the female offspring at W13. Results are expressed as mean ± SEM, n=6. Unpaired student's t test was used to analyse the data.

Table 4.2: The effects of maternal SE on cotinine level and renal function in female offspring.

Female	P20		W13	
	Sham	SE	Sham	SE
Cotinine (ng/ml)	2.29 ± 0.3	5.57 ± 1.4*		
Urinary albumin/creatinine ratio (μg/mg)	6.4 ± 0.9	6.2 ± 1.6	25.3 ± 9.8	27.7 ±5.1
Serum enzymatic creatinine (μmol/l)	18.0 ±2.9	25.7 ± 5.6	13.7 ±1.4	14.5 ±1.3

Results are expressed as mean ± SEM, n=6-11. * $P < 0.05$ vs Sham.

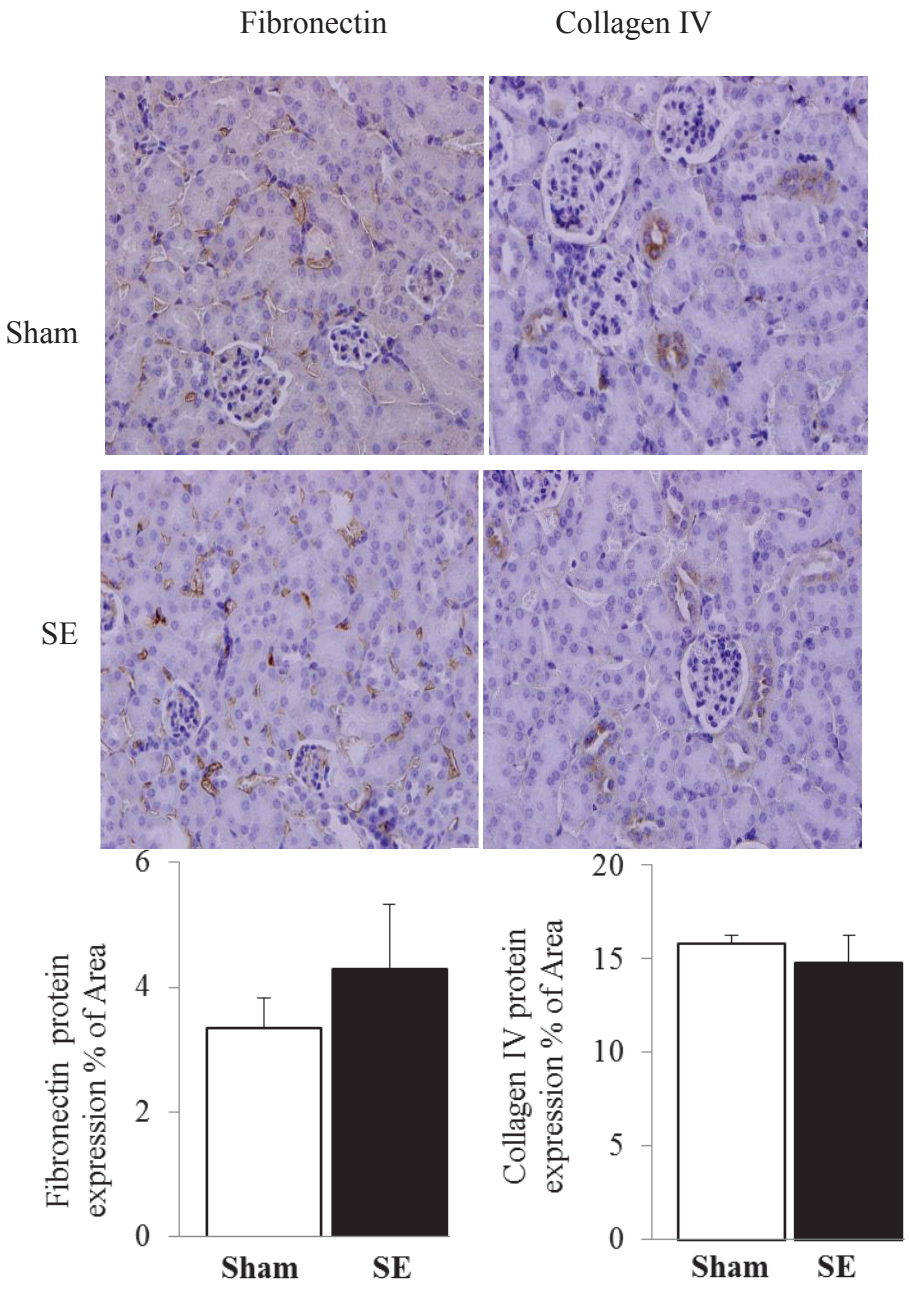


Figure 4.8: Renal fibtonectin (Left panel) and collagen IV (Right panel) protein expression in the Sham (top panel) and SE offspring (lower panel) at W13. Results are expressed as mean ± SEM, n=6. Unpaired student's t test was used to analyse the data. Mag. 40X.

4.3.5. IPGTT

IPGTT showed a significantly increased blood glucose level at 15, 30 and 90 min post-injection ($p<0.05$, Figure 4.9A). AUC also showed significant glucose intolerance (Figure 4.9B) in the SE offspring compared to the Sham offspring ($p<0.05$).

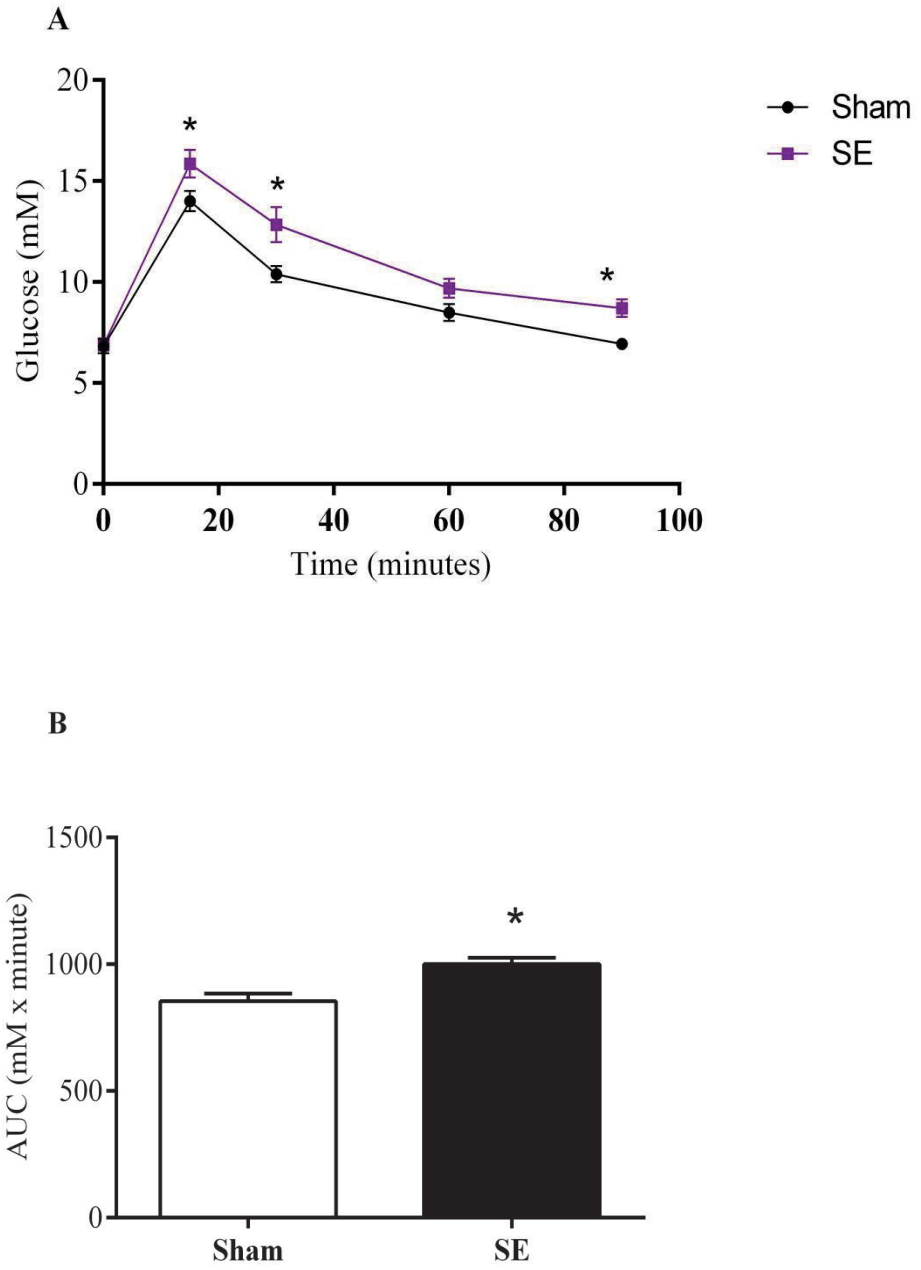


Figure 4.9: IPGTT results in the female offspring mice at 12 weeks. (A) Blood glucose level changes during the IPGTT. (B) AUC of (A) at adulthood (W12). Results are expressed as Mean ± SEM. n = 8-10. * $P < 0.05$ SE vs Sham.

4.4. Discussion

The major finding in this study is that maternal cigarette SE led to glucose intolerance in the female offspring; however, it did not have significant impacts on fetal and early postnatal renal development, neither were the kidney structure and renal function at adulthood affected, which is quite different from the outcome in the male offspring (Al-Odat et al. 2014) shown in Chapter 3.

Kidney volume is an indicator of nephron number (Taal et al. 2012). Male offspring had renal underdevelopment in addition to renal structural changes at early postnatal life. Moreover, at adulthood male offspring from the SE mothers still had structural changes in the kidney including decrease in nephron number and increase in nephron size, as well as deterioration in kidney function. However, such adverse maternal impacts on renal development and function outcome were not observed in the female offspring.

Our findings are in agreement with different studies that showed female offspring are less susceptible to diseases due to different intrauterine insults. For example, female but not male offspring from the rat dams exposed to hypoxia during pregnancy are protected from developing peripheral vascular resistance and cardiovascular disease (Hemmings, Williams & Davidge 2005). In addition, prenatal lipopolysaccharides exposure affects cognitive functions in male but not female offspring in rat model of schizophrenia (Wischhof et al. 2015).

Our results showed that maternal SE lead to reduced blood glucose level in the female offspring at P1 (Chen et al. 2004). This reduction in blood glucose level might be a result of fetal malnutrition due to maternal SE-induced placental insufficiency (Gagnon 2003), that can potentially predispose the offspring to metabolic syndrome which can in turn increase the risk of chronic kidney disease. It might also contribute to reduce podocytes apoptosis and depletion (Susztak et al. 2006) due to maternal SE-induced ROS generation in the developing kidney.

Maternal SE did not affect the mRNA and protein expression of renal growth and transcription factors in the female offspring at P1, including those essential for UB outgrowth and elongation and MM differentiation and transformation to epithelial during kidney development (Abdel-Hakeem et al. 2008), such as BMP4 and 7, FGF10 and WT1. In the male offspring, in addition to these four factors, we observed changes in other growth factors, including FGF2, 7, GDNF, Pax2, and WNT11. At P20, FGF2, FGF10 and WNT4 mRNA expression were increased in the males but not in the female offspring at the same age. As such, the change of a network of growth factors are required to cause kidney underdevelopment and reduced nephron number followed by deterioration in the kidney function, which are lacking in the females. Therefore, unlike the male offspring, these limited changes in growth factors did not affect kidney weight, glomerular number and kidney function, nor led to renal hypertrophy in the female offspring, suggesting an adaptive mechanism in the female offspring during fetal programming to maintain normal renal structure and functions.

TGF-β is a fibrogenic factor (Neugarten & Golestaneh 2013), and BMPs growth factors are a subfamily of the TGF-β superfamily. They are expressed in the nephrogenic mesenchyme and collecting ducts. It is essential in kidney development for regulating cells proliferation and apoptosis (Adams et al. 2007). Moreover, BMP7 has an anti-fibrotic and anti-inflammatory properties in the renal tissues (Meng, Chung & Lan 2013); whereas BMP4 regulates cell proliferation, differentiation and apoptosis in the developing kidney (Weber et al. 2008). In line with the fact that renal BMP7 expression is downregulated under disease conditions such as diabetic nephropathy and ischemic acute kidney injury (Meng, Chung & Lan 2013), our results showed insignificant trend of reduction in BMP7 mRNA expression in the female SE offspring. This might be a mechanism underlying reduce TGF-β and its related fibrotic gene expression (Meng, Chung & Lan 2013), as well as maternal SE induced kidney apoptosis and renal injury in a previous study (Jagadapillai et al. 2012). However, because the BMP4 can functionally substitute BMP7, the insignificant increase in BMP4 mRNA expression may compensate the limited reduction in BMP7 during kidney development (Oxburgh et al. 2005) which was observed in our results (Figure 4.3).

Nevertheless, there were no changes in fibronectin and collagen IV mRNA expression in the kidneys in both male and female SE offspring compared to the Sham offspring at W13. Compared with a pronounced increase in renal MCP-1 mRNA expression in the males, the non-significant 60% increase in MCP-1 mRNA levels in the females at W13 may still suggest an increased renal inflammation due to maternal SE. However, there were no significant differences in urinary albumin/creatinine ratio at W13 and serum enzymatic creatinine at P20 and W13 suggesting female offspring are protected from maternal SE; whereas males have already shown significant renal functional declining at W13. We postulate that the pathological changes in the males may be attributed to reduced nephron number and glomerular size change during early developmental period, which were not seen in the female offspring.

Female offspring are less susceptible to renal underdevelopment due to maternal SE compared to the male offspring, It is known that gender is a risk factor for developing kidney disease due to the differences in renal structure, glomerular hemodynamics and hormones metabolism between the males and females (Zhang & Rothenbacher 2008). For example, the kidney size and weight in animals is greater in males than the females after correcting for body weight, which is believed to be due to androgen effect to increase proximal tubule size (Silbiger & Neugarten 2008). In addition, the response of kidney glomerular hemodynamic to angiotensin II leading to increase glomerular filtration rate is only observed in the males, but not in the females (Silbiger & Neugarten 2008). Furthermore, sex hormones are involved in the normal growth of the body tissues (Padmanabhan et al. 2006). In addition, sex hormones can modulate different molecular and cellular events through affecting the biosynthesis and activity of different cytokines and growth factors including TNF-α, TGF-β and Collagens (Metcalfe & Meldrum 2006; Montezano et al. 2005; Neugarten & Golestaneh 2013; Silbiger & Neugarten 2008). Thus a possible explanation to this gender difference is that the adaptive changes in renal growth and transcription factors in the females can be a mechanism to overcome the adverse maternal SE impact to prevent fetal renal underdevelopment. It has been suggested that the protective effect of estrogen in females might be behind the difference in the life span between males and females

(Vahter, Gochfeld, et al. 2007) as a result of susceptibility to diseases. Both Estrogen receptor (ER)-α and ER-β are expressed in the glomeruli in the kidney suggesting the kidney is influenced by estrogen (Elliot et al. 2007; Gluhovschi et al. 2012). Estradol inhibits the stimulating effect of TGF-β on collagen IV gene transcription hence reducing renal injury (Neugarten & Golestaneh 2013). A proposed mechanism of estrogen controlling glomerulosclerosis in the female offspring is through the ER-mediated upregulation of matrix metalloproteinase degrading enzyme, MMP-9 expression and activity in mesangial cells. This influence ECM degradation and turnover which provide a protection to the females against renal glomerulosclerosis (Potier et al. 2001). Estrogen can also limit the effects of inflammation on renal vascular injury and endothelial dysfunction (Stenvinkel et al. 2002). This was consistent with a study where inflammatory response is gender-specific in rats due to cadmium exposure (a main component of the cigarette smoke) (Kataranovski et al. 2009). In this study, female offspring did not develop significant inflammatory response due to maternal SE as the male did (Chapter 3), although it has been reported in a study that the cigarette smoke has an anti-estrogen effect in women due to changes in estrogen physiology and metabolism. The level of cigarette smoke exposure via breast milk may be too short and too mild to affect estrogen level in the female offspring.

