FOOD-CT-2005-007036

EARNEST

EARly Nutrition programming- long term follow up of Efficacy and Safety Trials and integrated epidemiological, genetic, animal, consumer and economic research

Instrument: Integrated Project

Thematic Priority 5.4.3.1: Food Quality and Safety

Final public report on activity 3.2

The impact and mechanism by which fat-rich diets cause adverse fetal programming

Period covered from 15.04.2005 to 14.10.2010

Start date of project: 15.04.2005 Duration: 5,5 Years

Organisation Name of Lead Contractor for this report: KCL

Abstract

Introduction An adverse nutritional environment in pregnancy may confer detrimental long-term consequences to the child. Maternal obesity presents a suboptimal environment to the developing child and has become more prevalent over recent decades, in parallel with the rise of the worldwide obesity epidemic. **Aim** The aim of this project was to develop a novel, rodent model of maternal diet-induced obesity and to use this to examine effects of maternal obesity on offspring cardiovascular and metabolic function in C57BL/6J mice, wistar rats, and apolipoprotein E knockout (apoE$^{-/-}$) mice. **Methods** Maternal obesity was induced by feeding a highly palatable diet rich in fat and simple sugars. Offspring cardiovascular function was examined using radiotelemetry and small vessel myography. Glucose tolerance and insulin resistance was assessed using glucose tolerance tests and euglycemic hyperinsulinemic clamp. Atherosclerosis was examined by histology and magnetic resonance imaging and mitochondrial function using enzyme assay and qPCR. **Results** Exposure to maternal diet-induced obesity during gestation and suckling produced a hyperphagic, metabolic syndrome-like phenotype in the offspring, characterised by obesity, hypertension and abnormal glucose and insulin homeostasis. A cross-fostering study to examine the relative contributions of the *in utero* and suckling periods to this phenotype revealed that cross-fostering itself produced profound effects on offspring cardiovascular and metabolic function. Nevertheless there was evidence that exposure to maternal obesity during the suckling period may be important in the development of hypertension and non-alcoholic fatty liver disease. No effects of maternal obesity on the development of atherosclerosis were observed. The offspring of the obese dams developed impaired mitochondrial function in muscle and heart. **Conclusions** This report provides supportive evidence for persistent effects of maternal obesity on offspring cardiovascular and metabolic function. All objectives were met.

Introduction

Obesity amongst pregnant women is highly prevalent worldwide presenting an increasing challenge to health care. The WHO Global Burden of Disease database identifies approx. 20% of women of reproductive age being obese (http://apps.who.int/bmi/index.jsp). Studies in pregnant women and their children have suggested that exposure to maternal obesity and associated metabolic dysfunction may increase the risk of metabolic syndrome in the offspring (Boney *et al* 2005, Catalano *et al* 2009, Hull *et al* 2008). Nevertheless, the mechanisms which may contribute to an association between maternal obesity and metabolic and cardiovascular complications in the offspring are unclear. Animal experiments offer great advantages, including controlled breeding conditions and relative short life-span. In this project we have establish a novel, rodent model of maternal diet-induced obesity,– standardised at two different units King's College London and Katholieke Universiteit Leuven. Our initial aim was to examine the effect of maternal obesity on offspring cardiovascular and metabolic function in C57BL/6J mice, Wistar rats, and apolipoprotein E knockout (apoE$^{-/-}$) mice rodent model. We investigated the maternal milieu in search for causative vectors of programming and offspring structure and gene expression in search for potential mechanisms. Cross-fostering was conducted in a separate study to determine the relative contribution of exposure to an altered environment *in utero* versus postnatal period in offspring phenotype. Apolipoprotein E knockout mice (apoE-/- mice), which are atherosclerotic prone, were applied to the model of maternal diet-induced obesity to examine if maternal obesity leads to accelerated development of atherosclerosis in the offspring of obese dams.

