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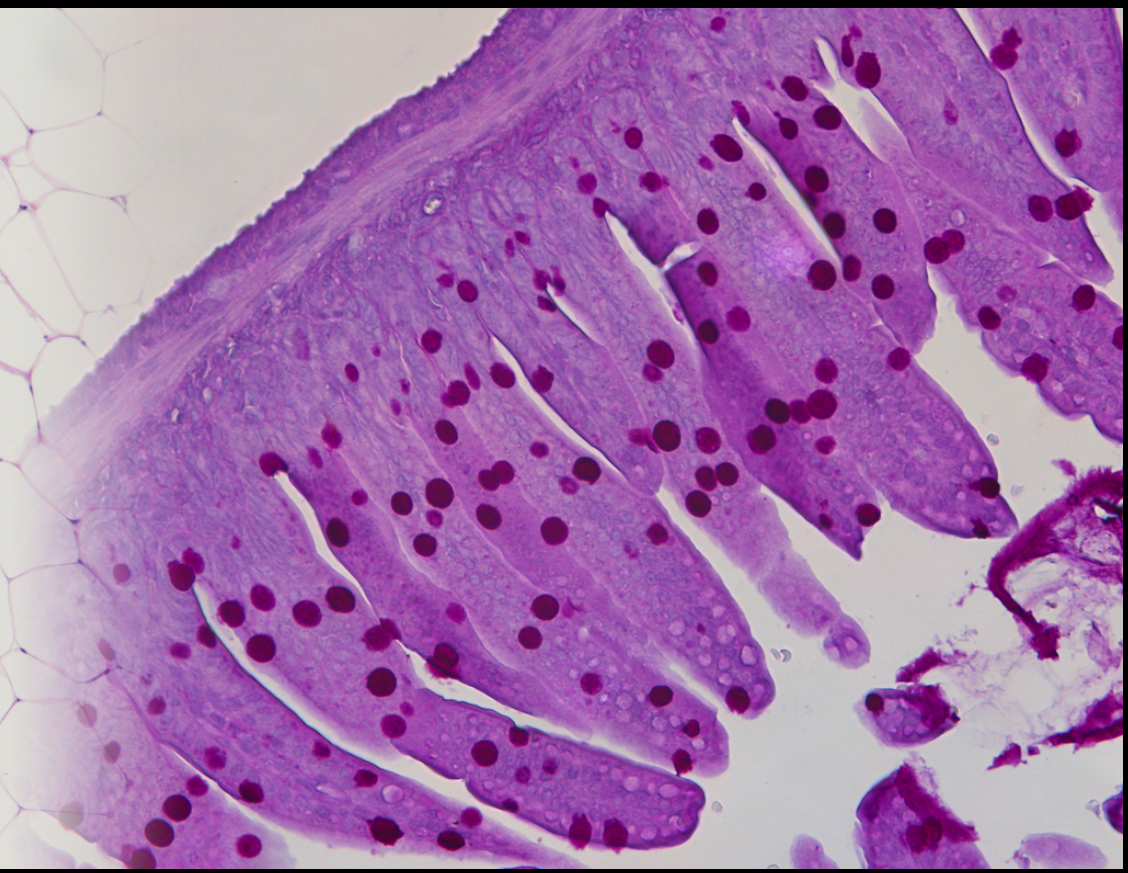

Sílvia Ribó Gené

Al meu pare que sempre em repetia: si vols, pots

Agraïments

Una plana buida per omplir lliurement d'agraïments a tota la gent que ha estat al meu costat lluitant per aquest doctorat. No és fàcil posar-los en un ordre específic, i ves a saber si en un altre moment seria diferent.

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Abstract

Abstract

Childhood obesity and overweight can often cause severe complications, including hypertension, dyslipidemia, insulin resistance, type 2 diabetes, and non-alcoholic fatty liver disease, amongst other disorders. Several studies have shown that early postnatal nutrition is of great importance in modulating newborn health outcomes. In this work, we have studied the role of nutrition during early stages of life in long-term metabolic health applying two different approaches: a) transgenerational transmission of impaired metabolic health induced by accelerated early weight gain caused by postnatal overnutrition and b) short and long-term metabolic effects on offspring of maternal diet supplementation with betaine.

Breast milk composition is important in modulating growth and health of the infant. Amongst the many nutrients that breast milk contains one worth highlighting is glycine betaine (or betaine). In addition to decreasing levels of fat in the liver, previous data demonstrated that maternal supplementation with betaine during breastfeeding also improves glucose homeostasis and modulates offspring early-life gut microbiota composition. Breast milk also contains essential bacteria that can influence gut microbiota composition of the breastfed infant. We observed beneficial short and long-term metabolic effects of betaine on offspring and protection against adult diet-induced obesity. We have analyzed ilea and gut microbiota of mice supplemented with betaine, and with or without antibiotics at different stages of life. Analyzing the microbiome we found that microbial community composition was modulated by betaine supplementation in 2-week-old offspring. Changes in the microbiome caused by antibiotic administration during early life were significantly correlated to higher adiposity and development of obesity during adulthood. Antibiotic treatment annulled completely long-term betaine-induced effects on body weight. Moreover, glucose tolerance was no longer improved when combining antibiotics with betaine treatment.

Rapid weight gain during early life has been associated with several components of the Metabolic Syndrome. Previously we developed a mouse model of neonatal overfeeding and rapid weight gain by litter size reduction. Neonatal overnutrition (ON) altered the metabolism of the exposed individuals (F0). Furthermore, offspring (F1) and grand-offspring (F2) of postnatal overfed male mice also developed metabolic complications

during adulthood. In agreement, it has been shown that environmental exposure on males can affect health in subsequent generations. Here, we hypothesized that epigenetic modifications, including DNA methylation, histone modifications, and noncoding-RNA, might be involved in the inheritance of diabetes risk in our model. We analyzed sperm methylome of F0 and F1 generations, and in the liver of 8-day-old mice of F1 and F2 generations, observing significant changes in methylation of specific DNA regions. We found 912 probes differentially methylated when comparing control and ON mice throughout the three generations, between the two tissues. Our results suggest that methylation of the male germ line caused by nutritional challenges during early life may carry information that influence metabolism across multiple generations. We then analyzed gene expression by qPCR of these genes in the liver of 8-days-old mice finding differences in some genes.

Resum

L'obesitat i el sobrepès infantil poden causar sovint complicacions greus en la salut, incloent hipertensió, dislipèmia, resistència a la insulina, diabetis tipus 2 i esteatosis hepàtica no alcohòlica, entre d'altres. Diversos estudis han demostrat que la nutrició post-natal precoç és de gran importància en la modulació de la salut del nounat. En aquesta tesis, hem estudiat el paper de la nutrició durant les primeres etapes de la vida en la salut metabòlica a llarg termini aplicant dos enfocaments diferents: a) efectes metabòlics de suplementar de la dieta materna durant la lactància amb betaïna sobre la descendència a curt i llarg termini i b) transmissió transgeneracional del fenotip d'intolerància a la glucosa induïda per un augment accelerat de pes en etapes primerenques de la vida, causat per l'excés de nutrició post-natal.

La composició de la llet materna és important per modular el creixement i la salut metabòlica de l'infant. Entre els nutrients que conté la llet materna, cal destacar la glicina betaïna (o betaïna). A més de disminuir els nivells de greix en fetge, diverses publicacions demostren que suplementar la dieta materna amb betaïna durant la lactància també millora l'homeòstasi de la glucosa i modula la composició de la microbiota intestinal del nounat. Al suplementar amb betaïna l'aigua de femelles durant la lactància vam observar efectes beneficiosos en la descendència a nivell metabòlic a curt i llarg termini. També vam poder observar que la betaïna protegia contra l'obesitat induïda per una dieta rica en greixos en l'etapa adulta. Se sap que la llet materna també conté bacteris essencials que poden influir en la composició de microbiota intestinal del lactant. S'ha analitzat la microbiota de l'ili i cec de ratolins suplementats amb betaïna, i amb o sense antibiòtics en diferents etapes de la vida. Analitzant el microbioma trobem que la composició de la comunitat microbiana dels ratolins de dues setmanes de vida estava modulada per la suplementació de betaina. Els canvis en el microbioma causats per l'administració d'antibiòtics durant la lactància estan significativament correlacionats amb una major adipositat i risc de desenvolupar obesitat durant l'edat adulta. El tractament amb antibiòtics en els nostres ratolins va anul·lar els efectes induïts per betaïna a llarg termini sobre el pes corporal. A més, la

tolerància a la glucosa no estava millorarada quan es combinaven els antibiòtics amb el tractament amb betaïna.

L'augment ràpid de pes durant les primeres etapes de la vida s'ha associat a diversos components de la Síndrome Metabòlica en l'adult. Prèviament en aquest laboratori hem desenvolupat un model murí de sobrealimentació neonatal i augment de pes ràpid a partir d'una reducció de la mida de la ventrada. L'excés d'alimentació neonatal (ON) va alterar el metabolisme dels mascles exposats (F0). A més, els fills (F1) i els néts (F2) dels ratolins exposats a la sobrenutrició també van desenvolupar un metabolisme alterat durant l'edat adulta. En acord, s'ha demostrat que l'exposició ambiental sobre els mascles pot afectar la salut de generacions posteriors. Així, ens vam plantejar que les modificacions epigenètiques, incloses la metilació de l'ADN, les modificacions de l'histona i l'ARN no codificant, podrien estar implicades en l'herència del risc de diabetis en el nostre model. Es va analitzar el metilma d'esperma de les generacions F0 i F1, i el metiloma de fetges de ratolins de 8 dies d'edat de les generacions F1 i F2, observant canvis significatius en la metilació de regions específiques d'ADN. Al comparar els ratolins control amb ON de cada generació i teixit, vam trobar 912 sondes diferentment metiladas. Els nostres resultats suggereixen que la metilació de la línia germinal masculina provocada per reptes nutricionals durant etapes primerenques de la vida pot portar informació que influeixi en el metabolisme en les següents generacions.

Contents

1. Introduction

1.1 Non-communicable diseases and global health

The World Health Organization (WHO) defines non-communicable diseases (NCDs) as those "chronic diseases that are of long duration and are a result of a combination of genetic, physiological, environmental, and behavioral factors". NCDs are non-infectious diseases and are not contagiously passed from one person to another. In addition to lasting for a long period of time, they have a slow progression, even though in some cases they result in rapid death, as seen in certain diseases including autoimmune diseases, cancer, or stroke, among others (Boutayeb and Boutayeb, 2005; Grabauskas, 1988; Hunter and Reddy, 2013).

Currently, NCDs are leading causes of death globally, being responsible for 70% of all worldwide deaths. Of these, 42% of the deaths occurred in people younger than 70 years old during 2012. Deaths due to NCDs are projected to increase, while total number of deaths from infectious diseases is projected to decrease by 2030 (WHO, 2015). NCDs include cancer, cardiovascular diseases (CVD), chronic respiratory diseases, musculoskeletal disorders, obesity, and diabetes. Among these, cancer, CVD, respiratory diseases, and diabetes were responsible for 63% of all NCD-related deaths during 2008 (Lachat et al., 2013; Popkin, 2006). People of all age groups, regions, and countries can be affected by NCDs, but a high percentage of deaths occur in low and middle-income countries (Boutayeb and Boutayeb, 2005).

According to the WHO, age, gender, genetics, environmental exposures like air pollution, unhealthy diet, and physical inactivity, which can lead to hypertension and obesity, are all factors that can increase risk of many NCDs (Williams et al., 2015). Targeting these main risk factors with appropriate interventions can prevent the majority of heart disease cases, strokes, cancers, and type 2 diabetes (T2D). Thus, modifying adult lifestyle is currently the main strategy to reduce the cases of NCDs among the population.

1.2 Prenatal origins of adult disease

Evidence now indicates that early-life factors play a crucial role in determining risk for development of NCDs, noteworthy nutrition during first stages of life, including *in utero*, lactation period, and childhood. This paradigm has been designated as the Developmental Origins of Health and Disease theory (Singhal, 2016). The relationship between early-life nutrition and long-term health outcomes was first revealed in the early 90s, when the Fetal Origins of Adult Disease (FOAD) theory was described (Barker DJ, 1990). This theory was the first to correlate *in utero* and postnatal nutrition with infant growth and later health outcomes. For instance, blood pressure and risk of hypertension in 50 year-old men and women exposed to famine while *in utero* correlated to placental and birth weight (Gluckman and Hanson, 2006). At that time, scientists already suspected that maternal nutrition had an important role, but were unable to provide sufficient supporting evidence (Barker DJ, 1990; Gluckman, 2008; Gluckman et al., 2005).

1.2.1 Developmental Origins of Health and Disease

The Fetal Origins of Adult Disease has evolved to what we now know as Developmental Origins of Health and Disease (DOHaD). This hypothesis proposes that long-term disease in the adult is in fact induced through adaptive responses during development of the fetus or infant readjusting to the environment (Gluckman, 2008; Isganaitis et al., 2015). This preparation of the fetus and infant is known as developmental plasticity, where changes in the environmental conditions during the developmental period result in different physiological or morphological states ensuring survival of the infant (Barker, 2004; Goldberg and Prentice, 1994; Symonds et al., 2009).

Over time this plasticity is lessened, and the ability to continue to adapt to a changing environment is progressively lost (Calkins and Devaskar, 2011). At some level this plasticity is also extended into childhood and organs keep continuously adapting to environmental changes (Calkins and Devaskar, 2011; Hochberg et al., 2011). In this way, exposure to environmental conditions during early-life, including stress and under- or overnutrition, influence risk of developing metabolic diseases, including cardiovascular

diseases and type 2 diabetes, during adulthood (Barker, 2004; Gluckman and Hanson, 2006; Gluckman et al., 2005, 2008). Several studies have identified specific critical time periods when nutrition modulates long-term health outcomes, in particular the prepregnancy, gestational, and postnatal periods (Esposito et al., 2009; Gluckman et al., 2005, 2008).

1.2.2 Prepregnancy environment

Even before pregnancy, maternal and also paternal body mass index (BMI) is known to alter pregnancy rates and outcomes, as well as embryo development (Bakos et al., 2011; Papachatzi et al., 2013). Overweight or obesity before pregnancy in both parents can result in macrosomic newborns and increase childhood obesity risk (Esposito et al., 2009; Weiqin et al., 2017).

Experimental and epidemiological studies have proven that maternal diet before conception, in particular intake of antioxidants, long chain polyunsaturated fatty acids, prebiotics, and probiotics, is linked to multiple health problems, amongst them asthma in preschool children and obesity later on. Other variables such as maternal age, owning pets or physical activity can also influence the development of diseases in a long-term manner (Castro-Rodriguez et al., 2008; Devereux et al., 2006; Renz et al., 2011). Childhood allergies and immune system diseases, which can manifest as early as the first months of life, can have an origin during the pre-pregnancy period, when risk factors including modern dietary patterns, environment pollutants, microbial patterns, and stress affect the development of the disease (Edwards, 2017; Hornsby et al., 2017; Jiménez-Chillarón et al., 2012; Munblit et al., 2017; Xu et al., 2014). These short-term associations with allergy inflammation also have implications in the development of cardiovascular disease and allergic diseases later in life (Phillips et al., 2016; Prescott, 2013).