Maternal smoking during pregnancy is a significant risk factor for developing type 2 diabetics in offspring (Montgomery & Ekbom 2002). We have previously shown that both male and female offspring develop glucose intolerance and insulin resistance even while consuming low fat diet in adulthood (Al-Odat et al. 2014; Chen et al. 2011). Glucose intolerance in the female offspring in this study is consistent with our previous study. The mechanism underlying glucose disorders will be investigated in Chapter 6.

The main strength of this study is that the maternal SE started long before the conception which is important in follicular atresia and changing the structure and physiology of the gametes before fertilization (Sobinoff et al. 2013). SE was continued during the critical kidney morphogenesis stages during gestation and early postnatal life. Our study supported the theory of the gender differences on the prevalence and

progression of renal injury by maternal SE. However, although female offspring renal function and structure are normal at W13 in this study, it can still be postulated that additional insult in later life, eg diabetes may still make the SE female offspring more vulnerable to increased renal damage compared with those from non-smokers, which requires further investigation.

Chapter Five

5

The Effects of Maternal L-Carnitine Supplementation on Ameliorating Renal Underdevelopment and Dysfunction, and Glucose Intolerance in Male Mice Offspring of Cigarette Smoke Exposed Dams

5.1. Introduction

Smoking is associated with renal dysfunction and progression of kidney disease (Briganti et al. 2002) and impaired glucose tolerance (Frati, Iniestra & Ariza 1996) in humans. Second hand SE is also associated with renal dysfunction in children with CKD (Omoloja et al. 2013). In addition, obesity, glucose intolerance, insulin resistance, hyperlipidemia and endothelial dysfunction are considered as risk factors for kidney disease due to their close link with the onset of CKD (Chalmers, Kaskel & Bamgbola 2006; Raimundo & Lopes 2011). Indeed, glucose metabolism and insulin resistance play a pathogenic role in the development and progression of kidney disease even in non-diabetic patients (Ikee et al. 2007).

In agreement with this notion, our previous work has shown that maternal SE caused renal underdevelopment, adulthood renal dysfunction and glucose intolerance in the male offspring in a rodent model (Al-Odat et al. 2014). Another study in our laboratory discovered that the underlying mechanism of this renal underdevelopment and dysfunction due to maternal SE is through the increase in renal oxidative stress and mitochondrial dysfunction at both birth and adulthood by impairing the anti-oxidant enzymes capacity such as sodium dismutase and GPx-1 (Nguyen et al. 2015). Furthermore, another study in our lab also confirmed that mitochondrial dysfunction and damage caused by increased oxidative stress by maternal SE is a critical mechanism leading to renal pathology in adult offspring (Stangenberg et al. 2015).

Diet supplementation with antioxidants such as Vitamin C, Vitamin E and β-carotene, has been shown to have beneficial role in decreasing the effect of oxidative stress-caused damage in the lymphocyte DNA due to cigarette smoking (Duthie et al. 1996).

LC has been shown to have anti-inflammatory (Miguel-Carrasco et al. 2008), anti-apoptotic (Ishii et al. 2000) and anti-oxidative (Gulcin 2006) effects. As such, the beneficial effect of LC supplementation has been demonstrated in rodent models of cardiac fibrosis due to arterial hypertension (Zambrano et al. 2013), kidney pathology due to atherosclerosis (Salama et al. 2012), intestinal ischemia reperfusion injury (Yuan et al. 2011), chronic renal failure due to partial nephrectomy (Sener et al. 2004), and neurotoxicity induced by sodium azide (Ahmed & Farouk Fahmy 2013).

In addition, it has been shown that LC is necessary for fetal development and maturation of the brain and lung during intrauterine development (Arenas et al. 1998). A previous work in our lab demonstrated that maternal LC supplementation during gestation and lactation reversed increased renal oxidative due to maternal SE in the male offspring (Nguyen et al. 2015). However, its effect on kidney development and glucose tolerance in such offspring is not known. Therefore, the aim of this study was to investigate the impact of maternal LC supplement during gestation and lactation on the deleterious effect of maternal SE on kidney underdevelopment, renal dysfunction, and glucose intolerance in the male offspring.

5.2. Methodology

5.2.1. Animal model and tobacco cigarette smoke exposure protocol

The animal experiment was approved by the Animal Care and Ethics Committee at the University of Technology, Sydney (ACEC#2011-313A). All protocols were performed as described in Chapter 2, section 2.1.2.

Briefly, female Balb/c mice (6 weeks old, n=36, Animal Resources Centre, Perth, Australia) were housed as described in Chapter 2, section 2.1.2. After 1 week of acclimatization, the mice were weighed and divided into two groups with equal BW, the Sham exposure group (control, n=12) and the cigarette SE group (SE, n=24). SE was performed as described previously (Al-Odat et al. 2014). After 6 weeks of cigarette SE, the female mice were mated and SE continued as described in Chapter 2. When pregnancy was confirmed by significant increase in body weight, the pregnant females were housed individually. SE was not performed on the day of delivery. Neither the male breeder nor the offspring were directly exposed to cigarette smoke at any time during the study.

A subgroup of the SE dams was treated with LC (SE+LC, n=12) via drinking water (1.5mM) which was started at the time of mating and continued throughout gestation and lactation. LC dose and administration were adopted from a study by Ratnakumari and colleagues (Ratnakumari et al. 1995) . A day after birth, litter size was adjusted to 3 to 4 pups per dam.

5.2.2. IPGTT

IPGTT was performed at W12 of age in the male offspring. After 5 hours fasting, a baseline glucose level was taken from the tail blood (Accu-Chek, Roche Diagnostics, Indianapolis, IN, USA). Then glucose solution was administered intraperitoneally (2 g/kg , n = 9-10) and blood glucose levels were measured at 15, 30, 60, and 90 min post-injection (Chen et al. 2011). The AUC of the glucose levels was calculated for each mouse.

5.2.3. Tissues collection

Male offspring mice were scarified at three different time points; P1, P20 (weaning age) and at W13 (mature age). Briefly, the pups were weighed and anaesthetized with sodium thiopental (0.1ml/g, i.p., Abbott Australasia PTY. LTD, NSW, Australia). Blood was collected through cardiac puncture. The plasma was stored at -20 ^{o}C for later analysis. Urine was collected directly from the bladder and stored at -20 ^{o}C. Mice were killed by decapitation. The kidneys were harvested, weighed, snap frozen in liquid nitrogen and then stored in -80^{o}C. One of the kidneys was fixed in 10% formalin (Sigma, VIC, Australia).

5.2.4. Real-time PCR

Total RNA was extracted from kidney tissues using RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturers' instructions.

The kidney tissues were homogenized in lysis buffer using hand-operating homogenizer (Qiagen, Valencia, CA). The total RNA purification was done using automated QIAcube robot machine (Qiagen, Valencia, CA). The DNA in the total RNA samples was digested using DNase I, Amp Grade enzyme (Life Technologies CA, USA). The RNA samples were quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The first strand of cDNA was synthesised from 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics,

Mannheim, Germany). The cDNA samples were quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and then tested for integrity.

qPCR was performed in a 10 µl volume using pre-optimized SYBR Green primers (Table 2.1) and SensiFAST^{TM} SYBR Hi-ROX Kit (Bioline, Toronto canada). The reaction plate was run in ABI7900HT Sequence Detection System (SDS 2.4, Life Technologies, CA, USA).

The results were analysed by relative quantitation using RQ Manager Software (RQ 1.2.1, Applied Biosystems) using ΔΔCt. The average mRNA expression of the Sham (Control) group was used as the calibrator and 18S rRNA was used as the housekeeping gene.

5.2.5. Kidney histology and IHC staining

Fixed kidney samples in 10% formaldehyde were embedded in paraffin and sectioned in 2µm and 4µm. Kidney structure of the offspring was examined using H&E and PAS staining of the 2µm kidney sections.

For H&E staining, the kidney sections were de-waxed and hydrated. The sections were then incubated in haematoxylin for 3 minutes followed by de-colourization in acid ethanol. The sections were then counterstained in eosin then dehydrated, cleared and mounted. Glomerular number was estimated by counting the developed glomeruli in 3-4 non-consecutive H&E stained kidney sections from the same animal. For this purpose, 6-8 different animals were used from each group.

For PAS staining, the kidney sections were de-waxed and hydrated. Then the sections were incubated with 0.5% periodic acid solution for 5 minutes, and then washed in running water followed by incubation in Schiff's reagent. The tissues were counterstained with haematoxylin, dehydrated, cleared and mounted. Glomerular size for each animal was measured in the PAS stained 6 different images of kidney sections using Image J (Image J, NIH, USA) and then averaged (Al-Odat et al. 2014).

For IHC staining, 4µm kidney sections were de-waxed then dehydrated. Heat induced epitops retrieval was performed, and then endogenous peroxidase was blocked by

incubating the sections in 0.3% H_2O_2 for 5 minutes at room temperature. The kidney sections were blocked using serum-free protein block (Dako, CA, USA) for 10 minutes at room temperature, and then incubated with rabbit anti-mouse primary antibodies against FGF2 (1:250 dilution), GDNF (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Pax 2 (1:1750 dilution) at 4^{o}C overnight. Negative controls were prepared by replacing the primary antibodies with rabbit IgG.

In the following day, the sections were exposed to Envision & system-HRP labelled polymer secondary anti-rabbit antibodies (Dako, CA, USA) for 30 minutes at room temperature. Secondary antibody was detected by the reaction with HRP enzyme and DAB+ (liquid DAB + substrate chromogen system, Dako, CA, USA). Then the sections were counterstained with Mayer's Haematoxylin, dehydrated, cleared and mounted.

The images of the kidney sections were captured. On average, 6 different non-overlapping fields of the same kidney section were captured and 6-8 mice were used from each group. Quantitation of the positive signals in the images was performed using Image J software (Image J, NIH, USA) and the percentage of the brown colour to the whole field was determined and averaged.

5.2.6. ELISA

Different commercial ELISA kits were used to measure urinary albumin and creatinine concentrations and serum insulin and cotinine levels. Urinary albumin and creatinine concentrations were measured using Murine Microalbuminuria ELISA kit (Albuwell M, Exocell Inc, PA, USA) and Creatinine Companion kit (Exocell Inc, PA, USA) respectively according to the manufacturer instructions. Insulin (Mouse) ELISA Kit (Abnova, Taipei, Taiwan) and cotinine Direct ELISA kit (Abnova) were also used according to the manufacturer's instructions.

5.2.7. Serum enzymatic creatinine

Serum enzymatic creatinine levels were measured by an automated analyzer (ARCHITECT, Abbott Australasia, NSW, Australia).

5.2.8. Statistical analysis

The differences between the groups were analysed using one-way ANOVA followed by Tukey's *post hoc* tests to compare the differences between the Sham, SE and SE+LC groups (Prism 6, Graphpad CA, USA). The results are expressed as mean ± SEM. $P<0.05$ is considered significant.

5.3. Results

5.3.1. Developmental parameters of the males mice offspring

At P1, body weight and kidney weight were significantly reduced in the SE offspring compared to the Sham offspring ($p<0.05$, Table 5.1). However, the kidney weight as percentage of the body weight was not different in the SE offspring at this time point. In addition, male offspring of the SE dams had increased blood glucose level ($p<0.05$). Maternal LC supplementation improved the effect of maternal SE on body weight and kidney weight ($P < 0.05$, Table 5.1) and prevented the increase in blood glucose level at P1 in the male offspring.

Body weight, kidney weight and percentage of kidney weight were similar among the three groups at P20 and W13 in the male offspring. Blood glucose level was similar at both ages as well (Table 5.1). Maternal LC supplementation had no significant impact on these parameters at both endpoints.

Table 5.1: Effects of maternal SE on growth and development in male offspring.

P1	**Sham**	**SE**	**SE+LC**
Body weight (g)	1.55 ± 0.05	1.35 ± 0.06*	1.58 ± 0.06$^{\#}$
Kidney weight (g)	0.0081 ± 0.0004	0.0069 ± 0.0004*	0.0086 ± 0.0010$^{\#}$
Kidney/Body (%)	0.52 ± 0.02	0.51 ± 0.04	0.55 ± 0.04
Glucose (mM)	4.01 ± 0.15	4.56 ± 0.21*	4.32 ± 0.19
P20	**Sham**	**SE**	**SE+LC**
Body weight (g)	9.97 ± 0.16	9.71 ± 0.14	9.74 ± 0.43
Kidney weight (g)	0.067 ± 0.001	0.062 ± 0.003	0.067 ± 0.002
Kidney/Body (%)	0.67 ± 0.01	0.64 ± 0.03	0.70 ± 0.03
Glucose (mM)	12.1 ± 0.43	12.1 ± 0.70	12.0 ± 0.47
W13	**Sham**	**SE**	**SE+LC**
Body weight (g)	25.5 ± 0.3	25.1 ± 0.6	25.3 ± 0.3
Kidney weight (g)	0.20 ± 0.01	0.19 ± 0.01	0.19 ± 0.01
Kidney/Body (%)	0.77 ± 0.01	0.76 ± 0.02	0.77 ± 0.02
Glucose (mM)	11.2 ± 0.63	10.5 ± 0.36	11.0 ± 0.54

Results are expressed as Mean ± SEM. n=8-20. One-way ANOVA followed by Tukey's *post hoc* tests were used to analyze the data among three groups at the same age. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE.

5.3.2. Renal histological changes

Kidney H&E staining showed significant reduction in the number of the developed glomeruli in the kidney of the offspring from SE dams compared to the offspring from the Sham exposed dams at P1, P20 and W13 ($P < 0.05$, Figure 5.1). Maternal LC supplementation significantly revered the effect of maternal SE at all 3 time points ($P < 0.05$).

Glomerular size was similar among the three groups at P1 (Figure 5.2). However, it was increased in the kidneys of the offspring from the SE dams at P20; whereas it was decreased at W13 ($P < 0.05$, Figure 5.2). Maternal LC supplementation significantly reversed these changes due to maternal SE to similar level as the Sham offspring at P20 and W13 ($P < 0.05$, Figure 5.2).

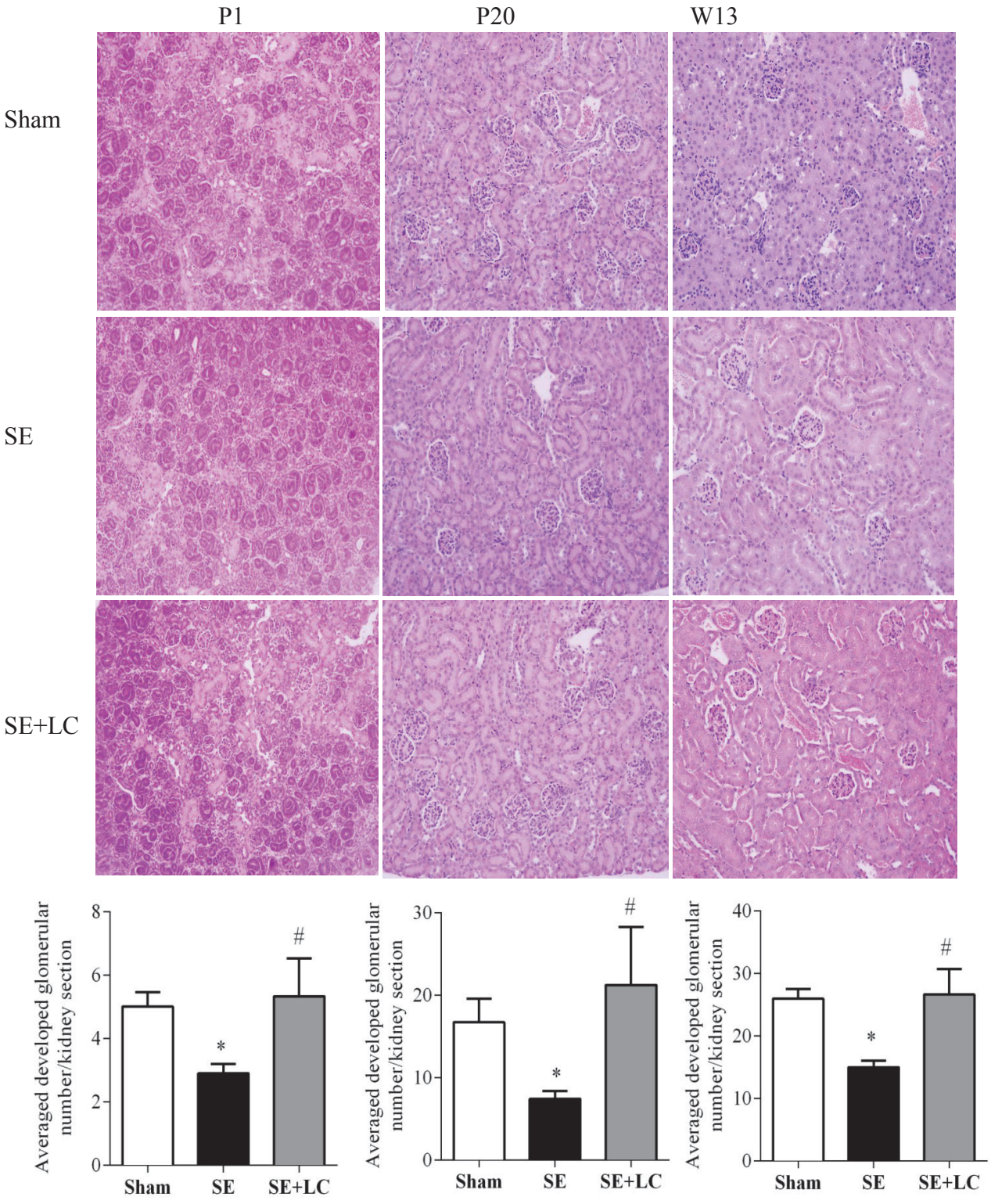


Figure 5.1: The number of developed glomeruli in the male offspring. H&E stained kidney sections from the offspring of the Sham dams (Top panel), SE dams (Middle panel), and SE+LC dams (Bottom panel) at P1 (Left column), P20 (Middle column) and W13 (Right column). Results are expressed as mean ± SEM, n=6-8 Mag. 20X. * $P < 0.05$ SE vs Sham, # $P < 0.05$ SE+LC vs SE.