Methods

Maternal Obesity protocols

3.2.1, 3.2.2, 3.2.3 & 3.2.5 Protocol 1. Female C57BL/6J mice (Samuelsson et al 2008) or Wistar rats (Nivoit et al 2009) were fed either a standard chow diet (7% simple sugars, 3% fat [w/w] energy 3.5 kcal/g, n=20, Special diet services SDS,), or a highly palatable obesogenic diet (10% simple sugars, 20% animal lard [w/w], energy 4.5 kcal/g, n=30, SDS). The obesogenic diet was supplemented by *ad libitum* access to sweetened condensed milk (55% simple sugar, 8% fat [w/w], Nestle) with added micronutrient mineral mix, according to fig 1.

3.2.4 & 3.2.5 Protocol 2. Cross Fostering (breeding according to protocol 1). 48-72 hours after birth, litters were cross-fostered to a dam on either the same diet as their biological mother or a dam on the opposite diet, producing 4 experimental groups (Fig 2). Offspring were weaned to control diet and metabolic and cardiovascular phenotype investigated.

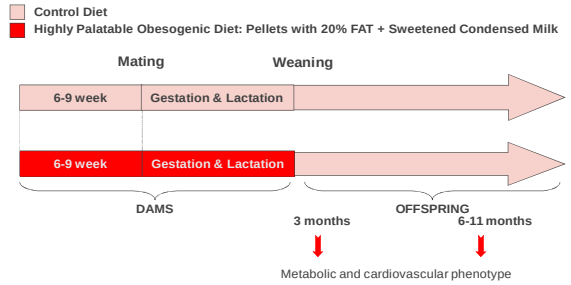


Figure1. Breeding protocol Female C57BL/6J mice, Wistar rats were fed either a control or a highly palatable diet (20% fat and sweetened condensed milk) 6-9 wks prior to mating. Offspring metabolic and cardiovascular phenotype were analysed at 3 and 6-11 M of age (Samuelsson *et al* 2008) (Nivoit *et al* 2009).

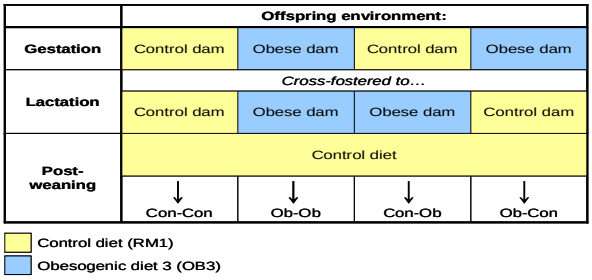


Figure2. Cross fostering protocol Offspring of obese dams cross fostered to control dams (Ob/Con), offspring of control dams cross fostered to obese dams (Con/Ob), offspring of control dams cross fostered to control dam (Con/Con) and offspring of obese dams cross fostered to obese dam

3.2.6 Protocol 3. apoE-/- (C57Bl/6J mice homozygous for the apoE gene) were breed at Charles River Laboratories according to protocol 1. After 11 weeks on the obesogenic diet (4g heavier, $P<0.05$) dams were mated and litters standardised and weaned. From 8 wks of age, mice were fed a 'Western' diet (829100, SDS) in order to accelerate the development of atherosclerosis (Fig 3).

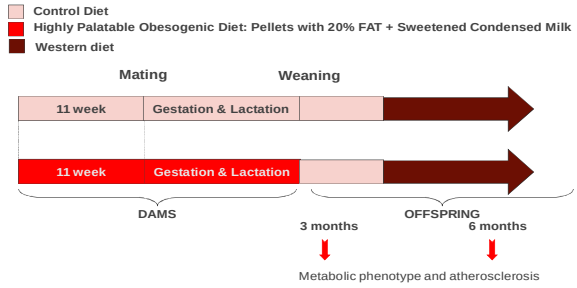


Figure3. ApoE-/- mice protocol Female ApoE-/- mice fed either a control (RM1) or a highly palatable (20% fat and sweetened condensed milk) diet 11 weeks prior to mating. Offspring are weaned to a control diet at 8 weeks of age mice were fed a western diet. Aspects of metabolic syndrome and atherosclerosis were investigated at 3 and 6 M of age.