1.2.3 Intrauterine environment

1.2.3.1 Maternal undernourishment during gestation

Maternal undernutrition during pregnancy results in in utero growth restriction (IUGR), affecting offspring long-term health outcomes (Davies et al., 2016; Mathias et al., 2014). Low nutrient supply that the fetus is exposed to predisposes crucial organs like the brain or the heart to receive more nutrients at the expense of somatic growth (Gluckman et al., 2008; Lillycrop, 2011; Symonds et al., 2009). Therefore, infants will most likely have low birth weight, this state and especially the rapid early weight gain during the first months of life has been associated with increased risk of childhood obesity and long-term adverse health outcomes like cardiovascular disease, obesity, and T2D in the adult (Lloyd et al., 2012; Martínez et al., 2014; Parikh et al., 2017).

These long-term effects of malnutrition during pregnancy on offspring have been observed in a well-nourished human cohort that suffered from a sudden famine, the Dutch cohort (Roseboom et al., 2006). During late World War II in the western part of the Netherlands, daily calorie intake was reduced to as low as 800 kcal per day. Women exposed to the famine during early gestation gave birth to infants with reduced weight (Roseboom et al., 2006). These infants had an accelerated early growth during infancy and had higher rates of obesity, cardiovascular diseases and altered lipid profiles in adulthood. In some cases cognitive functions were altered (Malo et al., 2013; Rasyid and Bakri, 2016; de Rooij et al., 2010). Those exposed to the famine in mid gestation had microalbuminuria and obstructive airways disease, as well as impaired glucose tolerance (Roseboom et al., 2006).

1.2.3.2 Maternal obesity or overweight during gestation

Maternal obesity prior to conception is a major risk factor in giving birth to neonates that weigh above the 90th percentile for their gestational age (Kim et al., 2015). While obesity before pregnancy in women is an indicator for overweight in the newborn and risk for disease in later life, high gestational weight gain (GWG) is also problematic in these pregnancies (Li et al., 2013). Even if BMI of pregnant women is within the normal range,

higher GWG is associated with complications at delivery and increased birth weight (Frederick et al., 2008; Lillycrop, 2011; Nohr et al., 2008).

It is known that blood glucose is higher in mothers with obesity, and can influence fetal growth and early infant overweight or obesity status (Frederick et al., 2008; Kerr et al., 2017; Weiqin et al., 2017). Other parameters altered in mothers with obesity or overweight (OWO) are increased circulating levels of fatty acids and triglycerides, elevated concentrations of leptin in blood (also found elevated in cord blood), higher levels of C-reactive protein and IL-6 indicative of a low-grade inflammatory state, and insulin resistance (Englich et al., 2017; Poston et al., 2011; Zhu et al., 2016). High levels of IL-6 in cord blood are associated with fetal adiposity and fetal insulin resistance in utero, suggesting that altered glucose metabolism may already be set during these early stages of development (Catalano et al., 2009; Poston et al., 2011).

One-carbon metabolism or folate metabolism consists of a wide group of metabolic pathways occurring inside the cell either in cytoplasm, mitochondria and nucleus of different tissues. It is designated one-carbon metabolism (1C) since it consists in the transfer of 1C units for biosynthetic processes, and it includes folate, choline, methionine, homocysteine, S-adenosyl-homocysteine (SAH), S-adenosyl-methionine (SAM), betaine, dimethylglicine, sarcosine, glycine, serine. Deficiency in folate is associated with developmental defects, such as neural tube defects at birth and in the adult a diet low in folate can induce anemia. In mitochondria of liver cells 1C metabolism directly controls levels of these three amino acids: serine, glycine and methionine (Figure 1). And levels of 1C metabolism components are associated with a variety of diseases including cardiovascular diseases, obesity, and metabolic disease in the adult (Ejaz et al., 2016; Ueland, 2011; Ueland et al., 2005). Components of the one-carbon metabolism are also altered by maternal obesity, glycine, serine, taurine and methionine both in the mother and the fetus. And these metabolites are associated with infant growth and childhood obesity (Isganaitis et al., 2015; Nathanielsz et al., 2015). Adverse effects of maternal obesity and maternal high fat diet can be partially rescued when supplementing the mothers diet during pregnancy and lactation with a 1C metabolite cocktail (choline, betaine, vitamin B12,

folate) in rodents (Carlin et al., 2013; Cordero et al., 2014).

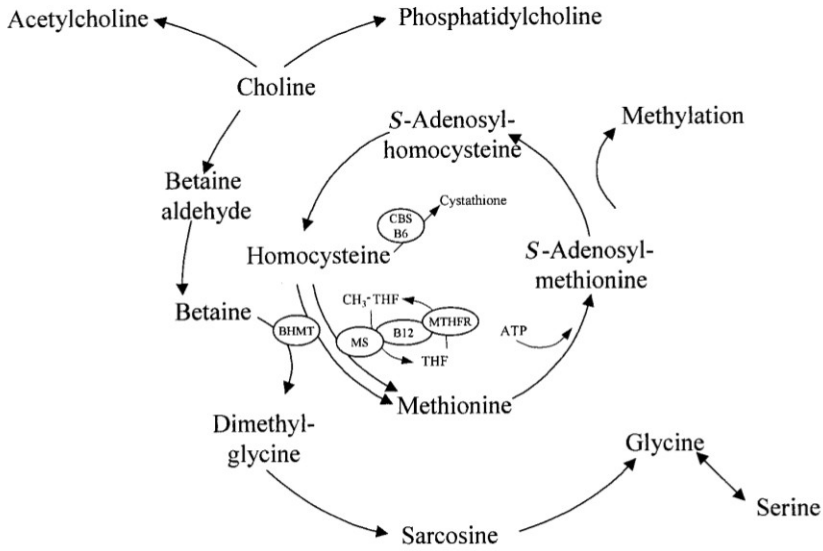


Fig. 1. Methionine cycle. This is one arm of the 1C-metabolic process. Methionine is synthesized from homocysteine, through demethylations it is converted to S-Adenosyl-methyionine and S-Adenosyl-homocysteine.
Betaine is implicated in transfering methyl groups to generate methionine from homocysteine.

1.3 Early-life environment and long-term obesity and metabolic disease

As famine during childhood is associated with cognitive impairment in later life, higher levels of nutrients also indicate risk of impaired health during adulthood (Wu and Chen, 2009; Zhang et al., 2017). Maternal diets poor in calcium or vitamin D are approached by supplementing her diet, and when breastfeeding these nutrients will also reach the newborn. For example, an excess of iron in early-life diet increases the risk of developing neurodegenerative diseases such as Parkinson disease (Hare et al., 2017). Inappropriate levels of 25-Hydroxyvitamin D during infancy are also correlated with impaired cognitive

functions during adulthood (Maddock et al., 2014). Adequate protein intake or fat content in the diet have been associated with improved development of the child.

Balanced nutrition during infancy will contribute to an adequate growth rate. On one hand undernutrition in infancy impedes a correct growth, and can induce impaired cognitive development (Kang et al., 2017). On the other, rapid weight gain in early stages of life increases risk of childhood obesity (Darnton-Hill et al., 2004; Munthali et al., 2017; Ong, 2017). This early-accelerated weight gain can induce long-term consequences including diabetes, metabolic syndrome and cardiovascular disease during adulthood (Daniels, 2009; Lloyd et al., 2012; Velkoska and Morris, 2011).

1.3.1 Early-life nutrition and disease

Since the Developmental Origins of Health and Disease concept emerged, more studies are associating postnatal nutrition with long-term health (Esposito et al., 2009; Gluckman et al., 2005; Jiménez-Chillarón et al., 2012). Early-life nutrition is defined as nutritional exposures during infancy, and in this thesis we delimited this to the breastfeeding period. Nutrition during this early stage of life is extremely important for both short- and long-term health outcomes. Receiving the necessary amount of nutrients is critical for a proper development and securing a good health in later stages of life (Horta and Victora, 2013; Horta Bernardo, 2013). The first nutrients that an infant receives after birth are through breast milk; thus, maternal nutrition during the breastfeeding period is of great importance.

As important as maternal nutrition during pregnancy, feeding of the newborn is crucial for an optimal development. As reviewed in the previous section, accelerated early weight gain of the newborn results in increased childhood obesity risk (Péneau et al., 2014; Taveras et al., 2009), which can increase risk of metabolic diseases during adulthood (Langley-Evans, 2015; Lanigan and Singhal, 2009). During infancy, the main source of nutrients comes from breastfeeding or formula feeding, until solid foods can be introduced. The World Health Organization (WHO) recommends exclusive breastfeeding for the first 6 months after birth because of its protective effects not only on the child but also the mother. When breastfeeding is held for at least 6 months, risk of childhood obesity

is significantly reduced, although there are some discrepancies in this area (Arenz et al., 2004; van Rossem et al., 2011; Yan et al., 2014). Therefore, postnatal nutrition is an important window of opportunity to break the obesity pattern passed from parents to infants, either through adjusting formulas to meet nutritional requirements, or by modifying breast milk composition through maternal diet.

1.3.2 Breast milk composition and offspring metabolic health

Along the period of lactation, breast milk composition varies from colostrum in the first few days postpartum to more mature milk in the following weeks and months. This first fluid is rich in immunologic components, and has low concentration of lactose, indicating that its primary function is more immunological than nutritional. In later stages of lactation, lactose concentration increases, as well as other macro and micronutrients (Ballard and Morrow, 2013).

Differences in breast milk composition also occur among mothers. Specific components of the milk, including oligosaccharide diversity and hormone levels, are associated with lower relative weight and total weight gain of the child (Alderete et al., 2015; Fields and Demerath, 2012). High protein intake during lactation is a risk factor for childhood obesity. Infants fed formula milk with high protein content have increased BMI when reaching 6 years of age compared to infants receiving a low protein formula milk. Breastfeeding results in similar infant weight and height measurements when compared to infants fed low protein formulas, suggesting protective effects of breastfeeding on infant BMI, and212 the necessity to review protein content in formula milk (Weber et al., 2014).

Diet of the lactating mothers can influence breast milk composition. For example, in rodents a high fat diet can increase fat content in milk (Purcell et al., 2011). However, other components (IL-6, TNFa, and leptin) that are high in milk from mothers with obesity or overweight did not correlate with weight gain of the infant at 1 month of age (Fields and Demerath, 2012). Breast milk also contains sugars (Ballard and Morrow, 2013); glucose and lactose have constant levels when milk is analyzed at 1 and 6 months (Goran et al., 2017). Fructose has been positively correlated with increased adiposity in 6 month-

old infants (Goran et al., 2017). But fructose is not a natural component of breast milk (Ballard and Morrow, 2013), and such as other metabolites it is most likely being absorbed from the mothers' diet. Different metabolites in cord blood and maternal plasma have been correlated to accelerated weight gain of the infant. Levels in plasma are relevant since they can be determining breast milk composition that will feed the newborn.

For instance, folate and its metabolites, such as choline, are in high demand during early growth and their levels are especially high in breast milk. Modern diets do not reach the minimum adequate intake of choline, folate, or vitamin B12. Therefore, pregnant and lactating women are often prescribed dietary supplements containing these vitamins (Fischer et al., 2010; Zeisel et al., 2003). Metabolites involved in the one-carbon metabolism (defined in 1.2.3.2) such as folate, have been correlated to higher or lower risk of childhood obesity associated also with mothers BMI (Wang et al., 2016). Lower levels in maternal plasma of metabolites involved in methionine metabolism such as dimethylglycine, N-acetylmethionine and betaine have been associated with postnatal accelerated weight gain, increased adiposity and size at birth (Isganaitis et al., 2015; Van Lee et al., 2016).

In this thesis we focused on a particular component of the methionine metabolism trimethyl glycine or betaine. Betaine is a tri-methylated amino acid synthetized from choline in the one-carbon metabolism. It has three main physiological functions; it can act as an organic osmolyte protecting cells under stress in microorganisms, plants and mammals. Also, in mammals, it can stabilize protein structures acting as a chaperone, and as a methyl donor converting homocysteine to methionine, s-adenosyl-methionine (SAM), s-adenosyl-homocysteine (SAH) and finally homocysteine in the methionine cycle, that occurs primarily in the liver (Figure 1) (Ueland et al., 2005). Betaine was first discovered in sugar beets in the 19th century (Craig, 2004). It is also found in other plants and foods common in the human diet as resumed in Table 1 (Zeisel et al., 2003). Betaine is used in treatment of homocysteinemia, and is also known to improve different symptoms of cardiovascular disease in combination with guanidinoacetate (glycocyamine, direct precursor of creatine). Betaine is absorbed through the intestine and is mainly metabolized

in the liver and kidney. Liver diseases such as hepatic steatosis, that can be induce by high fat diet, obesity or diabetes is ameliorated by betaine supplementation (Craig, 2004; Ueland, 2011).

TMG in foods	
Food	**TMG (mg/100g)**
Quinoa	630
Spinach	577
Wheat bran	360
Lamb's quarters	332
Beetroot	256

Table 1. Betaine found in common foods.

1.4 Early-life gut microbiota and health

Gut microbiome in the newborn begins to establish and define as early as delivery (Soderborg et al., 2016; Stinson et al., 2017). Afterwards breast milk insulin, glucose, oligosaccharide, and hormone levels will influence microbiota diversity (Lemas et al., 2016). What is already known is that with breastfeeding and later on with solid food introduction, gut microbiota is continuously changing and shaping in the infant (Pannaraj et al., 2017; Urbaniak et al., 2014).

Gut microbiota in adults has been correlated to metabolic syndrome and obesity (Geurts et al., 2014a; Tilg and Kaser, 2011). In particular, decreased levels of one bacterium species has been strongly linked to obesity in adulthood, Akkermansia muciniphila (Dao et al., 2015). This species feeds off the protective barrier in the small intestine, a mucus layer containing mainly mucins. This mucus will form a lubricative protective barrier; in the human intestine it can reach 200 μm thick. The cells responsible for secreting these mucins are known as Goblet Cells (Figure 12d, results), which are modified epithelial cells. Goblet

cells, and the protective intestinal barrier are crucial for protecting against systemic inflammation that leads to obesity and metabolic diseases (Edwards, 2017; Ganesh et al., 2013; Knoop et al., 2017).

The use of antibiotics in adulthood disrupts the gut microbiota, and also this protective barrier is destroyed and needs to be rebuilt again (Geurts et al., 2014; Knoop et al., 2017). But when antibiotics are administered in early-life it has been associated to accelerated weight gain that leads to obesity and long-term metabolic diseases (Bokulich et al., 2016; Cho et al., 2012; Cox et al., 2014).