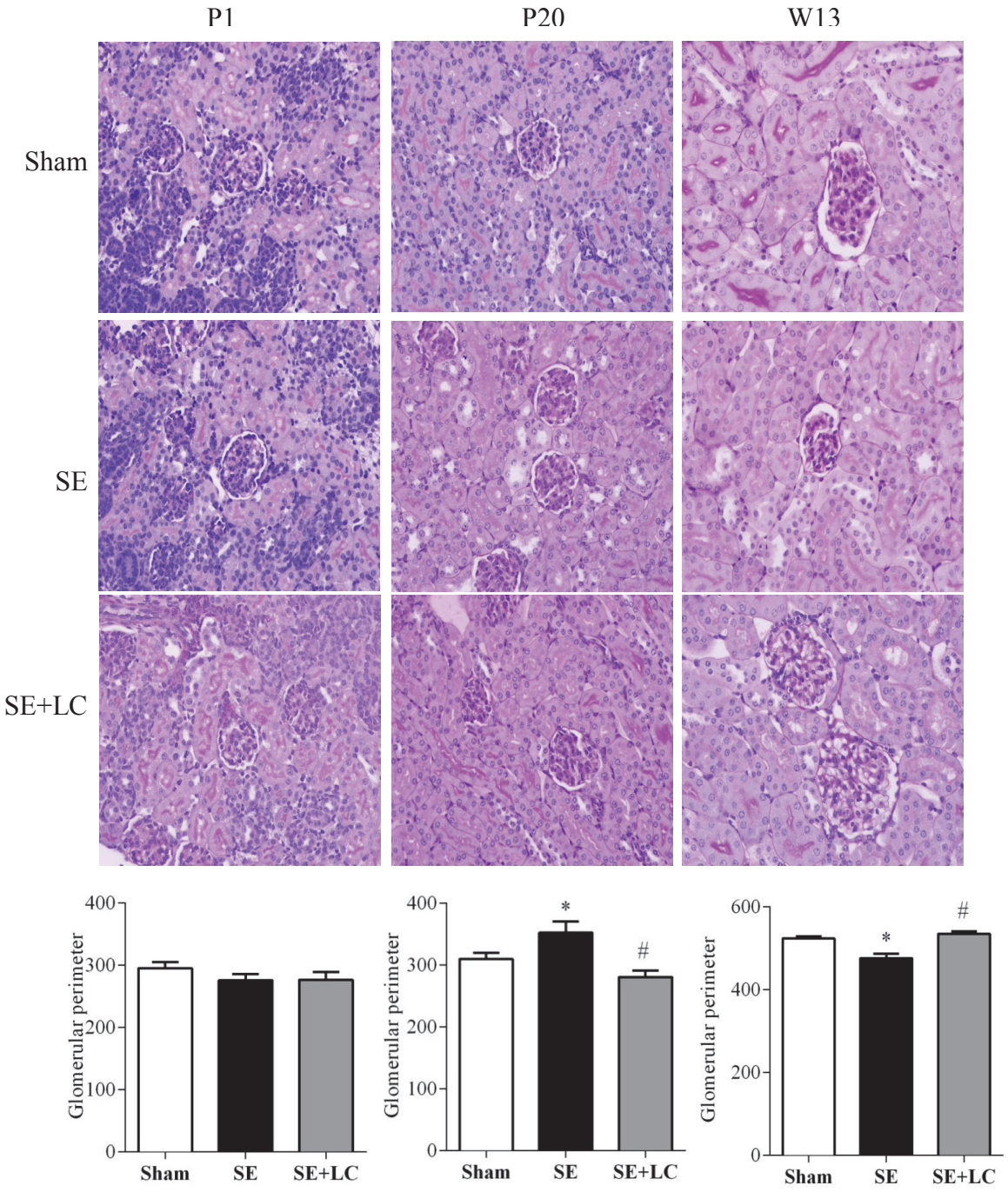


Figure 5.2: Glomeruli size in the male offspring. Kidney PAS staining showing glomerular size in the offspring of Sham dams (Upper panel), SE dams (Middle panel), and SE+LC dams (Lower panel) at P1 (Left column), P20 (Middle column) and W13 (Right column). Mag. 40X. Results are Mean ± SEM. n = 6-8. * $P < 0.05$ SE vs Sham, # $P < 0.05$ SE+LC vs SE.

5.3.3. mRNA and protein expression of renal growth and transcription factors

At P1, renal mRNA expression of FGF2, GDNF, Pax2, WNT11 and WT1 was upregulated, whereas FGF7 and FGF10 was downregulated in the kidney of the offspring from the SE dams compared to the offspring from the Sham dams ($P < 0.05$, Figure 5.3A). There was no change in the renal mRNA expression of BMP4, BMP7 and WNT4 at this time point. Renal mRNA expression of FGF7, FGF10, GDNF, pax2, WNT11 and WT1 were normalized in the kidney of the offspring from the SE+LC dams compared to the offspring from the SE dams ($P < 0.05$); however, maternal LC supplementation had no effect on FGF2 mRNA expression compared to the SE offspring (Figure 5.3A).

At P20, renal mRNA expression of the developmental factors FGF2, FGF10 and WNT4 were upregulated in the kidney of the offspring from the SE dams compared to the offspring from the Sham dams ($P < 0.05$, Figure 5.3B). These developmental factors were downregulated in the kidney of the offspring from the SE+LC dams compared to the offspring from the SE dams ($P < 0.05$, Figure 5.3B).

At W13, there were no changes in the renal mRNA expression of FGF2, GDNF and pax2 between the offspring from the SE dams and the offspring from the Sham dams (Figure 5.3C). However, renal mRNA expression of GDNF of SE + LC offspring was reduced by half compared to SE offspring although without statistical significance.

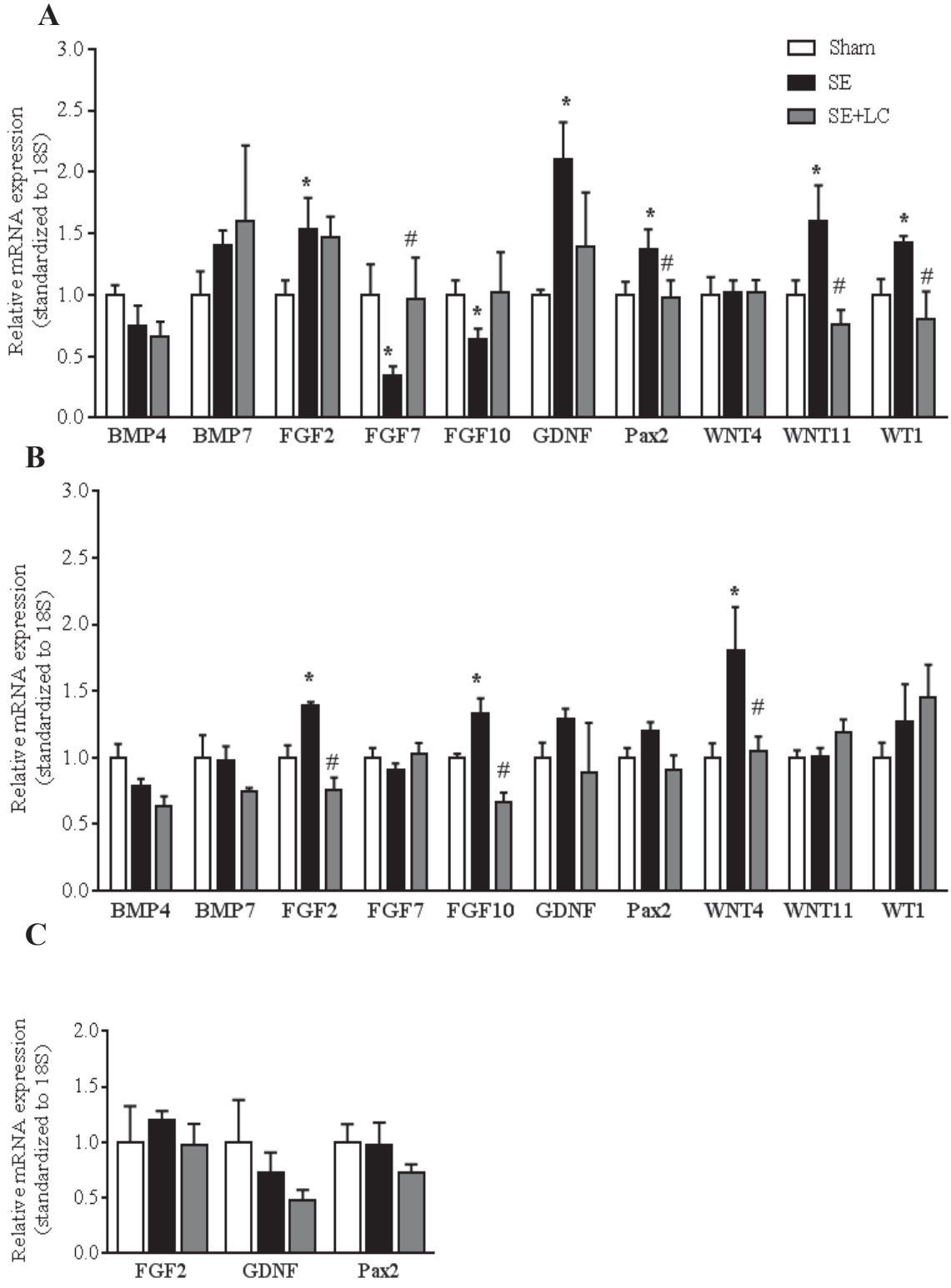
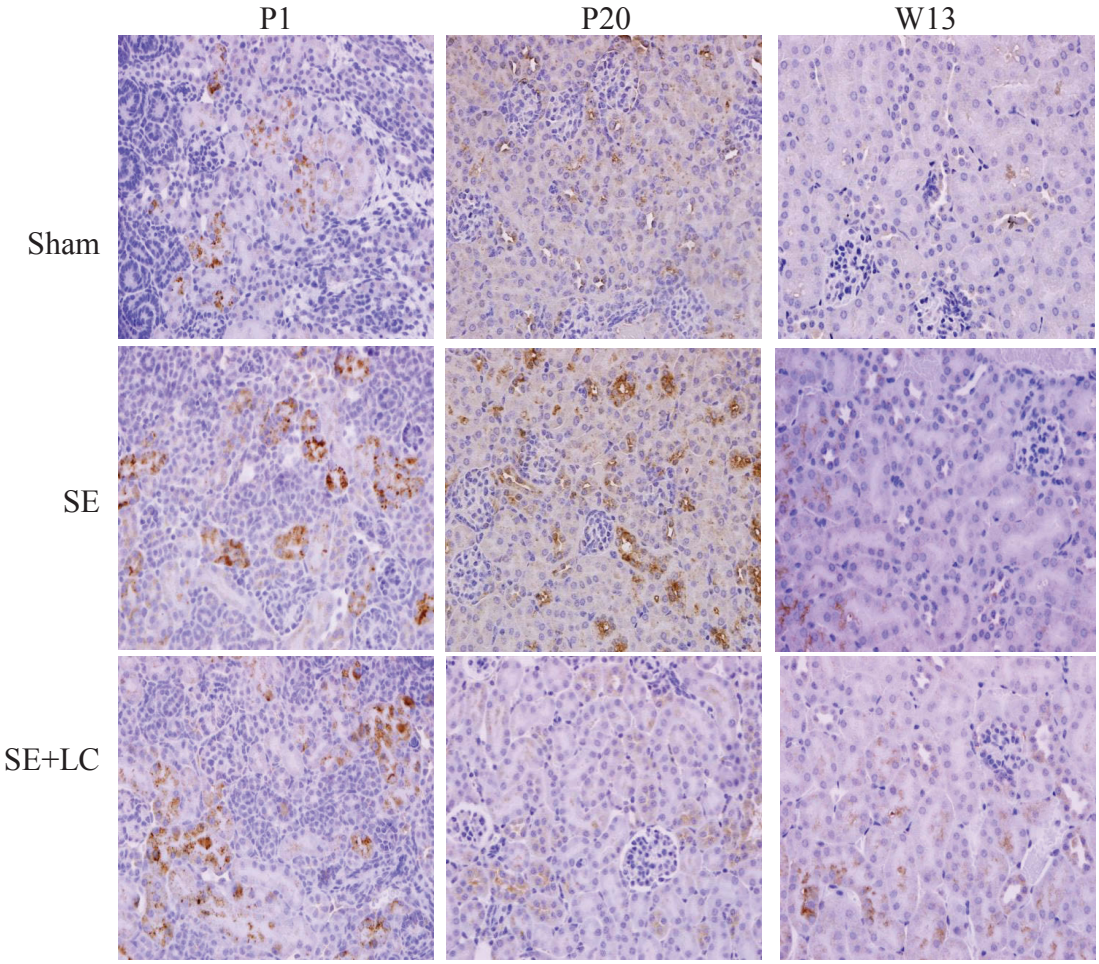


Figure 5.3: mRNA expression of renal growth and transcription factors in the male offspring mice at P1 (A), P20 (B) and W13 (C). Results are expressed as Mean ± SEM. n = 4-6. * $P < 0.05$ SE vs Sham, # $P < 0.05$ SE+LC vs SE.

Similarly, renal FGF2 protein level was increased in the kidney of the offspring from the SE dams compared to the offspring from the Sham dams at P1 and P20 ($P < 0.05$, Figure 5.4). However, maternal LC supplementation significantly reduced FGF2 protein expression at P20 ($P < 0.05$, Figure 5.4), but has no effect at P1.

Renal GDNF protein level was increased in the kidney of the SE offspring compared to the offspring from the Sham exposed dams at P1 ($P < 0.05$, Figure 5.5). There were no significant changes in the renal GDNF protein level in the kidney of the offspring from the SE dams at P20 and W13 (Figure 5.5). Maternal LC supplementation did not affect GDNF protein expression at P20 and W13 either (Figure 5.5). Both FGF2 and GDNF protein levels were also increased in the kidney of the offspring from the SE + LC dams compared to the offspring from the Sham dams at P1.

Renal pax2 protein level was increased in the kidney of the SE offspring compared to the offspring from the Sham dams at P1 ($P < 0.05$, Figure 5.6). Pax2 protein was reduced in the kidney of the SE+LC offspring compared to the SE offspring at P1 ($P < 0.05$, Figure 5.6). There were no significant changes in the renal pax 2 protein level in the kidney among the three groups at P20 and W13 (Figure 5.6).