Metabolic and Cardiovascular analysis (3.2.1-3.2.5)

BW and food intake were measured weekly. At weaning, milk samples were collected after oxytocin injection. Maternal milk and serum and offspring serum were analysed at the RIVM (Eugene Jansen, Bilthoven, NL). At 3 and 6 M of age cardiovascular function was assessed in mice offspring by remote radio-telemetry **(**TA11PA-C10, O.D 0.4 mm, DSI) and small vessel myography (MultiMyograph, Danish Myo Technology). Glucose tolerance test was performed in mice after ip. Glucose(1g/kg). Euglycemic –hyperinsulinemic clamp was carried out in rats according to Perrin et al (Perrin *et al* 2003) in awake and semi-restrained animals. Gene expression and mitochondrial function were determined by qPCR (Corbett Rotorgene ™ 6000). Adipose tissue, heart, liver and aorta were excised and fixed for standard histology preparations (Samuelsson *et al* 2008) (Oben *et al* 2010).

Results

Breeding protocol (Del. 57, Mil 25)

Maternal obesity protocol was established at the two units. In the first set of protocol 1, a high rate of cannibalism (50%) of the pups was observed in the obesogenic group. Mineral deficiency may be the origin of this and diet was therefore supplemented with mineral mix (AIN93G; SDS, UK). This reduced the rate of cannibalism to approximately 10%.

Maternal and Offspring phenotype (Del. 37, Mil 26)

Calorific intake and weight gain were significantly increased in mice (Fig 4A-B) and rats (Fig 5A-D) fed the OB diet compared with controls. At gestation day 18, obese mice were hyperinsulinemic and hyperleptinemic (Fig 4C) and at weaning obese dams demonstrated hyperleptinaemia, hyperinsulinemia, hyperglycemia, hypercholesterolemia (Fig 4D) with elevated milk triglycerides ($P<0.05$) and leptin ($P<0.05$). Offspring of obese dams (OffOb) were heavier and had at 3 month of age increased serum insulin and leptin levels compared with controls (Fig 6). At adulthood OffOb developed hypertension (Samuelsson et al 2008), endothelial dysfunction, insulin resistance (Nivoit *et al* 2009) (Samuelsson *et al* 2008), and non-alcoholic fatty liver disease (NAFLD) (Oben *et al* 2010).

Fig 4 MICE

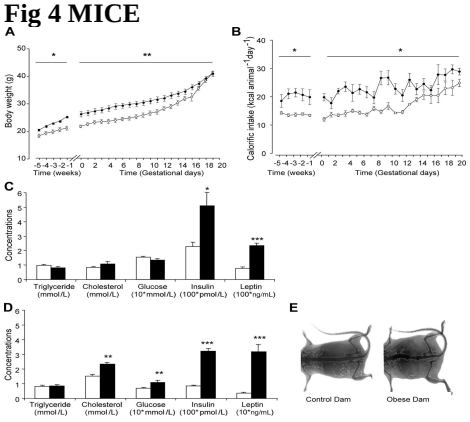


Fig 5 RATS

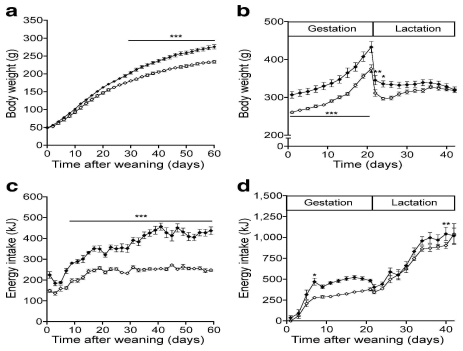
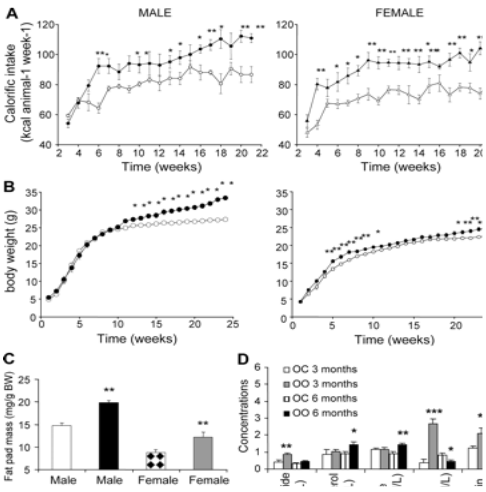