1.5 Intergenerational transmission of obesity

Childhood obesity is a condition that can be transmitted from the mother to the offspring, as seen before, during development in utero or during the infancy and more specifically the lactation period (Martínez et al., 2014). Early-life nutrition can also increase risk of developing metabolic diseases as diabetes type 2 in adulthood. Indeed, perinatal nutrition can also influence changes in DNA methylation that can be later transmitted to offspring (Vaiserman, 2017). This transmission is not due to mutations in the genome, but rather the epigenome.

1.5.1 What are epigenome and epigenetics?

The epigenome consists of chemical changes affecting the DNA and histone proteins. These changes can result in changes in the chromatin structure and function of the genome. They are involved in regulating expression of the genes, development, tissue differentiation and suppression of transposable elements. These chemical compounds are only attached to the sequence but do not form part of the DNA (Geiman and Muegge, 2010; 2017)

Therefore, the organism will only have one genome but as many different epigenomes as cell types. When these changes in gene expression are inherited during development or across generations one talks of epigenetics.

Epigenetic changes are those occurring in the phenotype without affecting the

genotype, but influencing how the information will be expressed and inherited (Bernstein et al., 2007; Mazzio and Soliman, 2012). Patterns of epigenetic modifications vary among individuals, different tissues in an individual and even different cells. Epigenetic modifications are implicated in gene expression of the tissue, turning on or off specific genes and therefore determining production of proteins in tissue- and time-specific manner (Mazzio and Soliman, 2012). Epigenetic modifications include DNA methylation, alteration of the histones, chromatin remodeling and more recently non-coding RNAs that can also influence gene expression (Corella and Ordovas, 2017). Of these epigenetic mechanisms DNA methylation is implicated in gene silencing at a long-term manner, while histone modifications are more variable and display short-term regulation. However, all mechanisms are tightly interconnected and remain as the cells divide (Lloyd et al., 2012).

When these epigenetic modifications occur in the germ cell line, they can be inherited into the following generations (Fernandez-Twinn et al., 2015; Mazzio and Soliman, 2012).

1.5.2 Environmental changes influence the epigenome

Epigenetic modifications can be consequence of certain environmental changes, which will influence gene expression without mutations in the genome. Among the environmental factors that can influence epigenetic modifications, the ones that have been most studied are diet, temperature changes, chemical pollutants and toxins and other external stresses (Feil and Fraga, 2012).

When these epigenetic changes appear during development, including conception, gestation and/or neonatal period, they affect a high proportion of somatic cells of the developing organism, as well as their germ cells. There is evidence that air contaminants have affected germ-line epigenome and they have been inherited in the next generations (Yauk et al., 2008). Longitudinal studies with twins have demonstrated that DNA methylation differences can be established after birth (Wong et al., 2010). When these epigenetic marks are established during gametogenesis, they can be inherited into the next generations.

In agreement, it has been shown that environmental exposure specifically during the perinatal window can affect health in subsequent generations and has been linked to a number of diseases, including cancer, cardiovascular diseases, diabetes, and obesity (Joss-Moore and Lane, 2016; Lillycrop, 2011).

Neonatal overfeeding has been linked to altered DNA methylation patterns of specific genes (Liu et al., 2013; Plagemann et al., 2009, 2010). These epigenetic modifications due to postnatal over-nutrition are transmitted both from the mother and from the father (Fullston et al., 2013; Jiménez-Chillarón et al., 2012). Transmission of metabolic phenotypes through male line suggests that the over-nutrition of the fathers might induce epigenetic modifications in germ cells/sperm that are subsequently transmitted to the following generations (Pauwels et al., 2017; Pentinat et al., 2010; Vickers, 2014). Additionally, several studies have demonstrated that feeding a high fat diet in rodent parents induced increased adiposity, overweight, impaired glucose tolerance and insulin resistance in the offspring (Carlin et al., 2013; Dunn and Bale, 2011; Morita et al., 2014).

2. Aims of study

1. To characterize the effects of maternal betaine supplementation during lactation on the offspring.

    1.1 To evaluate whether the intestinal microbiome contributes to betaine-induced long-term metabolic effects.

2. To evaluate the effects of a maternal obesity mouse model combined with betaine supplementation during lactation on the offspring.

3. To evaluate the effects of diet-induced obesity in adulthood on offspring of betaine supplemented dams.

4. To determine the metabolic impact of epigenetic modifications in a mouse model of overnutrition.

    4.1 To evaluate methylation patterns in successive generations of an overnutrition mouse model

    4.2 To evaluate gene expression in successive generations of an overnutrition mouse model.

3. Materials and methods

3.1 Study subjects

The human study was already previously reported (30). The study was approved by the Institutional Review Board (IRB), and subjects signed an informed consent. The trial was registered at cliniclatrials.gov (NCT02535637). Mother-infant dyads were exclusively breastfeeding when enrolled in the clinical trial. They arrived at the University of Oklahoma between 8:00 and 10:00 am. Then using a breast pump, they emptied the entire breast. The same day the infant had a whole-body dual-energy X-ray absorptiometry scan, at 1 month of age.

3.1.1 Human milk analysis

We determined one-carbon metabolites s-adenosyl-methionine (SAM), s-adenosyl-homocysteine (SAH), methionine, cystathionine, choline, and betaine by LC-MS/MS as previously described (Inoue-Choi et al., 2012). Also by LC-MS/MS 5-methyltetrahrofolate was measured as described (Arning and Bottiglieri, 2016). Total homocysteine was measured by LC-MS/MS. All data was collected and processed using Analyst software v1.4.2 (Applied Biosystems).

3.3 Animal procedures

3.3.1 Ethical Statements

All procedures with animals have been approved by the Committee of Animal Experimentation from the University of Barcelona and the *Conselleria de Ramaderia i Pesca de la Generalitat de Catalunya.*

We mated 8 week-old ICR (CD1) Outbred virgin females with 8 week-old ICR (CD1) Outbred virgin males (Envigo). Mice were housed on a 12-hour light-dark cycle with free access to food and water, unless otherwise specified.

3.3.2 Dams dietary supplementation model

Drinking water of the dams was supplemented during the lactation period with 1% (w/vol) of betaine, at delivery day for the betaine group (B). When indicated, antibiotics were administered in the dams' drinking water during the lactation period, starting at delivery day (1 g/L ampicilin and 0,5 g/L neomycin), alone (AB) or combined with betaine (ABB). Control group (C) had no supplementation in water.

All cohorts, control (C), betaine supplemented (B), antibiotic administered (AB) and antibiotic and betaine supplemented (ABB) were adjusted to 8 pups per dam, at delivery day. Pups from both groups are breastfed freely by the dam.

For maternal obesity mouse model, we fed dams a high-fat diet (45% kcal, D12451, Research Diets) for 8 weeks before mating, and maintained the diet during pregnancy and lactation. At delivery day, we randomly assigned them to control (MO-C) or betaine-treated groups (MO-B). For the diet induced obesity mouse model, 6 week-old mice were fed the same high-fat diet (45% kcal, D12451, Research Diets) until sacrifice day. Groups were then HF-C, for the control mice and HF-B for the betaine-treated offspring.

At 1 week of life, we measured milk intake of the pups. To do this, we separated them from the dam during 1h. We weighed them, considering this weight at time 0 (W0) and returning them to their cage. After 2h, we weighed the pups again (W1). The difference between both measurements (W1-W0) divided by the initial weight (W1), is the milk intake by the pup. Final result is expressed as percentage of body weight.

Weaning of the pups was done at 3 weeks of age, separating the pups from the dams in both groups. They were housed in cages of 4 animals/group, maintaining pups from each supplementation group separated.

We monitored the pups weight every two weeks until sacrifice day. Mice were fed *ad libitum* 2014 Teklad diet (Envigo). Mice were euthanatized when they were 2 week-old, 6 week-old and 24 week-old.

3.3.3 Neonatal overfeeding model

We mated 8 week-old ICR (CD1) Outbred virgin females with 8 week-old ICR (CD1) Outbred virgin males (Envigo). At delivery day cohorts were adjusted to 8 male pups for the control group (C), and to 4 male pups for the over nutrition or small litter size group (ON or SL). Pups from both groups were breastfed freely by the dam. This constituted the parental generation (F0).

In the successive generations 8 week-old males from F0 generation were mated with 8 week-old virgin females for both groups to form the F1 offspring generation. To obtain the F2 offspring generation we mated 8 week-old F1 males with 8 week-old virgin females. All cohorts (control and ON) were adjusted to 8 pups, to constitute the F1 and F2 offspring generations. Therefore, only males from the parental generation (F0) were exposed to overnutrition during the lactation period.

Weaning of the pups for all generations was done at 3 weeks of age, separating the pups from the dams in both groups. They were housed in cages of 6 animals/group, maintaining pups from control and ON groups separated. They had free access to water and food *ad libitum* 2014 Teklad diet (Envigo).

Dams had free access to water and food. Mice were fed *ad libitum* 2014 Teklad diet (Envigo).

3.4 Glucose tolerance analyses

3.4.1 Intraperitoneal glucose tolerance test

The intraperitoneal glucose tolerance test (ipGTT, 1,5 g/kg) measures the clearance by the body of an injected glucose load (Glucocemin, 33%). A healthy animal will normalize injection of glucose to basal levels after a 2h period. ipGTT was performed on unrestrained conscious mice after an overnight-fast. Blood glucose was measured with a Glucometer Elite (Menarini Group) at different time points (0min, 15min, 30min, 90min and 120min) after glucose injection.

Blood samples were obtained from the tail at two time points, one prior glucose

injection (0min) and the other after 15min of injection. Insulin was measured by ELISA kit (96 Well Plate Assay, Millipore), following manufacture instructions.

3.4.2 Intraperitoneal insulin tolerance test.

The intraperitoneal insulin tolerance test (ipITT, 0,6 U/Kg) evaluates insulin sensitivity by monitoring endogenous disappearance of glucose over time in response to an insulin injection. In healthy animals glucose levels will decrease as a result of insulin injection, staying within the normal ranges. In insulin resistant animals, tissues are not able to use it efficiently leading to higher amounts of glucose in blood. ipITT was performed on unrestrained conscious mice after a 4 hour fast. Blood glucose was measured with a Glucometer Elite (Menarini Group) before insulin injection, and at different time points (15min, 30min and 60min).

3.5 Sample collection

For the betaine supplemented experiment 24 week-old mice were euthanized using pentobarbital (40 mg/kg) as anesthesia. Cardiac blood was retrieved with a syringe, transferred to an eppendorf tube containing 5µl of 0,5M EDTA. They were then centrifuged at 10.000 rpm, 4ºC during 10min. We obtained 300-400µl of plasma that were transferred to a new tube and stored at -80ºC until analyzed.

Liver, inguinal and epididymal white adipose tissue (iWAT, eWAT); ilea and ceca were then retrieved. Tissues were weighed and snap frozen in liquid nitrogen, and stored at -80ºC until analyzed.

Mouse milk samples were harvested manually from dams under anesthesia (pentobarbital, 40 mg/kg) 14 days after delivery day; dams and pups were sacrificed after milk collection, and cecal content obtained for microbiome studies.

Body composition was determined using a 7.0T Bruker Biospect MRI system (Bruker Medical Gmbh, Germany).

Metabolic cages (PhenoMaster/LabMaster, TSE Systems GmbH, Germany) were

used to measure indirect calometry; O_2, CO_2, food intake, and locomotor activity were monitored for 48 hours, and data analyzed using 2-way ANOVA for repeated measures.

For the small litter size experiment, 8 day-old pups were sacrificed to obtain liver, inguinal white adipose tissue (iWAT), and brain. Also male mice were sacrificed at 20 weeks of age after being mated with control dams, and retrieved sperm, testis, liver, inguinal and epididymal white adipose tissue (iWAT, eWAT). Tissues were snap frozen in liquid nitrogen and stored at -80ºC until analyzed.

3.6 RNA extraction and cDNA generation and gene expression analysis

We extracted RNA from the different tissues and determined gene expression by quantitative Real Time PCR using Sybr Green, following the protocol described below.

3.6.1 RNA extraction protocol

We first homogenized tissue samples in 300µl of TRI Reagent in 1,5ml Safe Seal Microtube (Sarstedt), using ZROB05 0.5mm diameter zirconium oxide beads (Next Advance) and a Bullet Blender.

We then completed to 1ml of TRI Reagent to continue with RNA extraction. After incubating samples for 5 min at room temperature (RT) we added 200µl of Chloroform. When extracting RNA from iWAT or eWAT we added an extra centrifuge before adding chloroform to remove extra fat. After adding chloroform we centrifuged the samples, causing the mixture to separate in three phases: a red organic phase containing proteins, a white interphase containing DNA, and a colorless upper aqueous phase containing RNA.

In this case we only need the aqueous phase that we transferred to a new tube and add 2-propanol that will cause the RNA to precipitate.

RNA yield and quality was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Schwerte, Alemania).

NanoDrop will measure absorbance of all molecules present in the sample, and RNA, ssDNA and dsDNA all absorb at same wavelength. Therefore, we will accept as "pure" a 260/280-absorbance ratio similar to 2. If the ratio is appreciably lower it may indicate contamination by proteins, phenols or others that absorb strongly at 280nm. As a secondary measure of nucleic acid purity we used the 260/230 ratio. Expected 260/230 values are commonly in the range of 2.0-2.2, if the ratio is appreciably lower than expected it may indicate presence of contaminants that absorb at 230 nm.

3.6.2 cDNA generation protocol

Once RNA has been quantified and the quality assessed we synthesized complementary DNA (cDNA) using reverse transcriptase enzyme. We used Promega reactives. We started with 1µg of the RNA isolated, with 1µl of Random primers in a total volume of 10µl of water. After 5 min incubation at 70ºC we added to each sample the components described in Table 2.

Component	Volume
RNA inhibitor	1 µl
MLV-RT	1 µl
dNTPs	1 µl
Buffer 5x	5 µl
H_2O	2 µl

Table 2. Promega reactives to obtain cDNA from RNA

Following Promega's instructions we incubated the samples in a thermo cycler (DNA Engine, Peltier Thermal Cycler – BioRad) at 37ºC for 1h. After this, we added water until 200µl of volume.

3.6.3 Gene expression analysis qPCR

We measured gene expression by Real-time Polymerase Chain Reaction (PCR) technique, also known as quantitative PCR (qPCR). With qPCR the product is measured at each cycle. We can determine the initial quantity of the target gene by monitoring reactions during the exponential amplification phase. The fluorescence observed during the reaction is equal to the amplification of the target gene.

Each sample was duplicated in two wells, and in each set of wells the amplification of a different gene is carried. For this we used Sybr Green (GoTaq® qPCR MasterMix, Promega) as follows (Table 3).

Component	Volume (x well)
Sybr Green	5 μl
Forward Primer	0,06 μl
Reverse Primer	0,06 μl
H_2O	7,88 μl
cDNA	2 μl

Table 3. Promega reactives to perfom qPCR

We used Applied Biosystems 7500 Real-Time PCR System protocol (Table 4). Primer sequence can be found in Table 1 of the Appendix.