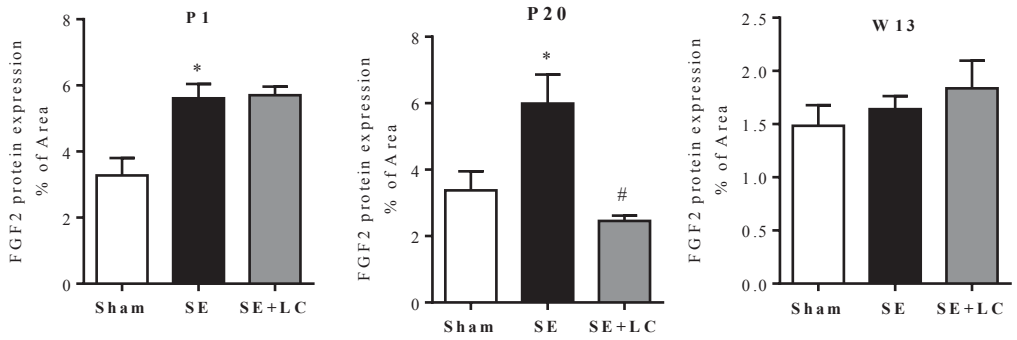


Figure 5.4: Renal FGF2 protein in the male offspring of the Sham dams (Top panel), SE dams (Middle panel) and SE+LC dams (Bottom panel) at P1 (Left column), P20 (Middle column) and W13 (Right column). Results are expressed as Mean ± SEM. n = 6. Mag. 40X. * $P < 0.05$ SE vs Sham, # $P < 0.05$ SE+LC vs SE.

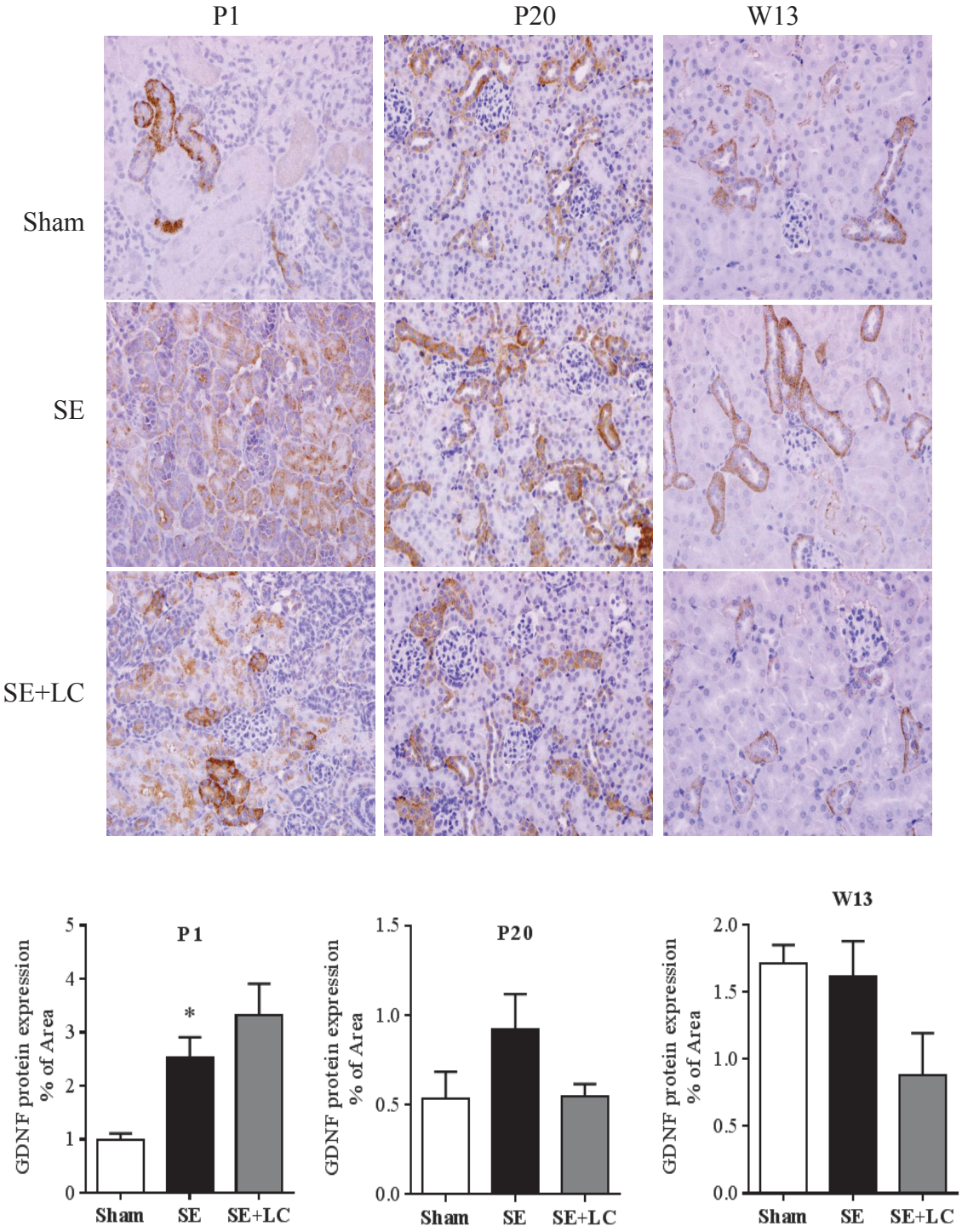


Figure 5.5: Renal GDNF protein expression in the male offspring of the Sham dams (Top panel), SE dams (Middle panel) and SE+LC dams (Bottom panel) at P1 (Left column), P20 (Middle column) and W13 (Right column). Results are expressed as Mean ± SEM. n = 6. Mag. 40X. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE

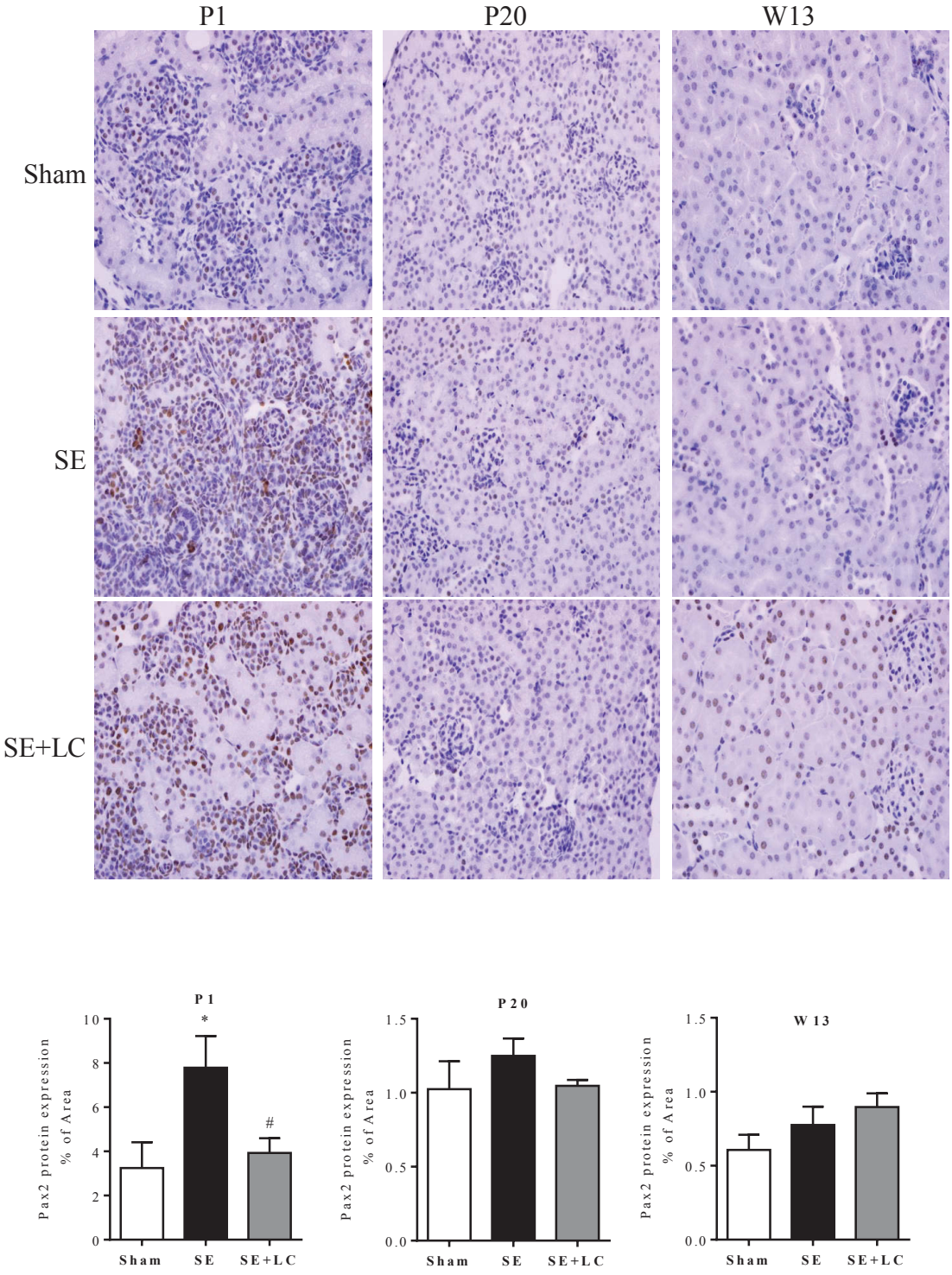


Figure 5.6: Renal Pax2 protein expression in the male offspring of the Sham dams (Top panel), SE dams (Middle panel) and SE+LC dams (Bottom panel) at P1 (Left column), P20 (Middle column) and W13 (Right column). Results are expressed as Mean ± SEM. n = 6. Mag. 40X. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE.

5.3.4. Renal markers of inflammation, injury and function

At W13, renal mRNA expression of kidney injury markers, including fibronectin (Figure 5.7A) and collagen IV (Figure 5.7B) were not changed in the kidneys from the SE offspring compared to the Sham offspring. Renal mRNA expression of MCP-1 was upregulated in the kidneys from the SE offspring compared to the Sham offspring ($p < 0.05$, Figure 5.7C). However, renal fibronectin, collagen IV and MCP-1 mRNA expression was downregulated in the kidney of the SE+LC offspring compared to the SE offspring ($P < 0.05$).

At P20, SE offspring and SE+LC offspring had significant increase in plasma cotinine level compared to the Sham offspring ($P < 0.05$, Table 5.2). At W13, offspring from SE dams had a significant increase in urinary albumin/creatinine ratio compared to the offspring from the Sham dams ($P < 0.05$, Table 5.2). Maternal LC supplementation improves the deterioration in albumin/creatinine ratio in the offspring from SE dams at adulthood.

There were no differences in the plasma insulin and plasma enzymatic creatinine levels among the three groups at W13 (Table 5.2).

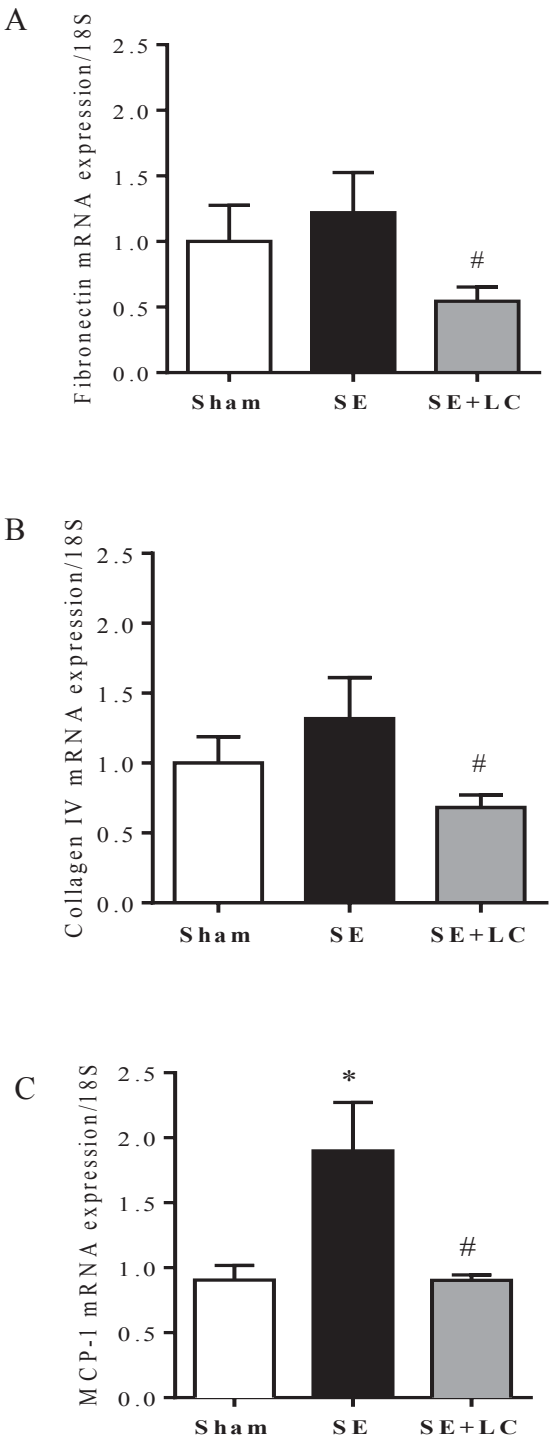


Figure 5.7: Renal mRNA expression of fibronectin (A), collagen IV (B) and MCP-1 (C) in the male offspring at W13. Results are expressed as Mean ± SEM. * $P < 0.05$ SE vs Sham, # $P < 0.05$ SE+LC vs SE. n=6-8.

Table 5.2: The effects of maternal SE and LC supplementation on plasma cotinine and insulin levels and renal function in the male mice offspring.

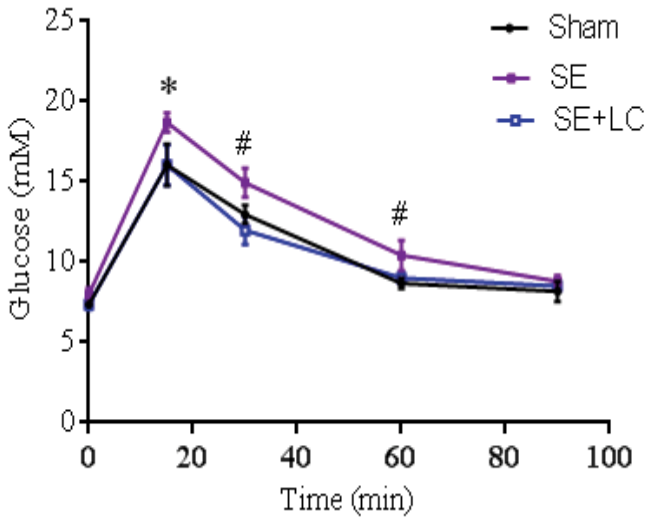
P20	**Sham**	**SE**	**SE + LC**
Cotinine (ng/ml)	1.35 ± 0.60	3.90 ± 0.42*	4.48 ± 0.17*
Week 13	**Sham**	**SE**	**SE + LC**
Insulin (ng/ml)	0.53 ± 0.02	0.54 ± 0.01	0.54 ± 0.01
Albumin/creatinine ratio (μg/mg)	43.0 ± 14.0	104.7 ±19.6*	81.5 ± 32.5
Enzymatic creatinine (μmol/l)	17.7 = 0.67	17.8 + 1.25	18.7 + 0.33

Results are expressed as Mean ± SEM. n = 8-10. * $P < 0.05$ vs Sham.

5.3.5. IPGTT

During the IPGTT, the baseline glucose levels were not different between the groups. However, SE offspring had highest blood glucose levels at 15, 30 and 60 minutes post glucose injection ($P < 0.05$, Figure 5.8A), and highest AUC ($P < 0.05$, Figure 5.8B). In addition, SE+LC offspring had normalized both glucose levels at 90 minutes post injection and the AUC ($p<0.05$).