Figure 4-5. Body weight and calorific intake in dams fed either the control (open symbols, n=8) or an OB diet (closed symbols, n=8). 4C Maternal plasma at gestational day 18 (n=9) or at 4D weaning (n=8 to 12). 4E, DEXA scan of control (15% body fat) and obese dams (34% body fat) at weaning. ***$P<0.001$, **$P<0.01$, * $P<0.05$ vs control (Samuelsson *et al* 2008) (Nivoit *et al* 2009)



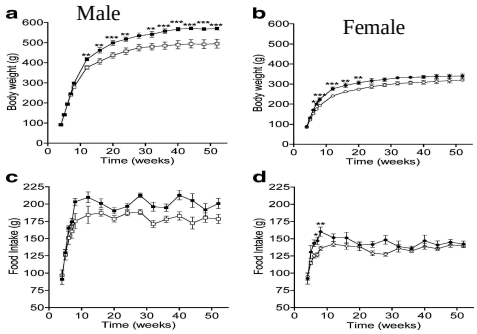


Figure 6. Calorific intake and body weight in male and female offspring of control (OC, open symbols) and obese dams (OO, closed symbols) from weaning (n=8 per group). C Offspring inguinal fat pad mass at 6 m of age and D serum analysis at 3 and 6 m of age. n=6 to 8 per group. ***$P<0.001$, **$P<0.01$, * $P<0.05$ vs OC. RM ANOVA t-test.(Samuelsson et al 2008)(Nivoit et al 2009)

Cross-fostering (Del. 33, 34 Mil 19)

Body weight and calorific intake was significantly increased in offspring of control dams lactating to obese dams (Con/Ob) compared with offspring of obese dams suckling on control dams (Ob/Con) (Fig 7A). Con/Ob had also increased central adiposity (P<0.05), with higher serum leptin (P<0.05). Systolic blood pressure and heart rate (Fig 8) were significantly higher in Con/Ob and Ob/Ob compared to Ob/Con and Con/Con (offspring of control dams lactating on control dams). In a separate study we also investigated the influence of cross fostering *per se* on the offspring phenotype. This was performed by comparing cross fostering controls (CF) with normal non-cross fostering controls (CON). Cross-fostering was associated with juvenile hyperphagia and obesity (Matthews *et al* In submission).

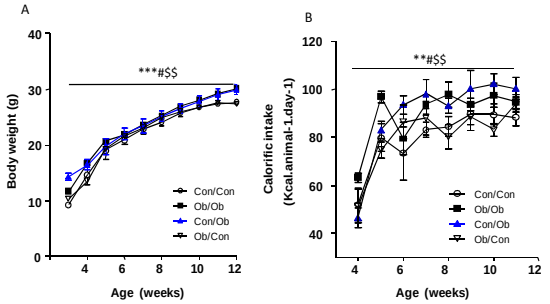


Figure 7. Body weight (A) and calorific intake (B) in C57BL6 male cross-fostering offspring n = 6-8 per group. ***P<0.001, **P<0.01 Con/Ob vs Con/Con, $$P<0.01 Ob/Ob vs Con/Con #*P*<0.05 Con/Ob vs Ob/Con, RM ANOVA.

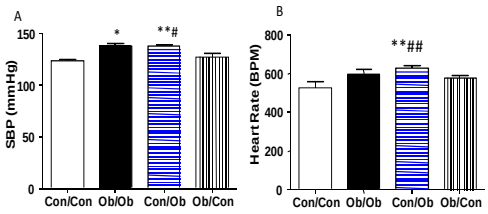


Figure 8. SBP and HR in female offspring Con/Con, white, Ob/Ob, black Con/Ob, blue stripes, Ob/Con, black stripes. Values expressed as means ± SEM, **P<0.01, *P<0.05 Ob/Ob vs Con/Con, ##P<0.001, #P<0.05 Con/Ob vs Con/Con, n=6-8, t-test.