Stage	Temperature (ºC)	Time
Hold Stage (1x)	95 ºC	2 minutes
Cycling Stage (40x)	95 ºC	3 seconds
	60 ºC	30 seconds
Melt curve stage as defined by AB System		

Table 4. qPCR protocol

3.7 Genomic DNA extraction and methylation analysis

3.7.1 Obtaining of genomic DNA

We extracted genomic DNA from liver with Promega® Reagents. We started with 50-100mg of tissue. We incubated the samples with Proteinase K (20mg/ml) on a rotating platform at 37ºC until the tissue disappeared. Then we precipitated the proteins and transferred the supernatant containing DNA to a new falcon tube. With 2-propanol we precipitated the DNA forming a medusa and then washed it with 70% ethanol. Once the DNA pellet is dry, we added 40-90µl of DNA Rehydration Solution depending on pellet size. After this DNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, Schwerte, Alemania). To assess the purity of DNA the ratio of absorbance at 260nm and 280nm is used, accepting a ratio of 1.8 as "pure" for DNA. Same as with RNA purity assessment, a second measurement of purity of DNA is done with 260/230 ratio, that should be in the range of 2.0-2.2.

3.7.2 Methylation analysis

Samples were analyzed following Agilent Microarray Analysis of Methylated Inmunoprecipitation v1.1 protocol. We obtained a general view of differentially methylated regions between C and ON of sperm and liver of the two different generations. Microarrays and bioinformatics analysis were done at Bioarray S.L. (Elche, Alicante).

We validated the microarrays by bisulfite conversion and pyrosequencing in collaboration with Dr. Jorg Töst (Laboratory for Epigenetics and Environment, Centre National de Génotypage, CEA-Institute de Génomique, France).

Further validations of targeted genes were done by bisulfite conversion and pyrosequencing in collaboration with Dr. David Monk (Imprinting and Cancer group, Cancer Epigenetic and Biology Program, Institut d'Investigació Biomedica de Bellvitge, Hospital Duran i Reynals, Spain).

3.8 Gut microbiota DNA extraction

We extracted DNA of cecum contents with the PowerSoil DNA Isolation Kit© (MOBIO Laboratories), using manufacturer's protocol with a minor variation. We used all of cecum content obtained from the animals, and we added an extra heating step to help dissolve the sample. In the last step, elution was done using 50µl two times instead of only one elution of 100µl. This was done to increase the yield and DNA concentration of each sample.

We then quantified the DNA concentration using NanoDrop Spectrophotometer (Thermo Scientific, Schwerte, Alemania). Considering purity values of ratio 260/280 as explained above.

Bacterial DNA was analyzed using Illumina MiSeq System to sequence the V3-V4 region of the 16S rRNA gene. We also performed qPCR to quantify presence of bacteria, using a different protocol (Table 5).

Stage	Temperature (ºC)	Time
Hold Stage (1x)	95 ºC	5 minutes
Cycling Stage (40x)	95 ºC	15 seconds
	60 ºC	40 seconds
Hold stage (1x)	72 ºC	5 min
Melt curve stage as defined by AB System		

Table 5. Bacterial qPCR protocol

To analyze the differences in gut microbiome we used UniFrac measurements that is a distance metric used to compare biological communities, in our analyses we used the unweighted variant that considers the absence or presence of organisms. In combination with principal coordinate analysis (PCoA) one can explain differences among microbial communities (Lozupone et al., 2011). To identify differences of organism classes between samples we used the linear discriminant analyses (LDA) effect size (LEfSE) method, that is an algorithm that characterizes the differences between biological conditions or classes within samples (Segata et al., 2011).

3.9 Histology analysis

Tissues for histology analyses were fixed in paraformaldehyde 4% at sacrifice. Samples were cut and stained by the department of Anatomical Pathology of Hospital Sant Joan de Déu.

3.9.1 Ilea samples

Ilea sections were stained using Periodic Acid Schiff (PAS) protocol and were then coded. Using Leica Application Suite v4.3.0 Software embedded in the LEICA DFC 300 FX microscope 10 villi per sample were photographed at 20x. We counted the goblet cells that are responsible for mucin and mucus production in the ileum, for each villus photographed. Using Fiji (ImageJ v2.0.0) we measured the villi height and crypt depth.

3.9.2 Adipose tissue samples

Adipose tissue samples were stained using hematoxylin and eosin protocol. Sections were coded and using Leica Application Suite v4.3.0 Software embedded in the LEICA DFC 300 FX microscope, 30 areas per sample were photographed at 20x. Using Fiji (ImageJ v2.0.0), area of the adipocytes was measured.

3.10 Biochemical analyses

3.10.1 Mouse milk samples

To analyze macronutrient components in mouse milk samples we diluted them 1:3 and analyzed them by mid-infrared spectroscopy in a Miris Analyzer (Miris AB). We determined relative betaine concentrations in mouse milk samples by liquid chromatography (Acquity UPLC BEH HILIC column, Waters) coupled to mass spectrometry (QqQ/MS 6490, Agilent).

3.10.2 Plasma samples

We determined plasma insulin by ELISA (Millipore), following manufacturer's instructions.

3.10.3 Triglyceride quantification in liver and fecal samples

We quantified triglycerides in liver and fecal samples (50mg) from chloroform-methanol extracts with Triglyceride Assay Kit (Sigma-Aldrich), following manufacturers instructions.

4. Results

4.1 Maternal dietary supplementation during breastfeeding

4.1.1 Introduction

Rapid weight gain during infancy increases the risk of childhood obesity (Esposito et al., 2009; Weiqin et al., 2017). The breastfeeding period is a critical window to decrease risk of childhood obesity and the risk of obesity and related metabolic diseases in later adulthood.

In collaboration with David Fields' group, we studied 34 exclusively breastfeeding mother-infant dyads within a range of pre-pregnancy BMI (18,5 – 47,2 kg/m^2). The focus was to quantify one-carbon metabolite levels in breast milk, and to determine potential associations with infant growth.

To study infant growth, we used weight-for-length z-score (WLZ) that is calculated according to tables from World Health Organization (WHO) website, age and sex determinant using the weight and length of each child. With z-score, data was normalized so that average was zero and standard deviation was 1. One month-old infants' WLZ, fat, and lean mass were higher in infants born to obese or overweight (OWO) mothers, compared to normal weight (NW) mothers (Table 6).

	All (n=34)	NW (n=15)	OWO (n=19)	p value
MOTHER				
Age (Years)	29,1 (5,1)	27,7 (5,5)	30,1 (4,7)	0,1820
Pre-pregnant BMI	27,3 (7,1)	21,6 (1,8)	31,8 (6,4)	**<.0001**
INFANT				
Birth				
Gender (female/male)	19/15	9/6	10/9	0,6675
Gestational age (weeks)	39,6 (1,2)	39,4 (1,1)	39,8 (1,2)	0,2945
Weight (kg)	3,53 (0,48)	3,39 (0,51)	3,64 (0,45)	0,1477
Length (cm)	51,6 (2,3)	51,6 (2,3)	51,6 (2,3)	0,9885
1 month				
Weight (kg)	4,67 (0,71)	4,45 (0,63)	4,84 (0,73)	0,1154
Weight gain (kg)	1,14 (0,44)	1,06 (0,36)	1,20 (0,50)	0,3607
Length (cm)	55,8 (2,1)	55,8 (2,1)	55,8 (2,2)	0,9552
WLZ	-0,36 (1,04)	-0,87 (0,91)	0,05 (0,98)	**0,0084**
Head circumference (cm)	38,3 (1,3)	38,4 (1,4)	38,2 (1,3)	0,6762
DXA fat mass (%)	24,1 (2,8)	22,9 (3,0)	25,0 (2,4)	**0,0266**
DXA fat mass (kg)	1,19 (0,29)	1,06 (0,27)	1,30 (0,26)	**0,0130**
DXA lean mass (kg)	3,64 (0,50)	3,45 (0,46)	3,78 (0,48)	**0,0497**

Table 6. Infants weight compared to mothers BMI. Values represent mean (SD). Bold font indicates p<0,05. [a]Two-tailed *t*-test between NW and OWO groups for continuous variables; Pearson chi-square for categorical variables (gender). NW, normal weight; OWO, overweight or obese; WLZ, weight-for-length z-score

When determining one-carbon metabolite levels in milk samples 1 month after birth, there were no differences in levels between NW and OWO mothers (Table 7). Of these metabolites, the only one that significantly correlated with WLZ at 1 month of life was betaine, observing a trend for SAH and cystathionine (Table 8). Betaine correlation in infants from OWO mothers was especially strong, with no association among infants with NW mothers (Figure 2).

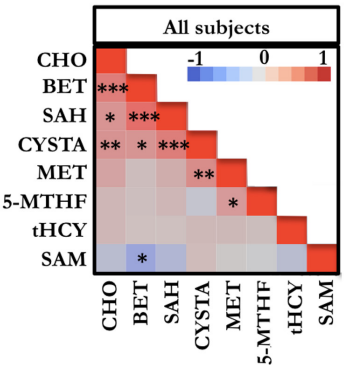
BREAST MILK METABOLITES	All (n=34)	NW (n=15)	OWO (n=19)	p value
Choline (μ mol/L)	96,8 (49)	96,3 (61,4)	97,1 (38,2)	0,541
Betaine (μ mol/L)	3,12 (2,65)	3,34 (3,30)	2,95 (2,09)	0,951
Methionine (μ mol/L)	4,39 (2,69)	4,70 (3,51)	4,15 (1,89)	0,732
SAM (nmol/L)	1469 (436)	1427 (483)	1503 (406)	0,542
SAH (nmol/L)	216 (81)	201 (83)	227 (80)	0,279
Cystathionine (nmol/L)	44,9 (34,3)	44,0 (35,4)	45,7 (34,3)	0,896
5-methyltetrahydrofolate (nmol/l)	29,5 (15,9)	27,0 (16,5)	31,5 (15,6)	0,420
Homocysteine (nmol/L)	207 (68)	215 (55)	201 (77)	0,529

Table 7. Breast milk metabolites. Correlation of quantified metabolites in breast milk and mothers BMI. NW, normal weight; OWO, overweight or obese. Values represent mean (SD). [a]Two-tailed *t*-test between NW and OWO groups

	All subjects				Normal weight				Overweight and Obese			
	Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted	
	r	p val	β std	p val	r	p val	β std	p val	r	p val	β std	p val
CHO	-0,09	0,593	-0,07	0,666	0,01	0,981	0,05	0,890	-0,35	0,142	-0,38	0,200
BET	**-0,40**	**0,018**	**-0,37**	**0,041**	**-0,09**	**0,759**	**0,07**	**0,904**	**-0,78**	**<0,001**	**-0,81**	**<0,001**
MET	0,08	0,649	0,15	0,441	0,16	0,557	0,11	0,760	0,09	0,718	0,18	0,664
SAM	0,04	0,822	-0,06	0,755	-0,10	0,716	0,16	0,720	0,07	0,772	0,09	0,776
SAH	-0,29	0,094	-0,31	0,068	-0,14	0,609	0,07	0,854	**-0,66**	**0,002**	**-0,83**	**0,001**
CYSTA	-0,34	0,050	-0,33	0,062	-0,34	0,216	-0,15	0,693	-0,43	0,070	**-0,64**	**0,042**
5-MTHF	0,13	0,449	0,10	0,568	0,32	0,248	0,17	0,614	-0,21	0,394	-0,18	0,577
tHCY	-0,00	0,996	0,09	0,629	0,13	0,643	0,15	0,650	0,05	0,836	0,08	0,797

Table 8. Multivariate correlations between milk metabolites and infant WLZ at 1 month of life. Metabolite levels were log-transformed and the correlation to 1-month WLZ was assessed. Bold font indicates $p<0.05$. ßstd, standardized beta coefficient from regression model; CHO, choline; BET, betaine; MET, methionine; SAM, s-adenosylmethionine; SAH, s-adenosylhomocysteine; CYSTA, cystathionine; 5-MTHF, 5-methyltetrahydrofolate; tHCY, total homocysteine. [a]Model adjusted for maternal age, pre-pregnacy BMI, gestational age, and birth WLZ.

a. Milk metabolites correlation with mothers BMI



b. Betaine content correlation with 1 month WLZ

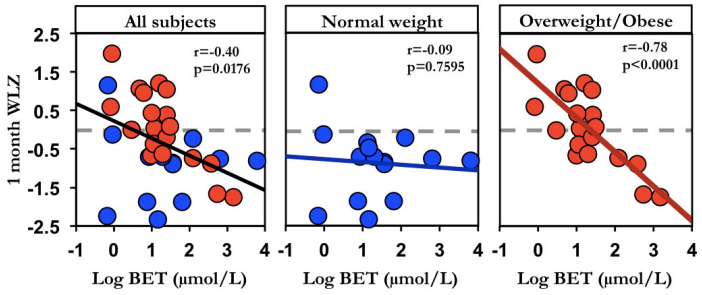


Fig. 2. Betaine content in milk correlates with 1 month WLZ. a) Heatmap of Pearson correlation coefficients among log-transformed milk metabolite levels. b) Bivariate plots between WLZ and milk betaine concentration at 1 month for all, normal weight (blue circles) and overweight/obese mothers (red circles). CHO, choline; BET, betaine; MET, methionine; SAM, s-adenosylmethionine; SAH, s-adenosylhomocysteine; CYSTA, cystathionine; 5-MTHF, 5-methyltetrahydrofolate; tHCY, total homocysteine.

Work from this laboratory has already suggested a protective role for betaine in adult obesity (Ejaz et al., 2016). With this in mind, and the results from breast milk analyses, we aimed to study the effect of betaine in earlier stages of life. We focused on the lactation period, where we could easily supplement the dams' diet with betaine and study the effects on the offspring at different time points: 2 week-old, 6 week-old and 24 week-old.

For this purpose, we randomly assigned dams to treatment group supplementing the drinking water with 1% of betaine at delivery day (betaine group or B), or the control groups (C) with no supplementation. Betaine supplementation was given exclusively to the mother during the lactation period. After weaning mice from both groups drank the same un-supplemented water, and were fed a chow diet. Experiment planning is shown in Figure 3.

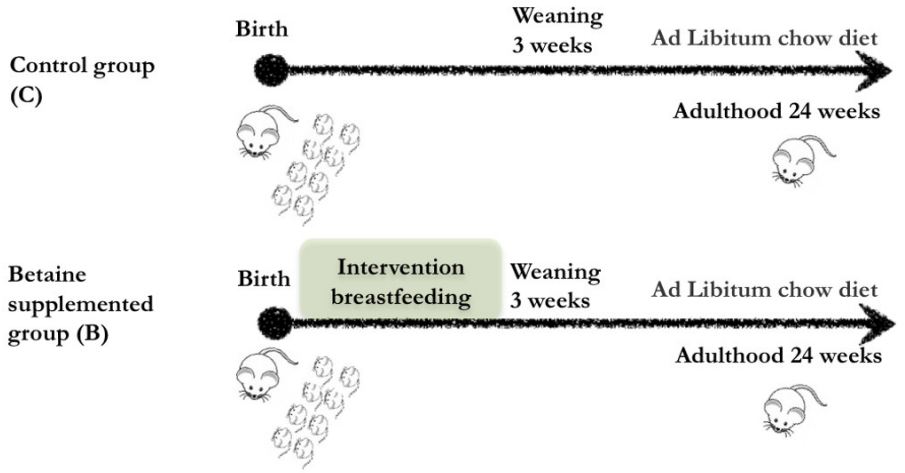


Fig 3. Maternal betaine supplementation. Betaine (1% wt/vol) was added to dams drinking water during lacatation. After weaning pups were feed normal diet ad libitum until adulthood.