A



B

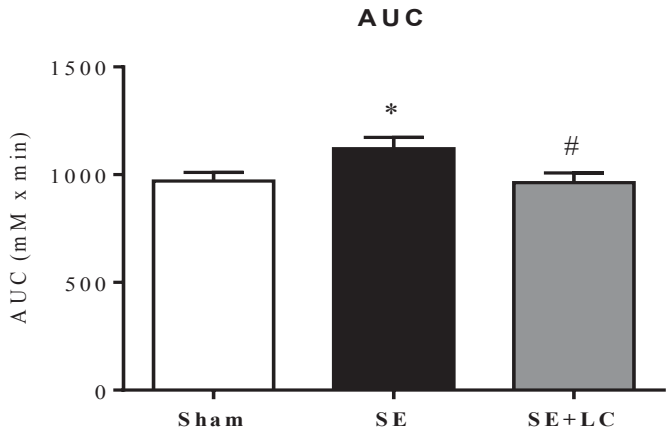


Figure 5.8: IPGTT results in the male offspring mice at 12 weeks**.** (A) Blood glucose level changes during the IPGTT. (B) AUC of (A) showing that offspring from the SE dams are glucose intolerant at adulthood (W12). Results are expressed as Mean ± SEM. n = 8-10. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE.

5.4. Discussion

The main finding in this study is that maternal LC supplementation during gestation and lactation has a beneficial effect in ameliorating kidney underdevelopment, renal dysfunction and glucose intolerance induced by maternal SE in the male mice offspring.

Exogenous LC supplementation has shown to have a protective role against chronic kidney failure in a rodent model (Sener et al. 2004). However, this study is the first to investigate the effect of maternal LC supplementation during gestation and lactation on the renal development and functional outcome in the offspring. Our results showed that maternal SE reduced body weight and kidney weight of the offspring at birth, which are consistent with the effects of maternal SE on offspring in humans (Chiolero, Bovet & Paccaud 2005). In addition, there was also remarkable increase in the serum cotinine level in the SE and SE+LC offspring, confirming the effect of maternal SE used in this study. In addition, the dosage of SE in mice used in our study is equivalent to 20-25 cigarettes per day in humans.

Although, newborn animals do not have the capacity to biosynthesise sufficient LC for their needs, they can source additional LC from the breast milk to fulfil the requirement (Matera et al. 2003). In humans, a previous study did not show any specific effect of maternal LC supplementation (10 mg/kg/d) on the growth and development of premature neonates (Seong et al. 2010). However, the limitation of this study is that only body weight gain was used to assess growth and development, which can't reflect the development of individual organs. Moreover, another animal study showed that dietary LC during pregnancy and lactation did not improve the growth of the offspring (Birkenfeld et al. 2005). This may be because that these studies were carried out in the normal animals without growth abnormalities, therefore it can only be concluded that LC does not affect normal fetal development and growth. However, the impact of LC on the development of offspring due to adverse maternal impact is unknown, although LC has been shown to have a beneficial role in embryogenesis *in vitro* (Abdelrazik et al. 2009) .

Our results showed that maternal LC supplementation during gestation and lactation improved the small body and kidney weight at birth due to maternal SE. Furthermore, it ameliorated the reduction in glomeruli number at every age measured in this study, in

addition to the abnormally large glomerular size at P20 and small glomerular size at W13 due to maternal SE. As a result, this led to improved renal function in the adult SE offspring evident by reduced albumin/creatinine ratio at W13.

The underlying mechanism of improved renal underdevelopment and dysfunction in the SE offspring could be through the restoration of several developmental factors that are linked to kidney underdevelopment in the SE offspring at different ages. At birth, renal mRNA expression of kidney developmental factor FGF 7, which is critical to modulate UB outgrowth and nephron number in the developing kidney (Qiao et al. 1999) was downregulated by maternal SE; while, maternal LC supplementation during gestation and lactation restored the mRNA expression of FGF 7. Moreover, the upregulation of developmental factors involved in the regulation of branching morphogenesis and nephron differentiation such as pax2 (Narlis et al. 2007), UB branching such as WNT11 (Yu, McMahon & Valerius 2004), and podocyte function regulation such as WT1 (Guo et al. 2002) by maternal SE was normalized in the kidneys of the SE+LC offspring. However, maternal LC supplementation had no effect on renal mRNA expression of other developmental factors including FGF2, and FGF10, which are involved in matenephric mesenchyme maintenance and increase of the stromal cells population within the MM (Dressler 2006), and UB branching (Michos et al. 2010) respectively. Maternal LC supplementation showed a trend to restore mRNA expression of GDNF, which initiates the UB formation and branching (Basson et al. 2006).

At P20, maternal LC supplementation restored FGF2, FGF10 and WNT4 mRNA expression in the kidney of the SE+LC offspring. WNT4 is a mesenchymal signal that promotes the mesenchymal cells transformation to epithelial cells (Kispert, Vainio & McMahon 1998). At W13, maternal LC supplementation downregulated GDNF mRNA expression in the kidneys of offspring from SE + LC dams compared to those from SE dams without statistical significance. The effect of maternal LC supplement to increase GDNF level at adulthood may be an over adaptive response to the maternal SE effect on renal underdevelopment in offspring, which requires further investigation. Renal protein expression levels of these developmental factors confirmed these changes in mRNA expression.

The protective effect of LC in the SE offspring is probably due to its anti-oxidant, anti-apoptotic and anti-inflammatory properties (Chen et al. 2009; Nguyen et al. 2015; Rabie et al. 2012). The ECM of the renal glomerulus constitutes glomerular basement membrane and mesangial matrix. Renal glomerular and tubular interstitial fibrosis due to the accumulation of proteins such as fibronectin and collagens in the ECM is an early progressive status towards CKD (Martinez et al. 2009). Our results showed a beneficial role of maternal LC supplementation in downregulating renal mRNA expression of fibrogenic factors, including fibronectin and collagen IV, which had the trend to be increased by maternal SE. This anti-fibrogenic effect of LC is in agreement with a previous study on the effect of LC in reducing kidney fibrosis *in vitro* and in a rat model of hypertension (Zambrano et al. 2014). Additionally, maternal LC supplement is associated with the downregulation of the pro-inflammatory factor MCP-1 mRNA expression in the kidney of the SE offspring at adulthood. This effect is consistence with the anti-inflammatory effect of LC shown in a previous study (Lee et al. 2015).

Maternal SE-induced glucose intolerance in male offspring has been reported in our previous study (Al-Odat et al. 2014). In humans, dose-dependent association between prenatal and postnatal SE and the development of insulin resistance in 10 years old children has been reported (Thiering et al. 2011). In this study, as expected, SE offspring are glucose intolerant but with similar insulin level as the Sham offspring, suggests an impairment in insulin production by the β-cells in response to higher blood glucose level (Nguyen et al. 2015). This may be due to the effects of nicotine and CO toxicity on the pancreas in the offspring which is known to increase β-cells apoptosis, and decrease β-cell mass (Behl et al. 2013; Bruin et al. 2008). This can occur through binding of the nicotine to nicotine acetylcholine receptors in the fetal pancreas that leads to oxidative stress and mitochondrial damage leading to increased β-cell apoptosis as shown in a previous study (Behl et al. 2013).

In addition, our results showed a beneficial effect of LC in improving glucose metabolism in the SE offspring with improved glucose clearance during IPGTT (Reza López et al. 2013). This effect is similar to an observation in a study in obese and insulin-resistant ponies, where LC was supplemented into grass meals (1.3 g/100 kg BW, twice a day) of the pregnant mares (Schmengler et al. 2013). In this study, LC was shown to promote cellular glucose uptake and improve glucose oxidation. Cellular

glucose uptake is promoted by improving insulin sensitivity; while glucose oxidation is improved by regulating the mitochondrial acetyl-CoA/Free CoA ratio through facilitating transportation of the oxidation products, acetyl-CoA from the mitochondria to cytosol. Lowering the acetylCoA/CoA within the mitochondria can stimulate the activity of pyruvate dehydrogenase which in turn increases glucose oxidation (Schmengler et al. 2013).

In summary, the findings of this study suggest the beneficial role of maternal LC supplementation in the alleviation of renal underdevelopment and dysfunction as well as glucose intolerance due to maternal SE in the male mice offspring.

Chapter Six

6

The Effects of Maternal L-Carnitine Supplementation on Glucose Intolerance and Liver Profile in the Female Mice Offspring of Cigarette Smoke Exposed Dams

6.1. Introduction

Cigarette smoking is associated with increased systemic inflammation, insulin resistance, hyperinsulinemia, glucose intolerance and dyslipidemia in humans (Facchini et al. 1992; Houston et al. 2006; Niskanen et al. 2004). In addition, the association between maternal SE and the risk of such metabolic disorders in the offspring has also been reported in humans (Ino 2010; Seal, Krakower & Seal 2013). In this regards, the effects of maternal SE on the adiposity, glucose and lipid metabolic disorders in the offspring have been previously reported in a rodent model by our laboratory (Chen et al. 2011). Perinatal SE has been shown to result in increased pancreatic oxidative stress and β-cells apoptosis, which can predispose the offspring to the development of type 2 diabetes later in life (Bruin et al. 2008). In addition, maternal SE has been shown to cause liver oxidative damage in the offspring, which may be linked to liver insulin resistance and liver microsteatosis (Conceição et al. 2015). Furthermore, in a sex-specific manner, maternal smoking during the second trimester has been shown to change fetal liver protein levels of markers involved in inflammation, proliferation and apoptosis in the male offspring as well as those involved in glucose metabolism in the female offspring in humans (Filis et al. 2015), which may underlie lipid and glucose metabolic disorders in such offspring at adulthood.

The immune system and metabolic regulation are well integrated components of body homeostatic control system, and the proper function of each relies on the other (Hotamisligil 2006). As such, lipids are involved in the regulation of inflammatory and subsequent glucose metabolic processes (Hotamisligil 2006). It has been found that lipid accumulation in the liver leads to hepatic inflammation, which in turn leads to insulin resistance (Cai et al. 2005).

Recently, studies have documented that the female offspring are protected from renal pathophysiology and many other pathological conditions induced by different maternal conditions (Llorente et al. 2009; Woods, Ingelfinger & Rasch 2005). For example, neuronal degeneration and astroglial changes in the hippocampus and cerebellar cortex of neonatal rats due to maternal deprivation and separation were less marked in the females compared to males (Llorente et al. 2009). In addition, female rat offspring do not develop adult onset hypertension observed in their males littermates due to modest perinatal maternal protein restriction (Woods, Ingelfinger & Rasch 2005). Nevertheless,

our unpublished results indicated that female offspring of the SE mothers can still develop glucose intolerance at adulthood although renal pathology is not remarkable.

I have previously demonstrated that in the male offspring, maternal LC supplementation during gestation and lactation improved renal underdevelopment and dysfunction, as well as glucose intolerance induced by maternal SE. Therefore, the aim of this study was to evaluate the effect of maternal LC supplementation during gestation and lactation on glucose intolerance in addition to inflammation and lipid metabolic markers in the liver in the female offspring of the SE dams.

6.2. Methodology

6.2.1. Animal model

The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ACEC#2011-313A). All animal experiments were performed as described in Chapter 2, section 2.1.2. Briefly, female Balb/c mice (6 weeks old, n=36, Animal Resources Centre, Perth, Australia) were weighed and divided into two groups, the sham exposure group (control, n=12) and the cigarette SE group (SE, n=24). SE was performed as described in Chapter 2. After 6 weeks of cigarette SE, the female mice were mated and SE was continued as described in Chapter 2. When pregnancy was confirmed by significant increase in body weight, the pregnant females were housed individually. SE was not performed on the day of delivery. Neither the male breeder nor the offspring were exposed to cigarette smoke at any time during the study.

A subgroup of the SE dams was treated with LC (SE+LC, n=12) via drinking water (1.5mM) which started at the time of mating and continued throughout gestation and lactation. The LC dose and administration has been described in Chapter 5.

6.2.2. IPGTT

IPGTT was performed at W12 of age in the female offspring. After 5 hours fasting, a baseline glucose level was taken from the tail blood (Accu-Chek, Roche Diagnostics, IN, USA). Then glucose was administered (2 g/kg , n=9-10, ip) and blood glucose levels were measured at 15, 30, 60, and 90 min post-injection (Chen et al. 2011). The AUC of the glucose levels was calculated for each mouse.

6.2.3. Tissues collection

Female Offspring were scarified at P20 and at W13 as described in chapter 2, section 2.1.4. Briefly, the non-fasted pups were weighed and anaesthetized with sodium thiopental (0.1ml/g, i.p., Abbott Australasia, NSW, Australia). Blood was collected through cardiac puncture as described in Chapter 2. Blood glucose was measured and the plasma was stored at -20^{o}C for later analysis. Mice were killed by decapitation. The livers were harvested, weighed, snap frozen in liquid nitrogen and then stored at -80 ^{o}C.

6.2.4. Real-time PCR

Total RNA was extracted from liver tissues using RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturers' instructions as described in Chapter 2.

Liver tissues were homogenized in 350 µl lysis buffer using hand-operating homogenizer (Qiagen, Valencia, CA). Total RNA was purified using automated QIAcube robot machine (Qiagen, Valencia, CA). DNA in the total RNA extract was digested using DNase I, Amp Grade enzyme (life technologies CA, USA). The RNA samples were quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The first strand of cDNA was synthesised from RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The cDNA samples were quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and then tested for integrity as described in Chapter 2.

qPCR was performed in a 10 µl volume using pre-optimised Taqman probes (Life

Technologies, CA USA, Table 6.1) and Platinum® qPCR SuperMix-UDG kit (Life Technologies, CA, USA). The reaction plate was run in ABI7900HT Sequence Detection System (SDS 2.4, Life Technologies, CA, USA) as described in Chapter 2.

The results were analysed for ΔΔCt by relative quantitation using RQ Manager Software (RQ 1.2.1, Applied Biosystems). The average mRNA expression of the Sham group was used as the calibrator, and 18S rRNA was used as the housekeeping gene.

Table 6.1: probes sequences (Life Technologies, CA, USA) for rt-PCR.

Gene	NCBI references	Probe Sequence	Assay ID
18S	X03205.1	ACCGCAGCTAGGAATAATGGA	4319413E
ATGL	NM_025802.2	GGCCTGCCTGGGTGAAGCAGGTGCC	Mm00503040_m1
CPT-1α	NM_013495.2	TACCGTGAGCAGGTACCTGGAGTCT	Mm00550438_m1
DDit3	NM_007837.3	GAAACGAAGAGGAAGAATCAAAAAC	Mm01135937_g1
FASN	NM_007988.3	CAATTGTGGATGGAGGTATCAACCC	Mm00662319_m1
FOXO 1	NM_001191846	AAGAGTTAGTGAGCAGGCTACATTT	Mm00490672_m1
Glut 4	NM_009204.2	CTCTGCTGCTGCTGGAACGGGTTCC	Mm00436615_m1
IL-1β	NM_008361.3	TCCTTGTGCAAGTGTCTGAAGCAGC	Mm01336189_m1
IL-6	NM_031168.1	ATGAGAAAAGAGTTGTGCAATGGCA	Mm00446190_m1
IL-10	NM_010548.2	GAAGACTTTCTTTCAAACAAAGGAC	Mm00439614_m1
IL-22	NM_016971.2	TGGCCAAGGAGGCCAGCCTTGCAGA	Mm00444241_m1
PGC-1α	NM_008904.1	CGCAACATGCTCAAGCCAAACCAAC	Mm00447183_m1
PPAR-γ	NM_0011273330.1	ATGCTGTTATGGGTGAAACTCTGG	Mm01184322_m1
TLR4	NM_021297.2	CCCTGCATAGAGGTAGTTCCTAATA	Mm00445273_m1
TNFα	NM_013693.2	CCCTCACACTCAGATCATCTTCTCA	Mm00443259_g1
Xbp1	NM_001271730.1	AGCGCTGCGGAGGAAACTGAAAAAC	Mm00457357_m1

ATGL: adipose triglyceride lipase, CPT: carnitine palmitoyl transferase, DDti3: DNA damage-inducible transcript 3, FASN: Fatty acid synthase, FOXO: Forkhead box protein, Glut: Glucose transporter, IL: interleukin, PGC: Peroxisome proliferator-activated receptor gamma coactivator, PPAR: Peroxisome proliferator-activated receptor, TLR: Toll-like receptor, TNF: tumour necrosis factor, Xbp: X-box binding protein.

6.2.5. ELISA

NEFA, TG and insulin concentrations in the plasma were measured using HR Series NEFA-HR kit (WAKO, Osaka, Japan), in-house TG assay (TG reagent, Roche Diagnostics, Mannheim, Germany) and Insulin ELISA Kit (Abnova, Taipei, Taiwan) respectively as described in details in Chapter 2.