Molecular Mechanisms (Del. 15, 16, 38, 61 Mil. 16-18, 27)

Adipocyte and heart hypertrophy were evident in offspring of obese dams (OffOb) with altered gene expression (Fig 10A-B, 11A). Heart and muscle (Shelley et al 2009) showed reduced mitochondrial function, and a down-regulation of PCG-1α and ATP-5b mRNA expression (Fig 11B). There was however no difference in the percentage of atherosclerotic plaque area between OffOb and OffCon ApoE-/- mice (atherosclerotic prone mice) (Fig 12).

ADIPOSE TISSUE

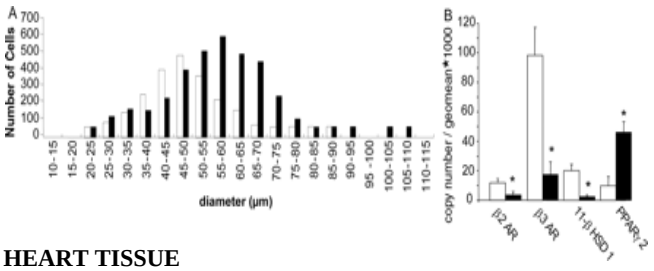
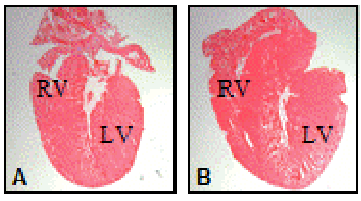


Figure 10. (A) **Adipose** cell size distribution and (B) mRNA expression of β_2 and β_3 adrenoreceptors and 11-β hydroxysteroid dehydrogenase (βHSD-1) and PPARγ type 2 gene in inguinal fat. (n=6 to 8) 0ffspring of control (OC, open bars) and obese dams (OO, closed bars) (B). *P<0.05 vs control (Samuelsson et al 2008).

HEART TISSUE



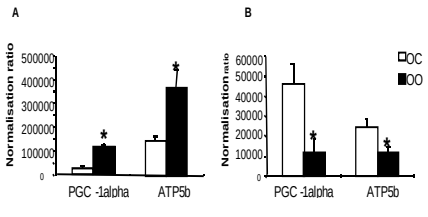


Figure 11. Heart (sagital) sections of male neonatal (d28) hearts from offspring of control (A) and obese (B) dams using hemotoxylin and eosin stain (x5). LV=left ventricle, RV=right ventricle. Mitochondrial related genes PGC-1alpha, ATP5b in (A) 28 day old and (B) 6 M old controls (open bars, n=4-6) and obese dams (closed bars, n=4-6). *P<0.05 vs control, t-test.

AORTA

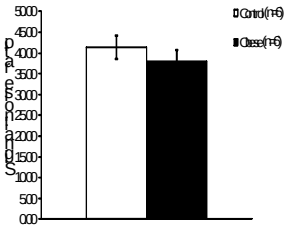


Figure 12 Artery Plaque contrast-to-noise ratio in the brachiocephalic artery of male offspring of control (OC) and obese (OO) dams at 6 months of age. Data expressed as mean±SEM.