4.1.3 Characterizing betaine effects during breastfeeding

4.1.3.1 Betaine early-life effects

We first analyzed milk from the lactating dams to assess that pups were indeed receiving betaine through the milk. We collected milk samples from both groups at day 15 after delivery. Analysis of the samples showed increased levels of betaine in the milk of the dams from the supplemented group without altering macronutrient composition (Figure 4a, 4b). We also analyzed betaine content in the 2 week-old pups' plasma, finding increased levels in the offspring of betaine-supplemented dams (Figure 4c). Milk intake in both groups was

similar (Figure 4d) and offspring body weight during lactation didn't seem affected by the supplementation (Figure 4e). Surprisingly, growth rate at 1-week old mice was slightly reduced in betaine group, but very significantly (Figure 4f).

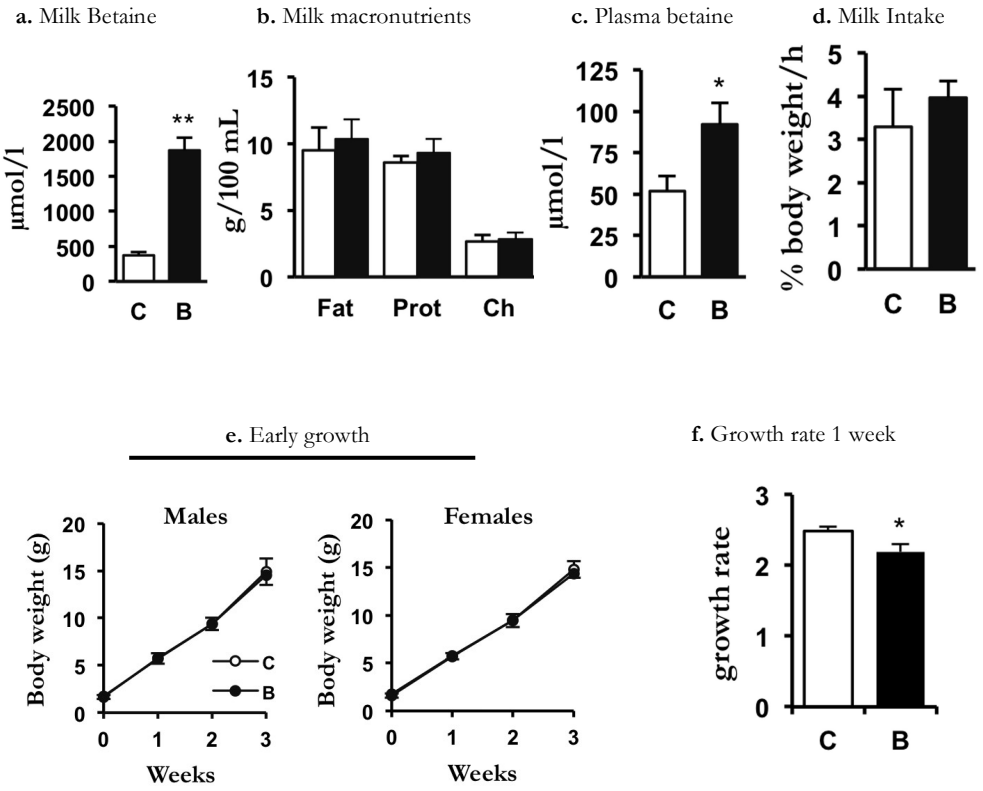


Fig. 4. Early growth analysis. Samples collected 15 days after delivery, during lactation and when supplementing betaine. a) Betaine content in milk from lactating dams 15 days after delivery. b) Macronutrients present in milk (Prot = protein, Ch = carbohydrates). c) Betaine content in plasma of 15 day-old pups. d) 1 week milk intake of pups. e) Body weight during the first 3 weeks of life of males and females. f) Growth rate during first week of life. Controls (C) white bars/circles (n=18), betaine-treated (B) black bars/circles (n=18) *p<0,05; **p<0,0001 Data are mean ± sem

Furthermore, when analyzing body composition at 6 weeks of age we found decreased fat mass in betaine-treated compared to control group (Figure 5a). We observed no differences in body weight of male mice from both groups (Figure 5b). White adipose tissue (both epididymal and inguinal, eWAT and iWAT) and liver from both groups had similar weight (Figure 5c, 5d). Other parameters like glucose tolerance, O_2 consumption, respiratory exchange ratio and activity levels were not different between the two groups at 6 weeks of age (Figure 5e, 5f, 5g, 5h)

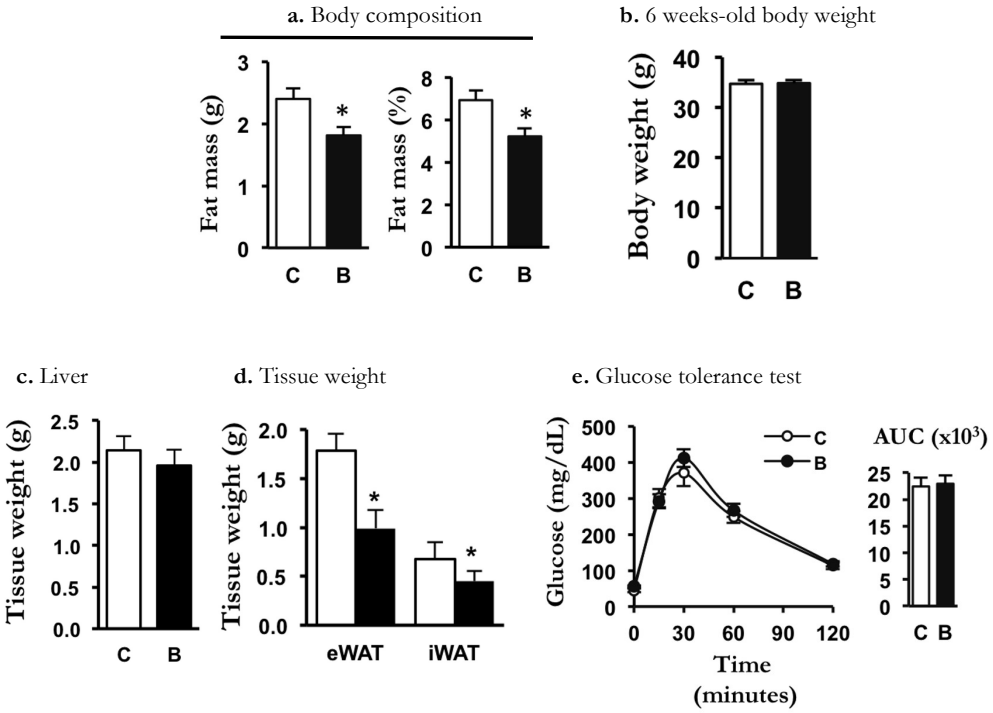


Fig 5. Betaine early-life effects. Growth parameters of 6 weeks-old mice. a) Fat mass (n=8/group) b) Total body weight c) Tissue weight at 6-weeks-old sacrificed mice e) Glucose tolerance at week 6. Control (C) white bars/circles (n=10), betaine-treated black bars/circles (n=12) *p<0,05 Data are mean ± sem

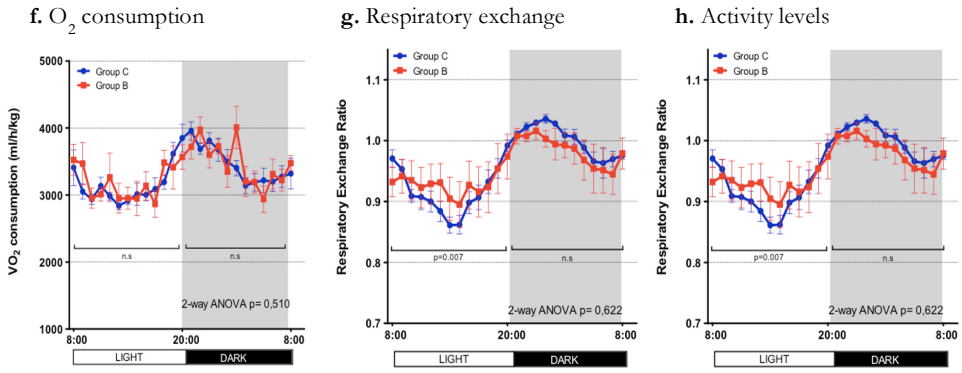


Fig. 5 (cont). Betaine early-life effects. **Growth parameters of 6 weeks-old mice.** f-h) O_2 consumption, respiratory exchange ratio and activity levels monitored in metabolic chambers (n=8 and n=5 for C (blue) and B (red) groups).
*p<0,05 Data are mean ± sem

4.1.3.2 Betaine long-term effects

To determine if supplementation of betaine during lactation has long-term effects on the offspring, we monitored pups weight and metabolic parameters until adulthood (24-week-old). Male mice from betaine-treated dams had lower body weight throughout the months compared to controls, increasing this difference with age (p<0,01, Figure 6a). Liver in male mice was of similar weight between both groups (Figure 6b). eWAT and iWAT weight was lower in the betaine-treated group (Figure 6b). In accordance, adipocyte size (Figure 6c) and inflammatory markers in eWAT were also decreased (p<0,05, Figure 6d).

Male mice from betaine group had improved glucose tolerance and basal insulin levels in betaine group were decreased when compared to control group (p<0,05, Figure 6e, 6f). When we assessed insulin tolerance we could not detect any difference in response to the insulin administration, however glucose levels at each time point were significantly lower from the betaine group (Figure 6g).

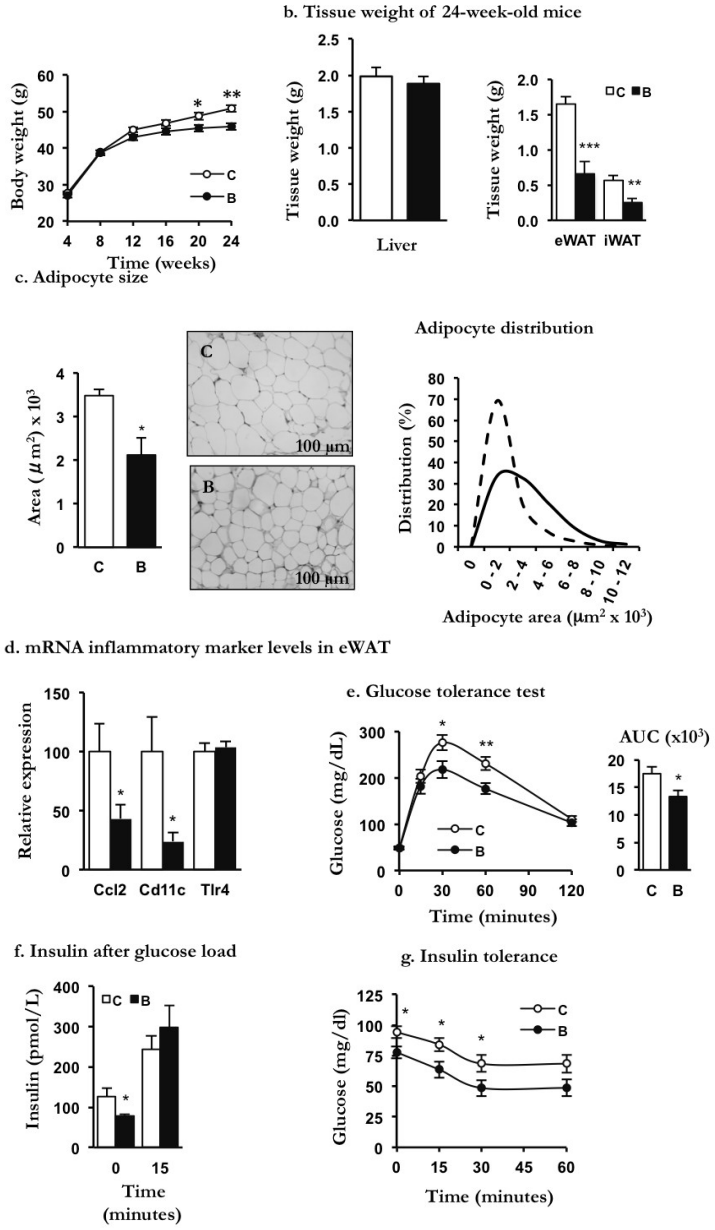


Fig 6. Betaine long-term effects. Supplementation of betaine during lactation has metabolic effects throughout adulthood. d) mRNA levels of inflammatory markers in eWAT. e) Glucose tolerance at week 24. f) Insulin levels at times 0 and 15 after glucose load. g) Insulin tolerance test at week 24. White bars/circles, n=16 controls; black bars/circles n=16 betaine-treated dams *p<0,05, **p<0,01 Data are mean ± sem

In 24 weeks-old female mice there were no differences in body weight or tissue weight between controls and betaine-treated groups (Figure 7a, 7b). Furthermore, glucose tolerance in females was not affected by betaine supplementation suggesting betaine effects are sex-dependent (Figure 7c).

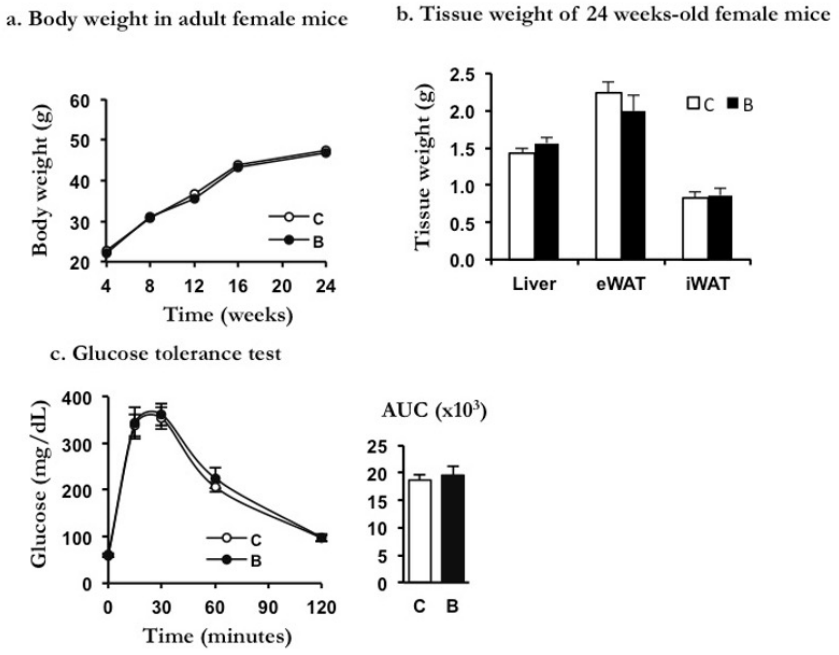


Fig. 7. Betaine long-term effects in females. Growth and metabolic parameters in 24-week old females. a) Total body weight during adult female mice. b) Tissue weight at sacrifice c) Glucose tolerance test. White bars/circles, n=12 controls; black bars/circles, n=10 betaine-treated dams

4.1.4 Betaine long-term effects when challenged with adult diet-induced obesity

Diet during adulthood is also an important factor in developing obesity. The abovementioned results brought us to ask if early-life betaine administration could have protective aspects against diet-induced obesity during adulthood. For this goal, we fed 6 week-old offspring from control or betaine-treated dams a HFD for 16 weeks (control, HF-C and betaine, HF-B, experimental design Figure 8). We observed a modest decrease in food intake that was not statistically significant (Figure 9a), along with no differences in fecal lipid content (Figure 9b). HF-B showed lower body weight gain compared to the control group both in males and females (Figure 9c).