6.2.6. Statistical analysis

The differences between the groups were analysed using one-way ANOVA followed by Tukey's *post hoc* tests to compare the differences between the Sham, SE and SE+LC groups (Prism 6, Graphpad CA, USA). The results are expressed as mean ± SEM. $P<0.05$ is considered significant.

6.3. Results

6.3.1. Body and liver weights of the females mice offspring

At P20 (weaning age), maternal SE did not significantly affect body weight and liver weight of the female offspring; however it significantly increased the percentage of liver weight/body weight and blood glucose level ($p<0.05$, Table 6.2) at this age. There were no changes in these parameters in the SE+LC offspring compared to the Sham offspring and SE offspring at P20.

At W13, body weight, liver weight, liver as percentage of body weight and blood glucose level in the SE offspring were similar as those in the Sham offspring (Table 6.2). However, maternal LC supplementation decreased liver/body weight% ($p<0.05$).

Table 6.2: Body and liver weights of the female offspring

P 20	**Sham**	**SE**	**SE + LC**
Body weight (g)	10.4 ± 0.54	9.59 ±0.18	9.45 ±0.28
Liver weight (g)	0.45 ± 0.03	0.45 ±0.01	0.43 ±0.02
Liver/Body (%)	4.27 ± 0.08	4.56 ±0.08*	4.44 ±0.08
Glucose (mM)	10.8 ± 0.4	12.6 ±0.4*	11.8 ±0.5
Week 13	**Sham**	**SE**	**SE + LC**
Body weight (g)	21.7 ± 0.3	20.9 ± 0.3	21.0 ± 0.2
Liver weight (g)	1.00 ± 0.03	0.96 ± 0.03	0.90 ± 0.02
Liver/Body (%)	4.54 ± 0.06	4.47 ± 0.5	4.29 ± 0.07$^{\#}$
Glucose (mM)	10.5 ± 0.4	10.5 ± 0.3	11.2 ± 0.2

Results are expressed as Mean ± SEM. n=8-10. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE.

6.3.2. IPGTT

During IPGTT, the baseline fasting glucose levels were not different between the groups. However, SE offspring had highest blood glucose levels at 15, 30 and 90 minutes post glucose injection ($P < 0.5$, Figure 6.1A), and highest AUC value ($P < 0.05$, Figure 6.1B). Blood glucose levels in the SE+LC offspring during IPGTT were similar as the Sham group, and same applies to the AUC value (Figure 6.1C). At 90 minutes post glucose injection, blood glucose levels in the SE+LC offspring were significantly lower than the SE offspring. No difference in non-fasting plasma insulin levels was found among the three groups at W13 (Figure 6.1C).

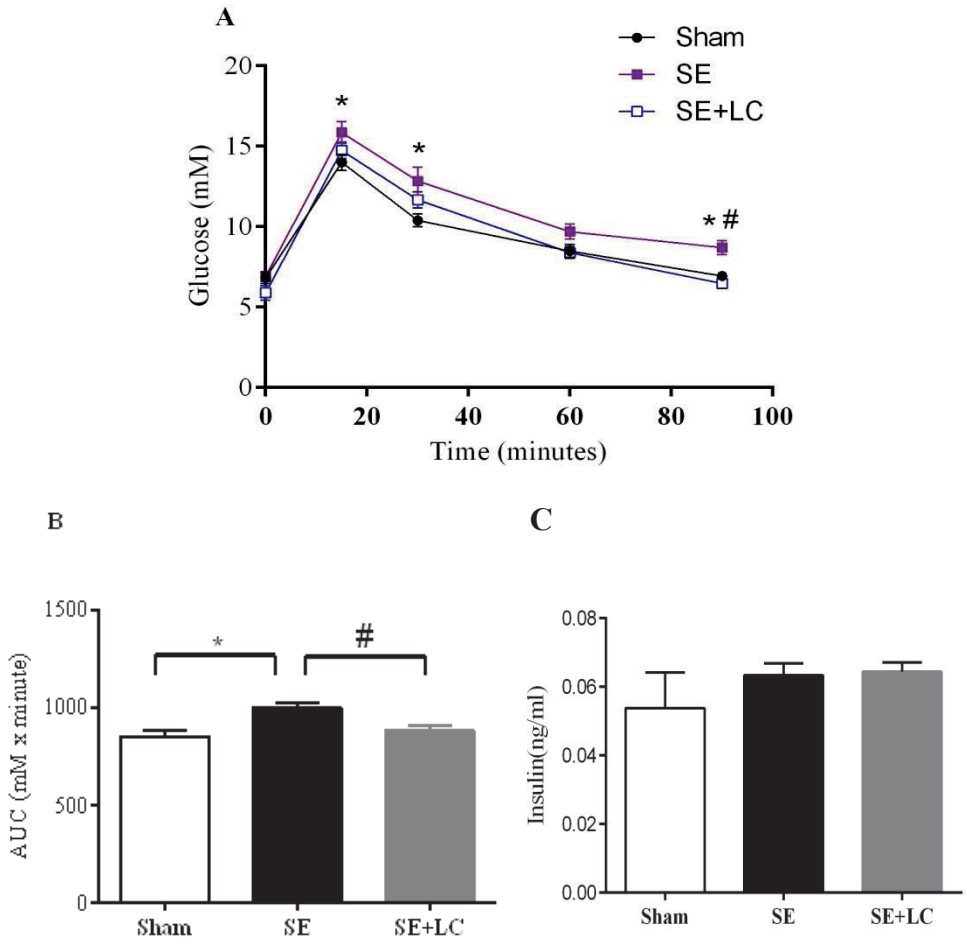


Figure 6.1: IPGTT and plasma insulin level in the female offspring mice**.**

(A) Blood glucose level changes during the IPGTT. (B) AUC of (A) at W12. (C) Plasma insulin level in the female offspring at W13. Results are expressed as Mean ± SEM. n = 6-10. * $P < 0.05$ SE vs Sham, # $P < 0.05$ SE+LC vs SE.

6.3.3. mRNA expression of the Inflammatory markers in the liver

At P20, no differences were observed in the liver mRNA expression of inflammatory markers including IL-1β (Figure 6.2A), IL-10 (Figure 6.2C), TNF-α (Figure 6.2E) and TLR4 (Figure 6.2F). However, IL-6 mRNA expression was upregulated by 38% (Figure 6.2B), and IL-22 mRNA expression was reduced by 64% (Figure 6.2D), although without statistical significance. The transcription factor X-box binding protein (Xbp) 1, which is involved in the regulation of immune cells function and cellular stress, was reduced by 68% by maternal SE without statistical significance (Figure 6.2G). Maternal LC supplementation seems to have no significant effect on the hepatic mRNA expression of these markers in offspring at this age.

At W13, hepatic mRNA expression of IL-1β (Figure 6.3A) and TNF-α (Figure 6.3E) was significantly upregulated, while the anti-inflammatory marker IL-10 (Figure 6.3C) was downregulated in the SE offspring compared to the Sham offspring ($p<0.05$). IL-6 mRNA expression was increased by 64% in the liver of the SE offspring compared to the control offspring without statistical significance (Figure 6.3B). No changes were observed in the liver IL-22 (Figure 6.3D), TLR4 (Figure 6.3F) and Xbp1 (Figure 6.3G) mRNA expression between the SE and control offspring at W13. Maternal LC supplementation normalized liver TNF-α mRNA expression in the SE offspring ($p<0.05$, Figure 6.3E). In addition, there was a trend for maternal LC supplementation to improve liver mRNA expression of IL-1β and IL-10 in the SE offspring without statistical significance.

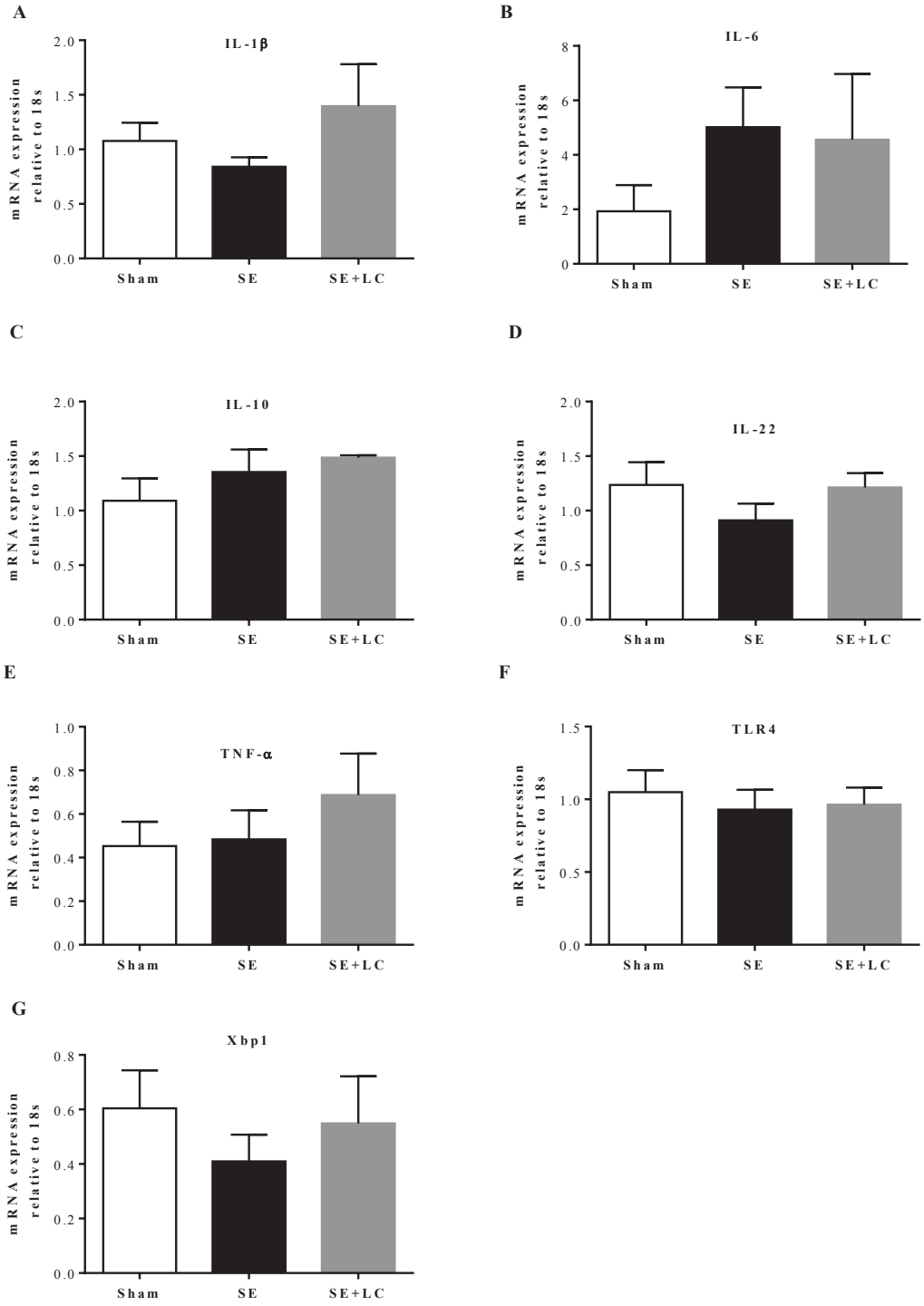


Figure 6.2: Hepatic mRNA expression of inflammatory markers of IL-1β (A), IL-6 (B), IL-10 (C), IL-22 (D), TNF-α (E), TLR4 (F) and Xbp1 (G) at P20. Results are expressed as Mean ± SEM. n=6.

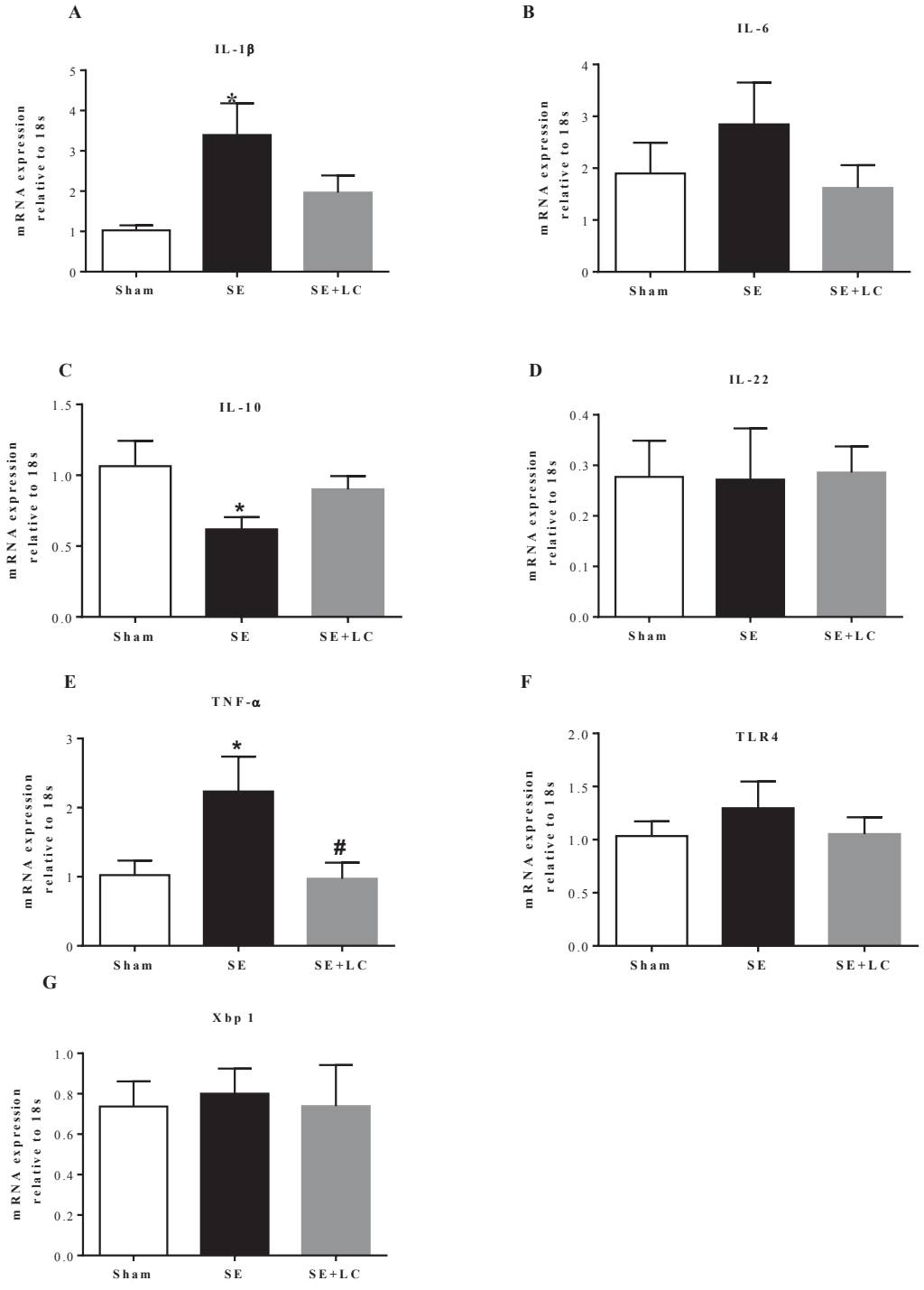


Figure 6.3: Hepatic mRNA expression of inflammatory markers of IL-1β (A), IL-6 (B), IL-10 (C), IL-22 (D), TNF-α (E), TLR4 (F) and Xbp1 (G) at W13. Results are expressed as Mean ± SEM. n = 6. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE.

6.3.4. mRNA expression of Lipid metabolic markers in the liver

At P20, lipid metabolic marker ATGL was downregulated in the liver of the SE offspring compared to the Sham offspring ($p<0.5$, Figure 6.4A). No significant change was found in the mRNA expression of PGC1α, FASN, FOXO1 and Glut 4 among the three groups at P20 (Figure 6.4D, E, F and H respectively). CPT-1α (Figure 6.4B), PPAR-γ (Figure 6.4C) and DDit3 (Figure 6.4G) mRNA expression was reduced by 57%, 54% and 73% respectively in the liver from the SE female offspring compared to the Sham offspring although without significance. Maternal LC supplementation appeared to improved liver ATGL mRNA expression in the SE+LC offspring which was at a similar level as the Sham offspring (Figure 6.4A).