Discussion & Conclusion

All deliverables and milestones were achieved. The strength of this study involves the consistency between models of maternal obesity (mice and rat) conducted in the two different centres (Kings' College London and Katholieke Universiteit Leuven). First, we have confirmed that maternal obesity induced by diet results in persistent metabolic dysfunctions in both rat and mice. Exposure to maternal obesity during gestation and suckling produced a hyperphagic metabolic syndrome-like phenotype in the offspring, characterized by obesity, and abnormal glucose and insulin homeostasis. Second, we have demonstrated that offspring of obese mice become hypertensive in adulthood with impaired endothelial function. Preliminary finding suggest blood pressure accompanied by increased sympathoexcitatory activation. Sympathetically mediated hypertension has been shown in several models of obesity related hypertension (Rahmouni *et al* 2008) but not if offspring of obese dams. In addition, juvenile OffOb mice demonstrated cardiac hypertrophy. This altered heart structure arises as a direct consequence of fetal and neonatal exposure to maternal obesity and not as consequence of offspring obesity. Previous in vitro studies have demonstrated that leptin (Hou *et al* 2010) and insulin growth factor I (Ito *et al* 1993) induce cardiomyocyte hypertrophy. Maternal hyperleptineamia and hyperinsulinemia may therefore be potential vectors for early cardiovascular dysfunction in the offspring. Offspring of obese dams also demonstrated reduced mitochondrial function (in muscle) and mitochondrial related genes in the heart in adulthood, which is a common indication of heart failure (Sihag *et al* 2009). There was however no impact of maternal obesity on measures of atherosclerosis in the apoE-/- offspring although other features of the metabolic syndrome were apparent. Offspring of both genders developed increased BW, associated with hyperphagia and adiposity. Exposure to a maternal obesogenic environment led to adipocyte hypertrophy (Samuelsson *et al* 2008) and insulin resistance (Nivoit *et al* 2009) in adulthood. Obesity, in particular abdominal obesity, has been identified as a risk factor for insulin resistance (Bjorntorp *et al* 1993) and adipocyte hypertrophy with enhanced pro-inflammatory secretion has been implicated in the development of insulin resistance and diabetes (Spiegelman *et al* 1996; de Souza *et al* 2001). Hyperphagia seems to precede the obesity and could therefore be an important mechanism leading to obesity. We have recently found a blunted anorexic response to leptin in juvenile OffOb, indicative of central leptin resistance (Kirk *et al* 2009). In addition, OffOb rats showed alterations of appetite-regulatory neurons in the hypothalamus (Kirk *et al* 2009). However this response seems to be selective for appetite as blood pressure responses to leptin were enhanced in the same animals (Samuelsson *et al* 2010). Maternal milk leptin might be the source for the offspring leptin resistance in OffOb rats, or more likely FFA and glucose (Kirk *et al* 2009). In the cross-fostering studies, whilst we have established the lactation period as being critical to the programming of both hyperphagia and hypertension following exposure to maternal obesity, we have also identified an inherent cross-fostering effect on offspring phenotype which calls into question the use of cross-fostering experiment design in general, paper in preparation for Journal of Physiology. The sexual dimorphism was apparent in the offspring phenotype exposed to maternal obesity or as a result of cross-fostering. The offspring adiposity and metabolic consequences were greater in the males than the females. This may suggest a role for testosterone or gonadal development in metabolic programming secondary to maternal obesity. Besides examining gender differences the potential hypothalamic involvement in both hypertension and hyperphagia will be explored in future studies. This work illustrates the important implications of maternal obesity on child future metabolic and cardiovascular health.

References: Boney CM et al. 2005, *Pediatrics;* **Björntorp P** et al 1993, *Adv Exp Med Biol;* **Catalano PM** et al. 2009, *Diabetes Care*; **de Souza CJ** et al 2001, *Diabetes;* **Hou M** et al 2010, *Clin Exp Pharmacol Physiol*; **Hull HR** et al. 2008, *Am J Obstet Gynecol;* **Ito H** et al 1993, *Circulation;* **Kirk S** et al, 2009. *PlosOne*; **Nivoit P** et al. 2009, *Diabetologia;* **Oben JA et al. 2010,** ***J Hepatol;*** **Perrin D et al. 2003,** ***Diabetologia;*** **Rahmouni K** et al 2005, *Diabetes;* **Samuelsson A-M** et al 2008, *Hypertension;* **Samuelsson A-M** et al 2010, *Hypertension;* **Shelley P** et al. 2009, *Am J Physiol Regul Integr Comp Physiol;* **Sihag S** et al 2009, *J Mol Cell Cardiol;* **Spiegelman BM** et al 1996, *Cell*.