Again very importantly, glucose tolerance was improved by supplementation (Figure 9d) and fasting glucose was decreased as well as insulin levels (Figure 9e, 9f). Hepatic triglyceride levels were reduced in the HF-B group (Figure 9g).

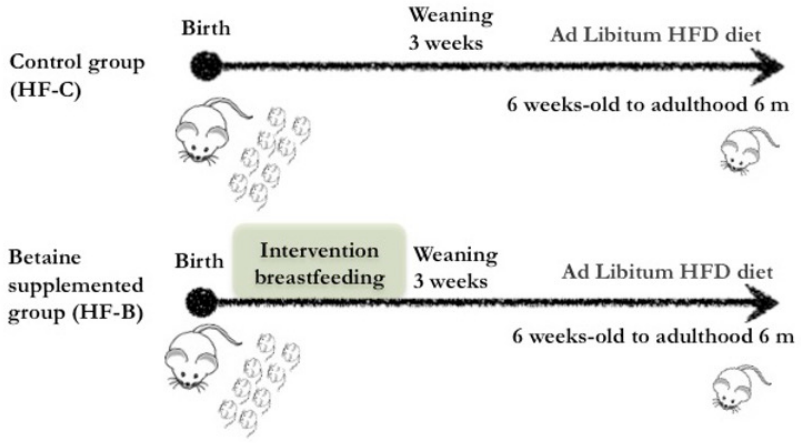


Fig. 8. Diet-induced obesity mouse model. Both betaine-treated and control 6 weeks-old offspring were fed a high fat diet (HFD) ad libitum during adulthood until sacrifice.

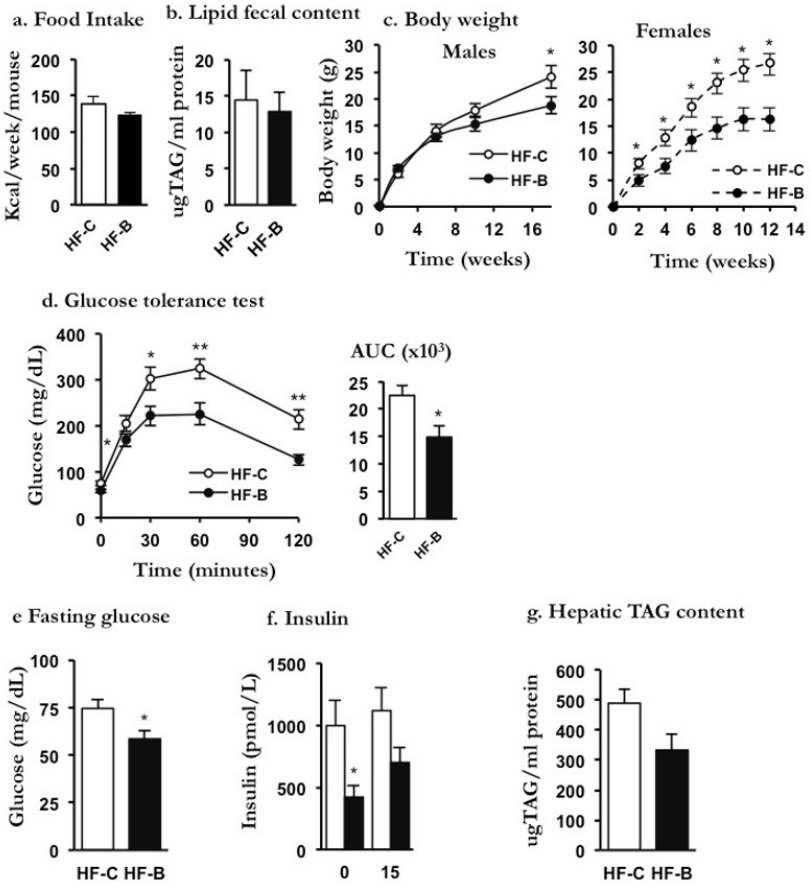


Fig. 9. Betaine administration during lactation has long-term effects when feeding high fat diet (HFD) during adulthood. a) HFD food intake during 16 weeks was similar between groups. b) Lipid fecal content was also similar between groups. c) Betaine treated mice had lower body weight even when challenged with HFD during adulthood. d) Tissue weight at sacrifice after 16 weeks of HFD. e) Glucose tolerance test. f) Fasting glucose before glucose load. g) Insulin levels at fasting and after glucose load. h) Hepatic triglicerid levels. White bars/circles controls; black bars/circle betaine-treated dams. Males n=14 HF-C, n=20 HF-B; females n=14/group. *p<0,05; **p<0,001 Data are mean ± sem

4.1.5 Offspring gut microbiota modulation by maternal diet supplementation

Since early-nutrition plays an important role in defining gut microbiome, we next tested if betaine supplementation could modulate the gut microbiota. Bacterial community diversity varies along the gastrointestinal tract; animal studies show that it reaches highest phylogenetic diversity in the cecum part compared to the rest (Gu et al., 2013). To analyze gut microbiota we retrieved cecum content from 2 week-old pups and 6 week-old pups.

To study the microbiome in the gut we used UniFrac measurements. In combination with principal coordinate analysis (PCoA) one can explain differences among microbial communities (Lozupone et al., 2011). With Principal Coordinate Analysis (PCA) of unweighted UniFrac distances we observed that microbial community composition at 2 weeks of age was modulated by betaine supplementation, but no differences were observed at 6 weeks of age (Figure 10a). Using LEfSE we were able to identify significant differences in bacterial groups in gut of 2 weeks-old mice, specifically *Akkermansia* spp. that was incremented in betaine-supplemented pups (Figure 10b). We confirmed this result by qPCR were we detected this increment in betaine-treated group (Figure 10c). Interestingly, betaine did not modify maternal gut microbiota (Figure 10d).

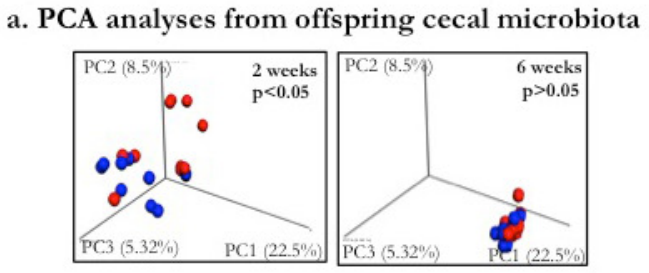


Fig 10. Gut microbiota analyses. a) Principal coordinate analyses (PCA) of unweighted UniFrac distances of cecal microbiota from 2 and 6 weeks offspring male mice of control (C, blue circles, n=10) and betaine-treated dams (B, red circles, n=10).

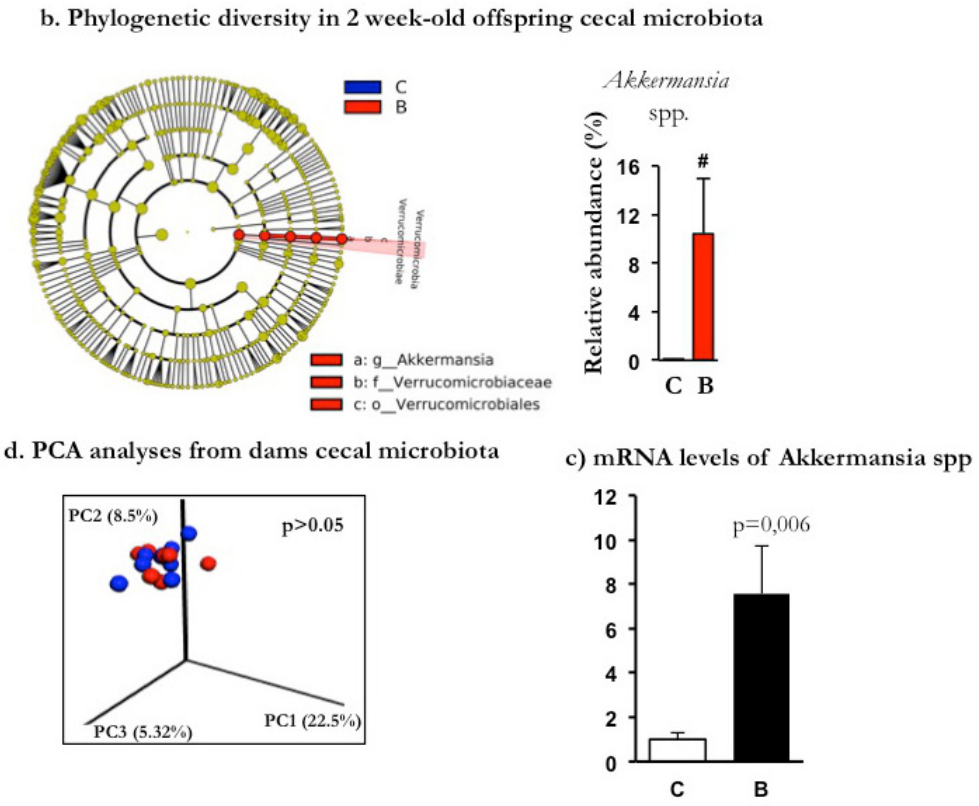


Fig 10 (cont). Gut microbiota analyses. a) Principal coordinate analyses (PCA) of unweighted UniFrac distances of cecal microbiota from 2 and 6 weeks offspring male mice of control (C, blue circles, n=10) and betaine-treated dams (B, red circles, n=10). b) Phylogenetic diversity in C and B groups. c) mRNA levels of Akkermansia spp in 2 week-old cecal samples. d) PCA of unweighted UniFrac distances of cecal microbiota from betaine-treated dams (B, red circles, n=4) and control dams (C, blue circles, n=4).

Considering these microbiota differences, and knowing it can affect gut histology (Gouyer et al., 2011) we next analyzed ileum histology from both groups (Figure 11).

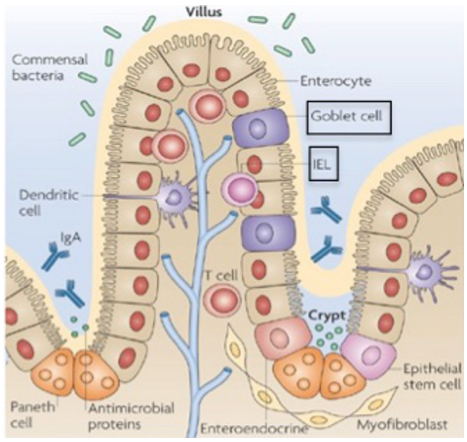


Fig 11. Ilea histology

To analyze changes in gut structure we measured villi length and crypt depth finding no differences between groups at 2 weeks old, 6 weeks old or 24 weeks-old mice (Figure 12a, 12b).

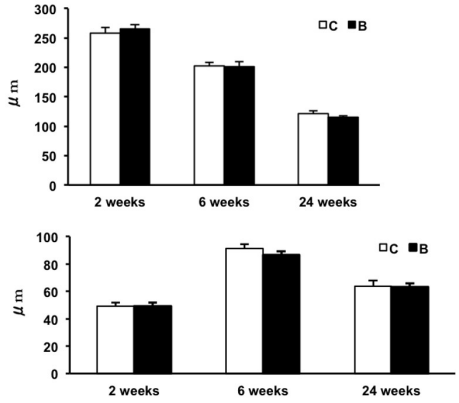
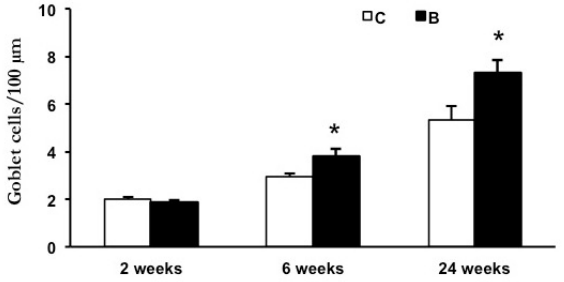


Fig. 12. Ileal structure. a) Villi height (VH) of 2 weeks-old, 6 weeks-old and 24 weeks-old male mice was measured. b) Crypt depth (CD) of 2 weeks-old, 6 weeks-old and 24 weeks-old male mice was measured *p<0,05. Data are mean± sem

W

When analyzing goblet cells, no differences were found between control and betaine male mice at 2-weeks of age. However, we did find increased goblet cell number in 6-week-old and 24 weeks-old betaine treated mice (Figure 12c, 12d).

c. Goblet cell count/100µm of villi



d. Fixed ileum section

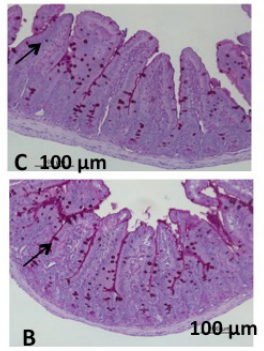


Fig. 12 (cont) . Ileal structure. c) Goblet cell count/100µm of villi of ilea from 2 weeks-old, 6 weeks-old and 24 weeks-old male mice. d) Fixed ileum section stained with hematoxilin/eosin, 10villi per mice were measured; arrows indicate goblet cells after PAS staining. Control white bars, Betaine black bars, n=16/group. *p<0,05. Data are mean± sem

As goblet cells are responsible for producing mucous, and they were incremented in 6-week-old and 2-week-old mice, we then analyzed *Muc2* (Mucin 2, oligomeric mucus/gel-forming) expression in ilea, principal protein found in mucous. Levels of *Muc2* were increased in betaine-treated groups without reaching significant differences, at 2 and 6-week-old mice. In 24-week-old mice, *Muc2* levels were significantly increased in betaine-treated mice (Figure 13a). To further analyze ileal barrier function we also determined gene expression of tight junction proteins. There were increased mRNA levels of *Ocln* (Occludin) at 24 week-old betaine-treated mice, (p=0,01, Figure 13b), finding no differences at 2 week-old and 6 week-old mice. *Zo1* (Tight junction protein 1) and *Zo2* (Tight junction protein 2) had similar mRNA levels at 2 week-old mice (Figure 13c, 13d). We observed a tendency (p=0,09) to increased levels of *Zo2* at 6 week-old mice, and no differences of *Zo1* (Figure 13c, 13d). mRNA levels of *Zo1* and *Zo2* were significantly increased in 24 weeks-old betaine-treated mice compared to control groups (p<0,01, Figure 13c, 13d).