At W13, PPAR-γ (Figure 6.5C), PGC1α (Figure 6.5D), FASN (Figure 6.5E) and FOXO1 (Figure 6.5F) mRNA expression was increased by 67% , 67%, 64% and 79% respectively in the liver of the SE offspring compared to the Sham offspring, although without statistical significance. Moreover, there is also a trend of maternal LC supplementation to ameliorate the effects of maternal SE on PGC1α (Figure 6.5D), FASN (Figure 6.5E) and FOXO1 mRNA expression. Glut 4 mRNA expression was downregulated in the liver of the offspring from SE+LC dams compared to offspring from SE dams ($P < 0.05$, Figure 6.5H).

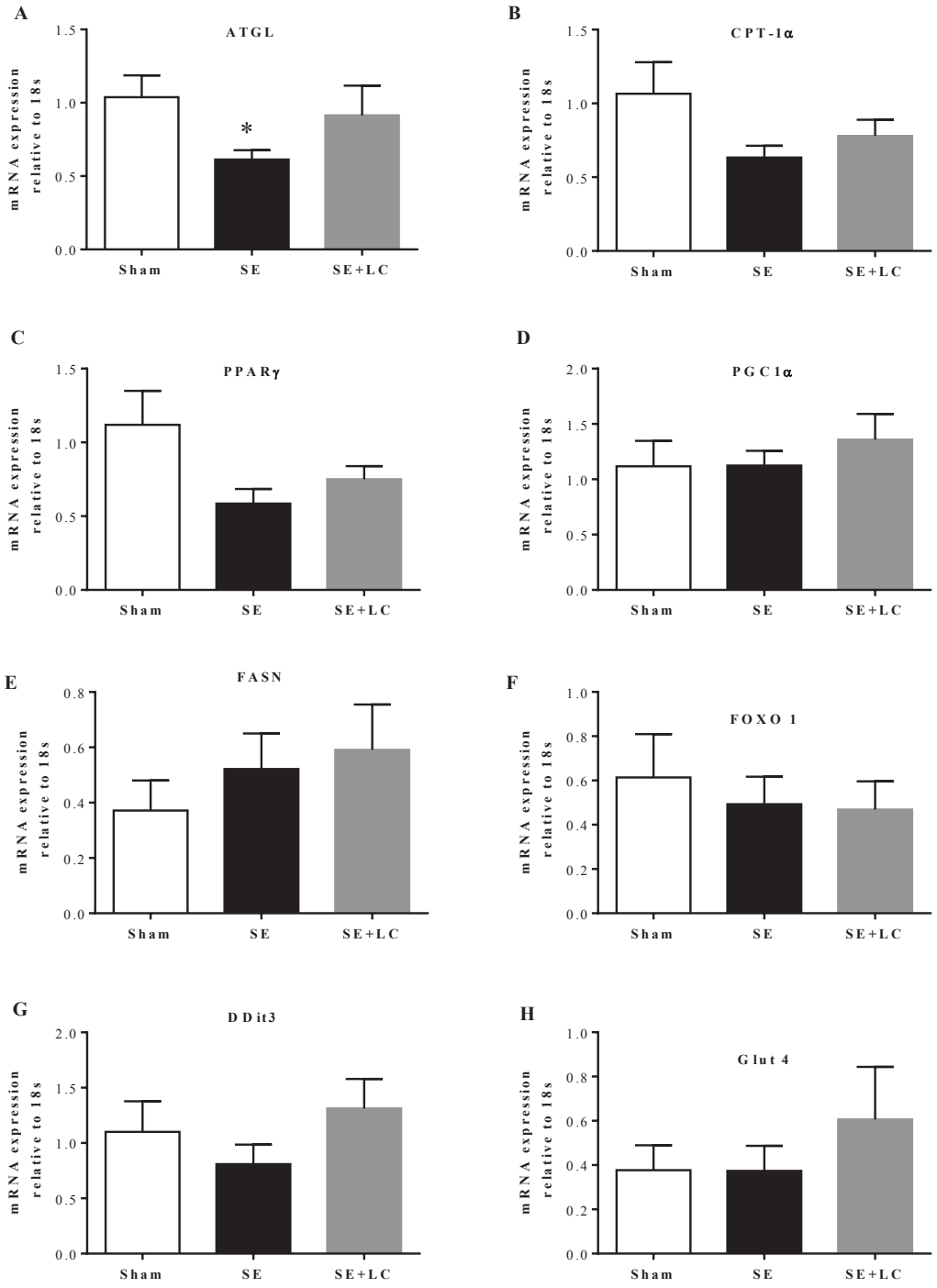


Figure 6.4: Hepatic mRNA expression of lipid metabolic markers of ATGL (A), CPT-1α (B), PPAR-γ (C), PGC1α (D), FAS (E), FOXO 1 (F), DDit3 (G) and Glut 4 (H) at P20. Results are Mean ± SEM. n = 6. * $P < 0.05$ SE vs Sham.

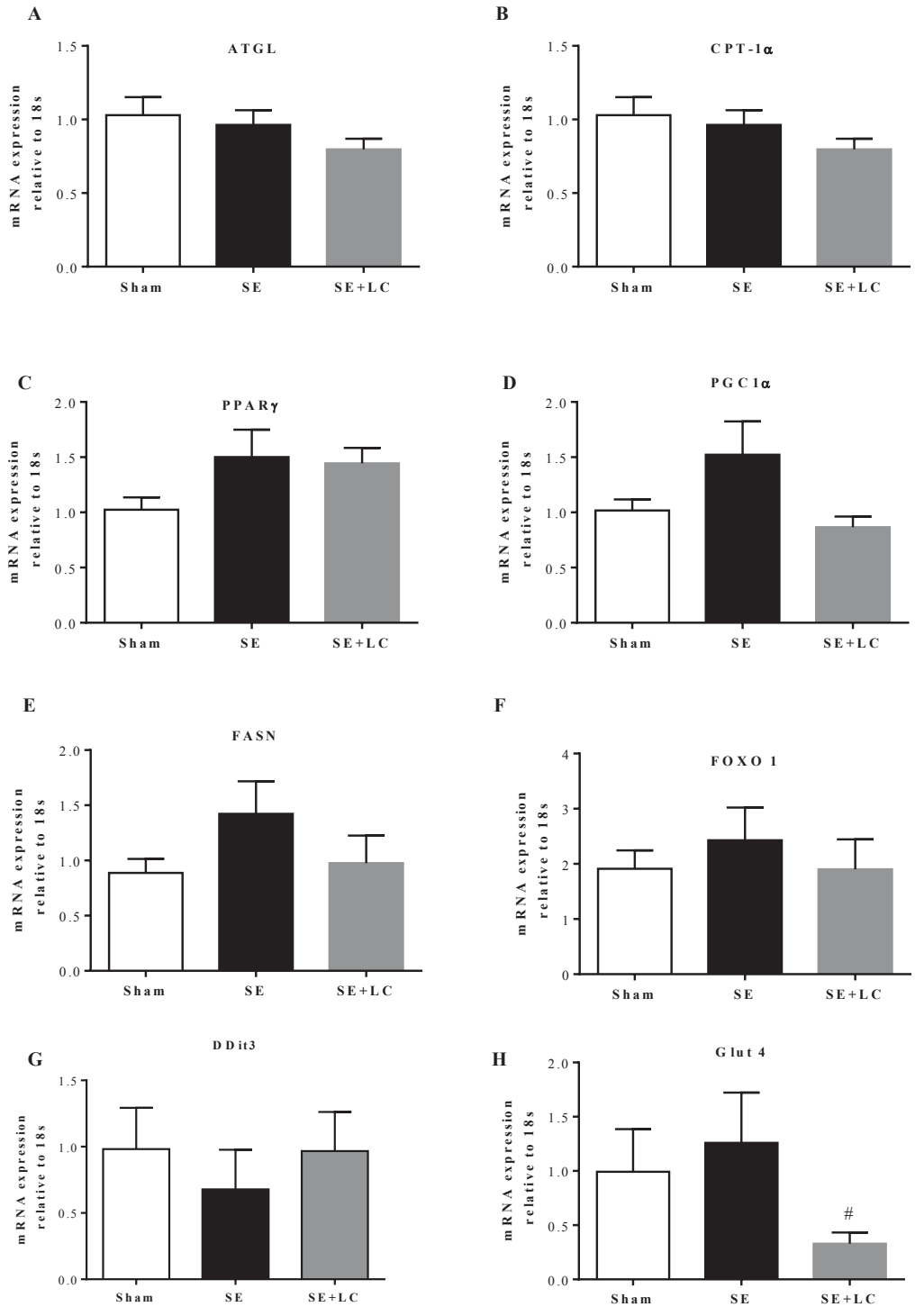


Figure 6.5: Hepatic mRNA expression of lipid metabolic markers of ATGL (A), CPT-1α (B), PPAR-γ (C), PGC1α (D), FAS (E), FOXO 1 (F), DDit3 (G) and Glut 4 (H) at W13. Results are Mean ± SEM. n = 6. Results are expressed as Mean ± SEM. n = 6. $^{\#}$ P < 0.05 SE+LC vs SE.

6.3.5. Plasma TG and NEFA levels

Plasma TG levels were significantly increased in the SE offspring compared to the Sham offspring at both P20 and W13. This increase in TG levels was normalised by maternal LC supplement in the SE+LC offspring at both P20 and W13 ($p<0.05$, Table 6.3). Plasma NEFA levels were not different among the three groups at any age.

Table 6.3: Plasma TG and NEFA concentrations in the female offspring at P20 and W13.

P 20	**Sham**	**SE**	**SE + LC**
Plasma NEFA (mM)	2.11 ± 0.3	1.95 ± 0.2	1.50 ± 0.1
Plasma TG (mM)	0.86 ± 0.2	1.90 ± 0.2*	0.66 ± 0.1#
W13	**Sham**	**SE**	**SE + LC**
Plasma NEFA (mM)	8.87 ± 0.5	8.14 ±0.7	7.18 ± 0.4
Plasma TG (mM)	1.43 ± 0.1	2.34 ± 0.1*	1.43 ± 0.2#

Results are expressed as Mean ± SEM. n = 6. * P < 0.05 SE vs Sham, # P < 0.05 SE+LC vs SE.

6.4. Discussion

The main finding in this study is that maternal LC supplementation during gestation and lactation ameliorates blood glucose level, glucose intolerance and hyperlipidaemia induced by maternal SE in the female offspring. This may be linked to improved metabolic markers in the liver.

Previously, we have shown that the female offspring are protected from changes in the body weight and kidney weight due to maternal SE at P20. This study showed an increase in the percentage of liver weight/body weight at weaning in the SE offspring compared with the Sham group. Ma et al. have previously demonstrated that maternal nicotine injection in female rats had no effect on the offspring liver weight (Ma et al. 2014). Increased liver to body weight in the current study is maybe due to postnatal catch-up growth commonly seen in the situation of maternal food restriction. This highlights the difference in the responses to nicotine alone from that to the cigarette smoke which contains more chemicals that can affect fetal growth. Maternal LC supplementation during gestation and lactation ameliorated the effect of SE on the offspring liver weight.

Blood glucose level was also increased in the SE offspring compared to the Sham offspring at P20 and LC supplementation was shown to ameliorate this increase. At W13, non-fasting blood glucose level was similar among the three groups. This is in contrast of the IPGTT results, where SE offspring had developed significant glucose intolerance whereas the SE+LC had normalised blood glucose clearance. This might indicate that non-fasting blood glucose level does not reflect the glucose metabolic status of the physiological system.

In humans, dose-dependent association between prenatal and postnatal SE and the development of insulin resistance, a risk factor for type 2 diabetes, has been reported in 10 year old children (Thiering et al. 2011). Neonatal nicotine exposure during lactation caused insulin resistance and metabolic syndrome at adulthood in rats (de Oliveira et al. 2010). In our previous studies, we have reported that maternal SE induced glucose intolerance in both male and female mice offspring (Al-Odat et al. 2014; Chen et al. 2011). In this study, plasma insulin level in the SE offspring was not different from the

Sham offspring at W13, however reduced tissue response cannot be excluded. Further measures on tissue insulin receptor downstream signalling are needed.

Exposure to cigarette smoke during pregnancy can lead to inflammatory response in the liver of the neonates (Diniz et al. 2013). The association between insulin resistance, hyperglycemia and increased inflammation in the liver has also been reported previously (Turpin et al. 2011) due to increased macrophage infiltration and its production of pro-inflammatory cytokine such as IL-1β, IL-6 and TNF-α (de Luca & Olefsky 2008; Fujimoto et al. 2010). IL-6 is associated with insulin resistance and impaired glucose tolerance, which may be mediated through its inhibitory effect on the transcription of Glut4 and PPAR-γ (Rotter, Nagaev & Smith 2003). Our results showed upregulation of IL-6 mRNA expression at P20 in the face of reduced PPAR-γ mRNA expression which may lead to the development of insulin resistance later on.

It has been reported that IL-22 can alleviate metabolic disorders such as improving insulin sensitivity, regulate lipid metabolism, decrease chronic inflammation in the liver and restore glucose intolerance and insulin resistance in obese mice (Wang et al. 2014). We found that mRNA expression of IL-22 was reduced due to maternal SE suggesting increased inflammation in the offspring from SE dams at P20 which may contribute to their metabolic disorders (Wang et al. 2014).

Anti-inflammatory cytokine IL-10 acts on macrophages to lower the pro-inflammatory cytokines such as TNF-α and IFN-γ (Pachkoria et al. 2008). Serum IL-10 is reduced in the patients with insulin resistance (de Luca & Olefsky 2008). The reduced IL-10 mRNA expression at W13 may suggest an attenuation of anti-inflammatory response due to maternal SE, in line with the upregulation of pro-inflammatory marker TNF-α and IL-1β in the SE offspring at W13. Increased mRNA expression of TNF-α at W13 is associated with significant glucose intolerance in the SE offspring. This is consistent with the role of TNF-α in insulin resistance and its related glucose metabolic disorders suggested by the literature (Moller 2000). Different mechanisms have been proposed, including the downregulation of different genes essential for glucose uptake such as Glut4, the direct effect on insulin signalling, the stimulation of lipolysis that elevates FFA, as well as the suppression on PPAR-γ expression and function which can in turn affect insulin sensitivity (Moller 2000). TNF-α can also increase plasma TG leading to insulin resistance (Rotter, Nagaev & Smith 2003). Moreover, IL-1β is suggested to

inhibit the transcription factor PPAR-γ (Tack et al. 2012). PPAR-γ has been shown to be involved in glucose homeostasis and insulin sensitivity in a tissue- and age dependent manner by controlling different genes involved in glucose homeostasis such as Glut4 (Ahmadian et al. 2013; Reza López et al. 2013). Therefore, PPAR-γ is a therapeutic target for insulin resistance (Barish, Narkar & Evans 2006). Here maternal SE downregulated hepatic PPAR-γ mRNA expression at early postnatal age, which may be an early event to predict later glucose metabolic disorders (de Luca & Olefsky 2008); whereas it was upregulated at adulthood suggesting an adaptive response to glucose intolerance at this age (Reza López et al. 2013). Xbp1 has a regulatory role in inflammatory response that leads to the activation of IL-1β (Cho et al. 2012). TLR4 activates the transcription factor Xbp1to regulate inflammation (Martinon et al. 2010). This network seems to be affected by maternal SE in the weaning offspring, where reduced mRNA expression of Xbp1 is associated with reduced mRNA expression of IL-1β and IL-22 at P20. One explanation may be due to high level of inflammatory factors delivered by breastmilk from the mother, which can suppress the innate immune response. As such, these changes were normalised at W13 with similar Xbp1 between the Sham and SE offspring.

Hepatic glucose production is increased in the patients with diabetes (Nakae et al. 2001). It has been reported that FOXO1 has a regulatory role in promoting hepatic glucose production (Matsumoto et al. 2006; Matsumoto et al. 2007). Our results showed a slight increase in FOXO1 mRNA expression at W13 in the female offspring which may play a role in their glucose intolerance.

TG and NEFA are the keys blood lipids. Smoking is a contributor to hypertriglyceridemia in humans (Craig, Palomaki & Haddow 1989). Perinatal SE alters plasma lipid profile in the adult offspring that could increase the risk of cardiovascular disease later in life (Ng et al. 2009). In agreement with this, our results showed that SE during pregnancy and lactation led to increased plasma TG in female offspring. This was also consistent with previous studies in humans and rodent (Ma et al. 2014; Power, Atherton & Thomas 2010). The exact mechanism for the effect of maternal SE on the increased serum TG level in the offspring is still unclear. One of the suggested mechanisms is attributed to the downregulation of enzymes and proteins involved in lipid metabolism (Kabagambe et al. 2009) that was observed at P20 in our study. One of

such enzymes is CPT-1α that catalases the transportation of the long-chain fatty acid into the mitochondria for oxidation. Our results showed a downregulation of liver CPT-1α mRNA expression in the offspring from the SE dams at P20. This downregulation may be responsible for the hyperlipidaemia observed in this study due to a decrease in fatty acid oxidation in the mitochondria (Kralisch et al. 2005). This is also supported by the downregulation of enzyme ATGL and transcription factor DDit3 mRNA expression in the liver suggesting impairment in mitochondrial β-oxidation in the hepatocytes. Our results also showed an increase in PGC1α mRNA expression at W13, which as expected led to increase PPAR-γ at the same age. This suggests an impairment of mitochondria biogenesis and mitochondrial dysfunction which in turn can negatively affect mitochondrial β-oxidation (Kalfalah et al. 2014). Another suggested mechanism underlying increased serum TG level by maternal SE in the offspring is increased *de novo* TG synthesis by FASN , which was upregulated in the SE offspring at 13 weeks as well (Ma et al. 2014).

Exogenous LC supplement was shown to have a protective role in rodent models against chronic kidney failure, heart and lipid peroxidation in atherosclerotic rats, hyperammonemia, adjuvant arthritis and liver damage induced by carbon tetrachloride (Dayanandan, Kumar & Panneerselvam 2001; Demirdag et al. 2004; O'Connor, Costell & Grisolía 1984; Sener et al. 2004; Tastekin et al. 2007). Our data showed that dietary LC supplementation in the SE mother significantly improved glucose intolerance in the female offspring from the SE mothers, which is consistent with our previous observation in the male offspring (Nguyen et al. 2015). Interestingly, Glut4 mRNA expression was decreased at W13 due to LC treatment which may be due to a reduction in PGC1-α mRNA expression observed at this age (Liang & Ward 2006). Dietary LC supplementation was reported to increase cellular glucose uptake and its oxidation (Schmengler et al. 2013). This may be by improving glucose disposal and altering expression of genes involved in glucose uptake signalling cascade such as the inhibitory effect of carnitine on gluconeogenic enzyme activity (Mingrone et al. 1999). In humans, dietary LC supplementation has been shown to improve hepatic inflammation and fibrosis by decreasing blood TNF-α and improving lipid profile and insulin resistance (Malaguarnera et al. 2010). In this regards, in a rat model of alcohol-induced liver disorder, it was also shown that dietary LC supplementation alleviated liver damage by decreasing TNF-α release by the Kupffer cells (Bykov, Järveläinen & Lindros 2003). In

consistence with this study, our results showed that maternal LC supplementation during gestation and lactation normalized mRNA expression of IL-1β, IL-6 and TNF-α as well as TLR4 in the liver of the SE+LC offspring at W13. This may be due to the anti-inflammatory property of LC (Miguel-Carrasco et al. 2008). This was also supported by normalization of the anti-inflammatory marker, IL-10 mRNA expression in the liver of the offspring at this age. Subsequently, liver insulin sensitivity can be improved.

In this study, maternal LC supplementation normalised blood TG level in the female SE offspring at P20 and W13. In agreement with this finding, other studies have demonstrated that LC supplementation reduced blood TG levels. For example, LC supplementation was shown to normalise increased serum TG level induced by high fat diet consumption in rats by reducing TG synthesis and/or release by the liver (Amin & Nagy 2009). Wu et al. also showed that LC supplementation decreased serum TG levels by improving mitochondrial fat β-oxidation (Wu et al. 2015). Furthermore, LC was shown to lower liver and plasma TG concentrations in ovariectomized aging rats (Clark et al. 2007). The mechanism of LC targeting dyslipidemia is through increasing mitochondrial transport of FFA and reduces FFA availability for TG synthesis (Hurot et al. 2002). To this regards, ATGL is a key regulator of TG hydrolysis in many metabolic tissues such as fat and liver (Turpin et al. 2011). One possible mechanism to explain reduced serum TG in our study may lie in the 60% increase in hepatic ATGL mRNA expression in the liver at P20 by maternal LC supplementation, which could increase the breakdown of TG influx into the hepatocytes (Pawlak, Lefebvre & Staels 2015). Furthermore, this was evident in our results that maternal LC supplementation during gestation and lactation normalized liver mRNA expression of key lipid metabolic markers such as PGC1α, FASN, FOXO1 and DDit3 in the offspring at W13. However, another study using LC supplementation during gestation and lactation failed to find changes in hepatic and plasma lipids in the offspring of sows (Birkenfeld et al. 2006). This may be due to that the dams in that study were fed nutritionally adequate diet. It may be also that the sows in that study have not been exposed to any kind of insult that can disturb the metabolic profile in the offspring; whereas in our study maternal SE increased inflammatory response and dyslipidemia in the offspring.

One of the limitations of this study is that only mRNA level of inflammatory and lipid metabolic markers were measured. So assessing protein levels of these markers is necessary to confirm these findings in the future.

In summary, maternal SE led to glucose intolerance and hyperlipidemia, maybe due to disorders of hepatic inflammatory and lipid metabolic marker in the female offspring. Dietary LC supplementation normalises blood lipid and glucose metabolism due to the inhibition of liver inflammation. This suggests that maternal LC supplementation can protect the offspring of smoking mother from metabolic disorders.

Chapter Seven

7

General Discussion and Perspectives

7.1. General discussion

The understanding of the pathogenicity of kidney disease is changing. Glomerulonephritis was thought to be the main cause of kidney disease in the past. However, hypertension and diabetes are the leading causes of kidney disease nowadays (Zhang & Rothenbacher 2008).

The developmental origin of adulthood disease attracts increased attention in recent years, which suggests the importance of intrauterine environmental factors and insults in the programming of adulthood diseases in offspring. As such, human and animal studies have linked maternal SE during critical time of fetal development to the health outcomes of the offspring, such as sudden infant death, congenital heart defects, behavioural problems, neurocognitive deficits and obesity (Cornelius et al. 2011; DiFranza, Aligne & Weitzman 2004; Mamun et al. 2012; Sullivan et al. 2015; Zhang & Wang 2013; Zugno et al. 2013). Maternal SE can lead to IUGR due to the effect of chemicals in the cigarette smoke on fetal-placental unit, hence disturbing fetal development. It has been reported that IUGR can lead to smaller kidney, where there is less nephron reservoir (Silver et al. 2003). This can in turn predispose the offspring to adulthood kidney disorders and CKD (Silver et al. 2003). However, the effect of maternal SE on renal development and renal function in the offspring, as well as offspring susceptibility to CKD in adulthood life has not been fully elucidated; neither were the underlying mechanisms. Therefore, we firstly examined the impact of maternal SE prior to, during gestation and lactation on renal developmental parameters, including nephron number and size, the expression of different growth and transcription factors, renal function, pro-inflammatory and injury markers in both genders at birth, weaning and adulthood.

Collectively, we showed that continuous maternal cigarette SE prior to gestation, during gestation and lactation leads to fetal kidney underdevelopment in male offspring with renal dysfunction at adulthood. Renal structural changes were also observed in the SE offspring including reduced nephron number and increased glomerular size at birth,

weaning and adulthood. In addition, mRNA and protein levels of renal growth and transcription factors involved in nephrogenesis including UB outgrowth and MM transformation to epithelial cells were altered by maternal SE, such as FGF2, GDNF and Pax2, which may contribute to renal underdevelopment in the SE offspring. As a result, deterioration in kidney function at adulthood was observed in the offspring of the SE dams, reflected by increase in urinary albumin/creatinine ratio indicating proteins leakage into the urine, and increased renal inflammatory responses. However, renal structural changes were subtle. Furthermore, maternal SE led to glucose intolerance in the male offspring at adulthood suggesting that they are at high risk of developing type 2 diabetes in the future, which is also a comorbidity of CKD. The finding in the male offspring suggests they are prone to the development of CKD in adulthood. This may be more so when additional insult is imposed on such offspring, such as diabetes mellitus, which can be future research direction on this topic.

Unlike the male offspring, female offspring are protected from renal developmental programming by maternal SE, which suggests the gender difference in the susceptibility to renal underdevelopment, renal dysfunction and CKD due to maternal SE. There was no change in the percentage of kidney weight in the female offspring at any time in this study. Female SE offspring did not have the same renal structural changes (glomerular number and size) as the male offspring. This may be due to preserved renal expression of early growth and transcription factors involved in nephrogenesis observed in the female SE offspring. As such, renal function and inflammatory response were also preserved in the female offspring of the SE mothers. This gender difference is likely due to the impact of sex hormones. It is believed that ovarian hormones are responsible for such renal protection in females (Dubey et al. 2002). For example, oestrogen has been shown to have a protective role in renal pathophysiology through different mechanisms, such as regulating renal homeostasis of protons and bicarbonate, as well as controlling different genes involved in renal development and function such as eNOS (Sabolić et al. 2007). As such, the renal disorders in the females are less severe than those in the males (Eriksen & Ingebretsen 2006; Kummer et al. 2012).

The RAS has also been suggested to play an important in fetal nephrogenesis and gender-specific developmental programming of kidney disease (Moritz et al. 2010; Ojeda, Intapad & Alexander 2014). During kidney development, angiotensin II acts on AT_1 receptor to mediate the growth and proliferation of renal tubules and branching morphogenesis. The AT_2 receptors in fetal kidney act to mediate apoptosis in the renomedullary interstitial cells. Reduced RAS activity during nephrogenesis has been shown to lead to reduced nephron number and decreased kidney size in the newborn (Moritz et al. 2010; Ojeda, Intapad & Alexander 2014).

Placenta is critical for fetal development including nutrients and gases transportation and hormones production (Jansson & Powell 2013). Thus abnormal placental function can play an important role in programming adulthood disorders (Burton & Fowden 2012). Maternal SE during pregnancy is associated with reduced placental functions and restriction in utero-placental blood flow leading to IUGR and its associated postnatal catch up growth and adulthood diseases (Jauniaux & Burton 2007). Moreover, it has been reported that abnormal placental shape and dimensions due to intrauterine environmental perturbations are also linked to adulthood disease (Jansson & Powell 2013). In addition, it has been suggested that placental dysfunction is responsible for higher risk of adulthood diseases in the males than the females (Dorey et al. 2014; Rosenfeld 2015). Placental insufficiency is the primary cause that leads to placenta dysfunction, which in turn reduce nephron endowment in the offspring by affecting the final branching of the ureteric tree (Dorey et al. 2014). Sex-specific placental impact on adulthood disease may also be due to structural differences (Dorey et al. 2014). However, there is a lack of evidence that suggests structural differences between male and female placentae, but the genes and proteins expression can be different (Clifton 2010). For example, some placental transcripts including human chorionic gonadotropin and interferon-τ are expressed in a sexually dimorphic manner, which are more abundant in the females. There are also sex-dependent differences in the global transcriptomic profile in the human placenta with female having more autosomal genes including immune-regulating genes such as IL-1 receptor-like 1 than the male, which enhance the female's immunity against infections (Rosenfeld 2015; Sood et al. 2006).

Although the female offspring are protected from renal pathophysiology due to maternal SE, they still developed glucose intolerance at adulthood, which suggests that they are still vulnerable to developing other metabolic disorders if not CKD. This may be due to the impairment of glucose homeostasis in the liver by maternal SE. Liver has been considered as a critical organ to regulate glucose metabolism and blood glucose level (Liu, Yuan, et al. 2012; Saltiel & Kahn 2001). To this respect, we found that maternal SE increased the percentage of liver weight in the face of glucose intolerance at adulthood in female offspring. Inflammation and hyperlipidemia were also observed at both weaning and adulthood, with decreased liver ATGL mRNA expression. ATGL plays an important role in lipolysis (Gaidhu et al. 2010), and ATGL deficiency has been found to impair insulin signalling in mice (Kienesberger et al. 2009). This downregulation by maternal SE is likely to lead to both dyslipidemia and glucose intolerance.

We have recently demonstrated that maternal SE increased oxidative stress in the kidney of the male offspring at birth and adulthood (Nguyen et al. 2015). This suggests that approaches to reduce oxidative stress may improve the kidney development and health outcome in the SE offspring. LC is an antioxidant that is essential for long chain fatty acid transportation across the mitochondrial membrane for β-oxidation. LC supplementation has been shown to have protective effect in different diseases and injury due to its anti-oxidative, anti-inflammatory and anti-apoptotic effects (Chao et al. 2011; Hua et al. 2014; Lee et al. 2015). Data in this thesis showed that maternal dietary LC supplementation during gestation and lactation improved low birth weight and small kidney weight in the male offspring at P1. It also ameliorated the reduction in glomerular number in those offspring at P1, P20 and W13, as well as abnormal glomerular size at P20 and at W13. Furthermore, maternal dietary LC supplementation normalized renal expression of several growth and development factors involved in renal genesis and development at P1 such as FGF7, Pax2, WNT11 and WT1, as well as FGF2, FGF10 and WNT4 at P20 contributing to normalised renal development. In addition, maternal dietary LC supplementation ameliorated inflammatory marker and fibrotic markers in the SE offspring at W13, resulting in improved renal function in the

male SE offspring at adulthood. Additionally, maternal dietary LC supplementation improved glucose metabolism in the male offspring at adulthood as well.

Interestingly, maternal dietary LC supplementation also improved hyperlipidemia and normalized hepatic inflammatory response by maternal SE in the female offspring, which may contribute to their improved glucose metabolism.

As the prevalence of maternal smoking is still a public health problem, the best recommendation for women is to quit smoking prior to pregnancy. On the other hand, LC supplementation during gestation and lactation may be a plausible approach to ameliorate maternal smoking related kidney disease and glucose metabolic disorders in the offspring.

7.2. Perspectives

The novel findings of this study present many new avenues for further investigation. For example, future studies can determine the epigenetic effects of maternal SE on renal development in the fetus; while epigenetics changes of the unfertilised eggs prior to gestation also needs further investigation in order to understand the impact of smoking prior to gestation to pre-determine renal pathophysiology in offspring. Epigenetic modifications have been shown to modulate the transcription of genes involved in the progression of CKD. Elucidation of epigenetic processes may facilitate perinatal identification of individuals at risk to enable early intervention to reduce such risk. Furthermore, the effect of maternal SE on the second generation of the offspring also needs to be followed up in order to understand the effect of maternal SE on the future generations. Whether LC supplementation prior to pregnancy can reverse or ameliorate the SE-induced epigenetic changes in the offspring require further investigation.

The effect of maternal SE on inflammatory response, oxidative stress as well as mitochondrial integrity in the pancreatic β cells in offspring requires further investigation. As there is gender difference in placenta morphology, future study can investigate the effect of maternal SE on placental changes in both male and female, as one of the underlying mechanism for gender difference in renal under development in the fetus. Changes in fetal renal RAS activity in the programming of kidney disease by maternal SE, as well as gender difference also warrens future investigation.

The therapeutic potential of BMP7 in treating CKD is exciting. BMP7 is an essential renal morphogen that can reverse TGF-β1-induced epithelial-to-mesenchymal transition, which is characterized by accumulation of myofibroblast and subsequent tubular atrophy leading to renal fibrosis (Zeisberg et al. 2003). Recombinant human BMP7 has been shown have a therapeutic potential in treating kidney interstitial fibrosis and chronic renal injury in humans (Davies, Lund & Hruska 2003). The mechanism is through the preservation of epithelial cells phenotype, inhibition of epithelial-mesenchymal transdifferentiation and inhibition of renal injury-induced epithelial cell

apoptosis (Wang et al. 2003). However, further study is essential in order to determine its dosage and treatment interval to develop potential therapies for kidney disease.

The optimal dietary LC supplementation during pregnancy also requires further validation in humans. In addition, whether direct supplementation of LC into offspring from SE mothers provides full protection from developing CKD need further investigation in the future. Future studies can also validate the beneficial role of IL-22 in reducing chronic inflammation and alleviating metabolic syndromes as a potential therapeutic intervention, as suggested by a recent publication (Wang et al. 2014).

8. References

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