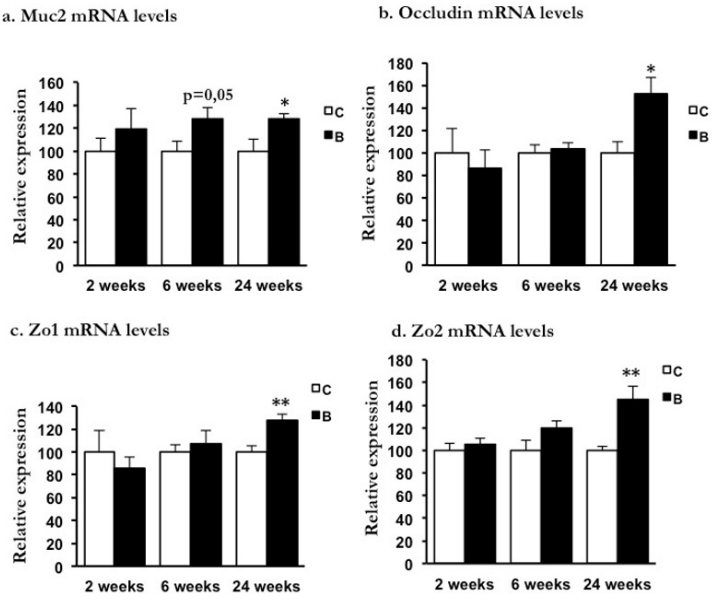


Fig. 13. mRNA levels of Muc2 and tight junction proteins in ilea. a) Muc2 mRNA levels. b to d) mRNA levels of tight junction proteins in 2, 6 and 24 weeks-old male offspring from betaine-treated dams (black bars, n=6/8) and controls (white bars, n=6/8). *p<0,05; **p<0,01

Changes in gut microbiota, and specifically *A. muciniphilla* are known to correlate to intestinal barrier function, low-grade systemic inflammation and metabolic dysfunction. Therefore, we analyzed mRNA levels of inflammatory markers in eWAT of both groups. *Ccl2* (C-C motif chemokine ligand 2) was significantly decreased in 6 week-old and 24 week-old betaine-treated mice ($p<0,05$, Figure 14a). *Cd11c* (Integrin subunit alpha X, *Itgax*) was decreased at both time points but only significantly at 24 week-old betaine-treated mice (Figure 14b). *Tlr4* (Toll-like receptor 4) was significantly decreased at 6 weeks-old mice, but levels were similar at 24 week-old betaine-treated mice (Figure 14c). We only determined mRNA levels of *Nos2* (Nitric oxide synthase 2) from 6-week-old male mice observing a decrease (Figure 14d). *Ccl2* was also decreased in other metabolic tissues in 6 week-old mice (brown adipose tissue; BAT, liver and skeletal muscle; SkM) ($p<0,05$, Figure 14e).

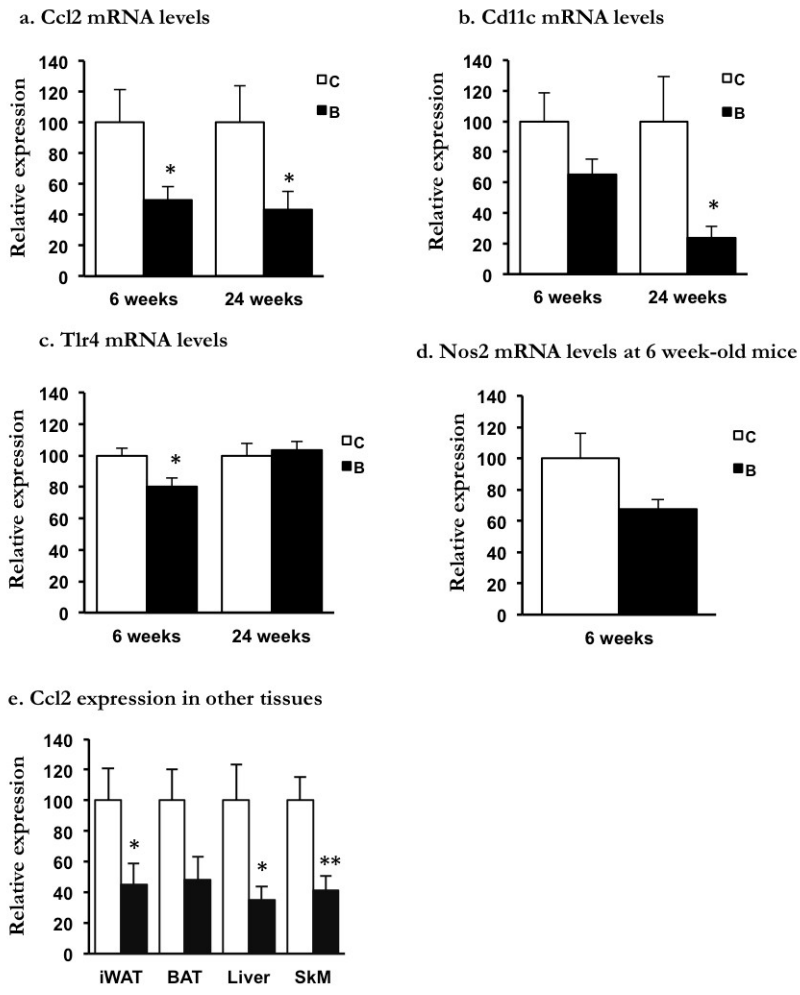


Fig 14. mRNA levels of inflammatory markers are decreased in eWAT offspring of betaine-treated dams. a) Ccl2 mRNA levels in eWAT of 6 weeks-old and 24 week-old offspring of control and betaine-treated dams. b) Cd11c mRNA levels eWAT of 6 week-old and 24 week-old offspring of control and betaine-treated dams. c) Tlr4 mRNA levels eWAT of 6 week-old and 24 week-old mice. d) Nos2 mRNA levels eWAT of 6 week-old mice e) Ccl2 mRNA levels in other tissues from 6 week-old male mice. 6 week-old controls, white bars (n=8), betaine-treated black bars (n=10); 24 week-old controls; white bars (n=16), betaine-treated; black bars (n=15). *p<0,05; **p<0,01 Data are mean± sem

4.1.6 Effects of antibiotics in offspring gut microbiota and consequences in long-term metabolic health outcomes.

We then asked ourselves if betaine had a role in defining the early gut microbiome, or if the microbiota contributed to betaine's long-term metabolic effects. Antibiotics are known to disrupt gut microbiota when administered in early-stages of life, even when given to the mother during lactation periods (Cox et al., 2014). For this we co-administered antibiotics to the dams along with betaine (Figure 15). We analyzed mice at 2, 6 and 20 weeks of age.

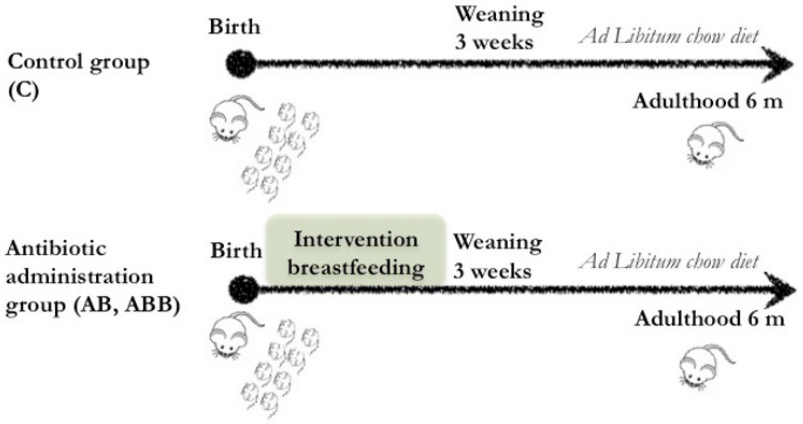


Figure 15. Maternal betaine supplementation and antibiotics administration during lactation. Antibiotics (ampicilin, 1g/l; and neomicin, 0,5g/l) were added to dams drinking water with (ABB) or without (AB) betaine (1% wt/vol). After weaning pups were feed normal diet *ad libitum* until adulthood.

Antibiotic treatment annulled completely betaine effects in a long-term manner. Body weight was no longer decreased in animals that received antibiotic, not even when combined with betaine (Figure 16a). Furthermore, eWAT depot was no longer decreased by betaine treatment, and liver weight was slightly increased in mice receiving antibiotics (Figure 16b). Glucose tolerance was no longer improved when combining antibiotics with betaine treatment, and basal insulin levels were increased (Figure 16c, 16d). In addition, inflammatory markers in eWAT were not decreased in the presence of antibiotics (Figure 16e).

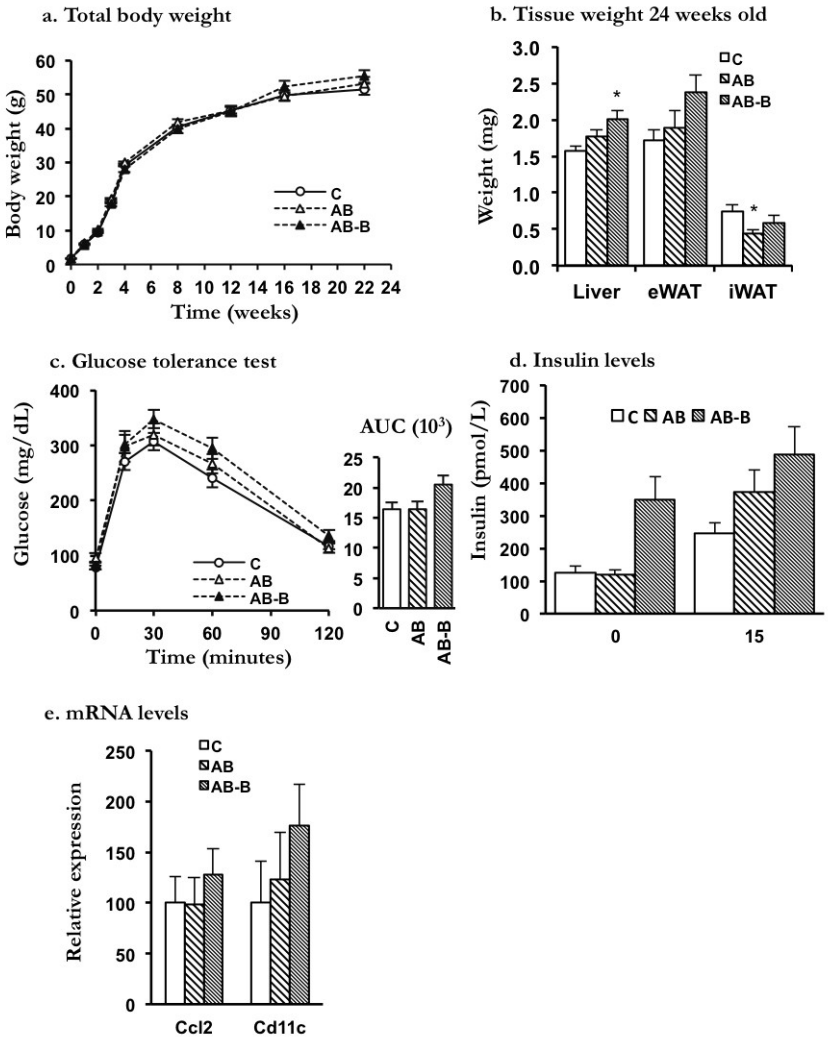


Fig 16. Growth parameters in offspring of antibiotic administered dams. a) Total body weight in adult male mice (n=16 controls, n=16 betaine). b) Tissue weight at 24 weeks sacrifice. c) Glucose tolerance test at 24 weeks. d) Insulin levels after glucose load. e) mRNA levels of inflammatory markers in eWAT. *p<0,05; **p<0,001 Data are mean± sem

4.1.7 Betaine administration during lactation protects the offspring against maternal obesity adverse effects.

As parental overweight or obesity is a risk factor for childhood obesity and accelerated weight gain in early-life (Weiqin et al., 2017), we then decided to study how betaine supplementation can interact with maternal overweight or obesity and infant growth. For this we fed dams a high-fat diet for 8 weeks before mating, and assigned them to control (MO-C) or betaine-treated (MO-B) at delivery as done before. After weaning offspring was fed a normal chow diet (Figure 17).

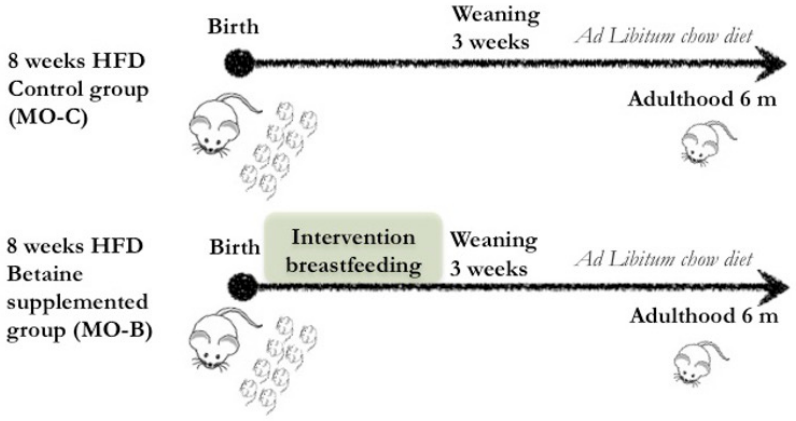


Fig 17. Maternal obesity mouse model. Dams were fed a high fat diet during 8 weeks before mating and throughout lactation. After weaning offspring was fed normal diet *ad libitum* during adulthood until sacrifice. Betaine was supplemented in dams water during lactation (1% w/vol).

4.1.7.1 Betaine early-life effects against maternal obesity adverse effects

Compared to normal weight mothers, maternal obesity resulted in increased weight gain during lactation (Figure 18a). Maternal betaine supplementation moderated offspring growth from overweight dams reaching similar weights as offspring from normal weight control dams of this same experiment. Again, there were no differences in milk intake from both groups (Figure 18b). At 6-weeks of age we observed decreased fat mass

in betaine-treated mice when compared to the control group, that almost reached significance (p=0,07, Figure 18c). Body weight was lower in betaine-treated mice (Figure 18d), as well as eWAT tissue weight. Liver and iWAT tissues weighed the same in both groups at 6-weeks of age (Figure 18e).

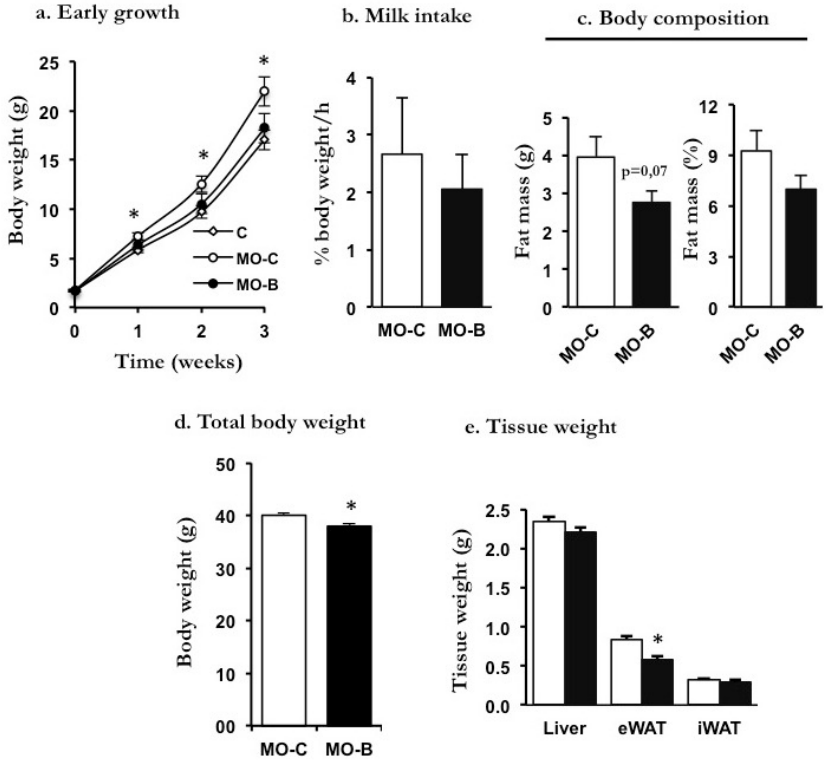


Fig 18. Betaine supplementation in a maternal obesity mouse model. a) During lactation offspring from betaine-treated dams weight less than control group. b) Milk intake in betaine-treated offspring was slightly lower c) Fat mass at 6 weeks of age d) Total body weight at 6 weeks of age. e) Tissue weight. Controls are white circles/bars (n=19), betaine-treated are black circles/bars (n=21) *p<0,05 Data are mean ± sem

4.1.7.2 Betaine long-term effects against maternal obesity adverse effects

As seen in normal weight mothers' offspring, MO-B offspring weighed significantly less at 24-weeks of age (Figure 19a). In accordance eWAT and iWAT tissue weight was also decreased compared to MO-C (Figure 19b). Liver weighed the same in both groups (Figure 19b). More importantly, betaine-treated offspring showed improved glucose tolerance and decreased insulin levels in the maternal obesity mouse model (Figure 19c, 19d) indicating betaine has beneficial long-term effects even on offspring from dams that are overweight or obese.

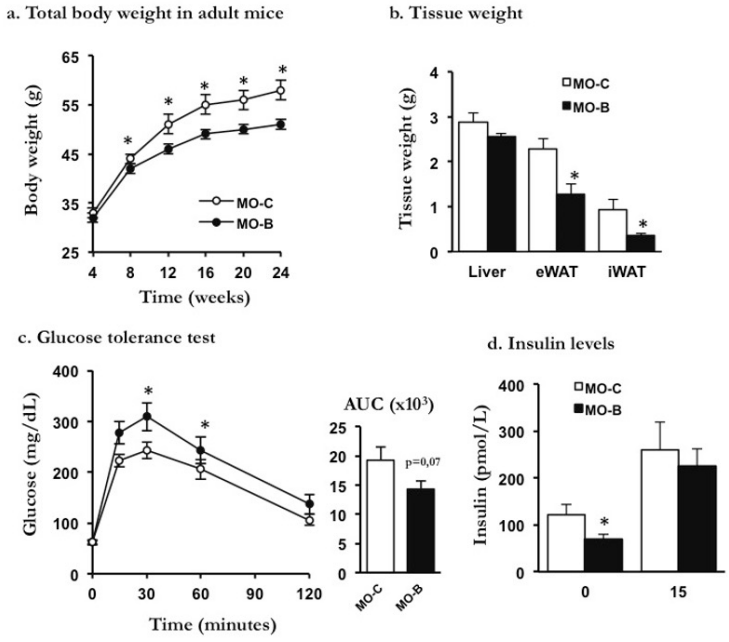


Fig 19. Long term effects of betaine on maternal obesity mouse model. a) Total body weight from 4 weeks to 24 weeks of age. b) Tissue weight in adult mice. c) Glucose tolerance test. d) Insulin levels in adult mice. Control mice white bars/circles (n= 11), betaine-treated black bars/circles (n=13). *p<0,05 Data are mean ± sem

4.2 Neonatal overfeeding

4.2.1 Introduction

As previously described, the environment in which the infant is growing will influence short and long-term health outcomes. Accelerated weight gain is strongly associated to childhood obesity. In turn, childhood obesity increases risk of obesity and metabolic disease during adulthood.

Furthermore, there is evidence that obesity has a strong heritable component and obesity-associated phenotypes can be transmitted from mothers and fathers to offspring. Many genetic variants are associated to the heritability of obesity (Burgio et al., 2015). But when the phenotype is due to environmental exposures epigenetic mechanisms are likely to be implicated in its' transmission (Pentinat et al., 2010).

4.2.2 Transgenerational phenotype inheritance

Our group has previously developed a mouse model of neonatal over-nutrition (ON) and rapid weight gain by litter size reduction (Figure 20a). Neonatal overfeeding altered the metabolism of the exposed individuals (ON). During the first weeks of age, body weight of ON mice was increased compared to controls. This difference was even more significant as animals aged (Figure 21a, 21b). In addition, ON mice developed glucose intolerance (Figure 21c) and insulin resistance (Figure 21d) by 4 months of age.

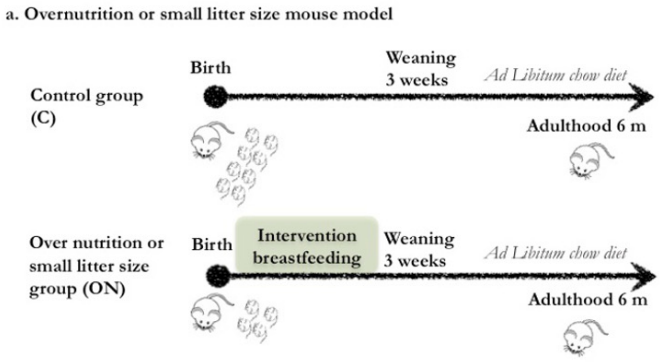


Fig 20. Neonatal overnutrition. a) Neonatal rapid weight gain was induced by reducing litter size causing overnutrition of the offspring. Control group (C) had 8 pups per litter, overnutrition group (ON) was reduced to 4 pups per dam.

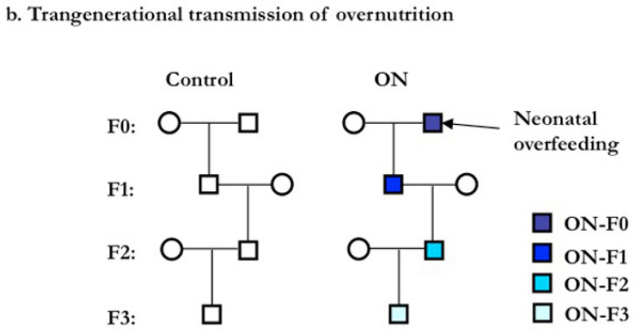


Fig 20 (cont). Neonatal overnutrition. b) Effects of neonatal overnutrition were transmitted through male line until 2nd generation and with less effects 3rd generation offspring.

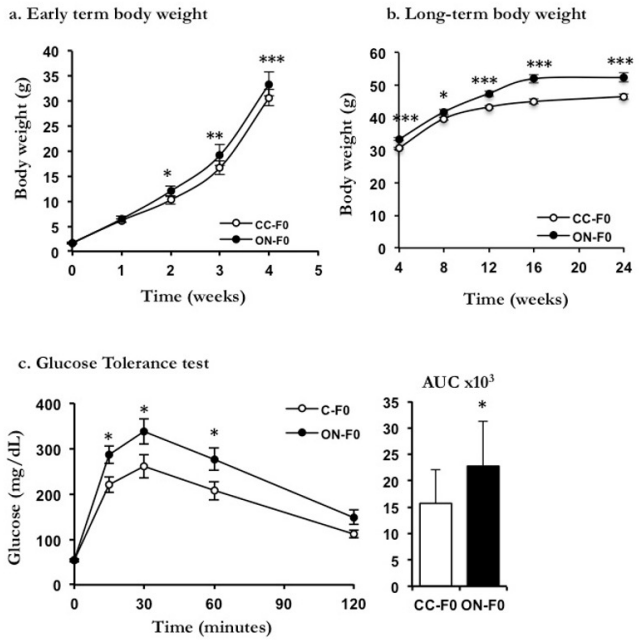


Fig 21. Postnatal over-nutrition causes rapid early weight gain and impaired glucose tolerance in adulthood. a) Total body weight gain during lactation (n≥6 in both groups). b) Total body weight during adulthood. c) Glucose tolerance test in 24-week male mice. Control male mice white circles/bars (n≥20), ON male mice black circles/bars (n≥20). *p<0,05; **p<0,01; ***p<0,001. Data are mean±sem Pentinat, et al. 2010

We next explored whether these phenotypes were transmitted to the following generation. Importantly, no intervention was done in the following generations (see material and methods, section 3.3.2) (Figure 20b). We focused on males only because they influence the phenotype of their offspring through the information contained in the sperm. In our experimental model, the males were removed from the cage upon confirmation of the pregnancy of the females. Hence, paternal physiology and/or behavior do not influence offspring phenotype and paternal transmission must occur through the information containing in the sperm: genome and epigenome. Since environmentally mediated transmission of metabolic disease occurred in several independent families, the epigenome is the likely carrier of information in our model. Maternal inheritance, on the other hand, is more complex than patrilineal transmission. Females influence their offspring phenotype through the combination of genetic, mitochondrial, metabolic, behavioral and also epigenetic modifications. Hence, exploring epigenetic inheritance through the maternal lineage is extremely difficult given the many channels of information.

Interestingly body weight of ON-F1 and ON-F2 mice was similar to that of control mice (Figure 22a). Despite having similar body weight, offspring (F1) and grand-offspring (F2) of the postnatal overfed male mice (F0) presented impaired glucose tolerance in adulthood (Figure 22b).

Our data strongly suggests that an environmental factor (nutrition) can induce modifications in the germ line that are transmitted to the following generations. However, these modifications are not permanent, as with successive generations this phenotype is diluted. Therefore, in this thesis we hypothesized that epigenetic mechanisms are implicated in the transmission of this metabolic phenotype.

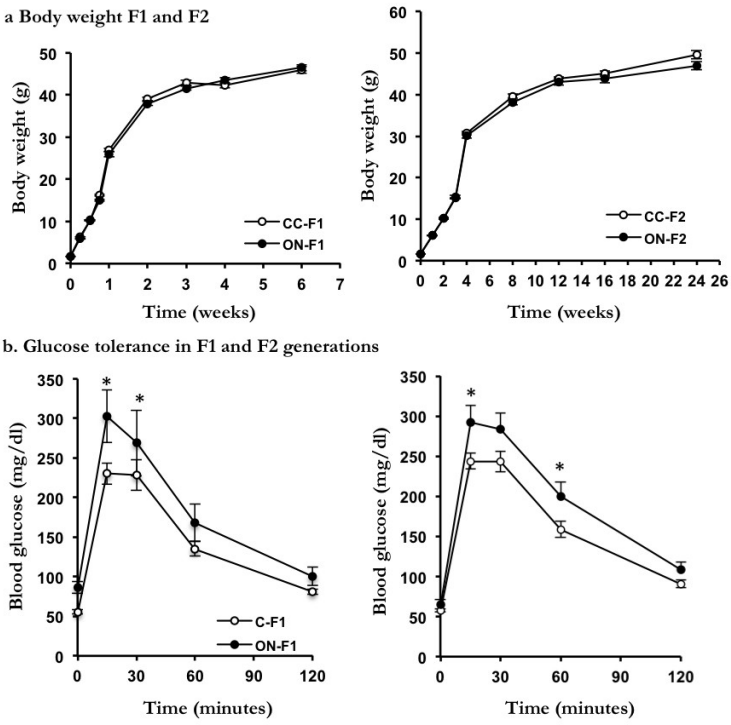


Fig 22. Postnatal over-nutrition in first generation causes higher adiposity depot and impaired glucose tolerance in adulthood. a) Total body weight during adulthood in first and second generation. b) Glucose tolerance test in 24-week male mice. Control male mice white circles/bars (n=30), ON male mice black circles/bars (n=30). *p<0,05; **p<0,01; ***p<0,001. Data are mean±sem. Pentinat, et al. 2010

4.2.3 DNA methylation in postnatal over-nutrition phenotype inheritance

There are different known epigenetic modifications, including DNA methylation, histone modifications, and noncoding-RNA that might be involved in the inheritance of diabetes risk. During germ-cell maturation, DNA methylation is extensively remodeled genome-wide (Bird, 1986; Yauk et al., 2008).. However, some elements, including Intracisternal A particles (IAPs), remain methylated along all stages of the embryo (Wong et al., 2010; Yauk et al., 2008). Furthermore, it was recently shown that a few methyl-marks are not

completely erased during the processes of gametogenesis and maintain substantial methylation (20-30%) (Seisenberger et al., 2012). Together, these data suggest that a few methyl –marks might be maintained during gametogenesis and be potential carriers of information across generations. We therefore focused on changes in DNA methylation between the different groups, and generations in our mouse model (Figure 20).

For our epigenetic study we retrieved liver from 8 days-old pups from F1 and F2 generations (Figure 23). At this age, liver is still developing and therefore reprogramming of DNA methylation is more likely occurring. Also, at this age mice have not developed open metabolic dysfunction and thus changes in methylation are likely primary rather than secondary to the effect of metabolic defects. Finally, we also retrieved sperm from adult F0 and F1 males.

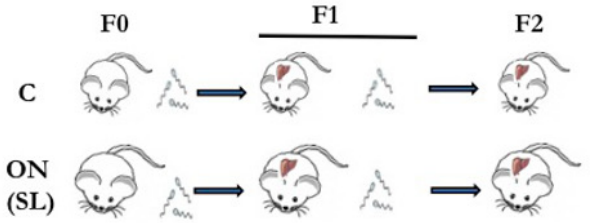


Fig 23. Overnutrition mouse model sampling. For methylation analysis we retrieved sperm from adult mice and liver from 8 day-old offspring

Using MedIP-microarrays from Agilent we analyzed the methylome of sperm and liver from the animals (Figure 24). We obtained a list of 19033 differentially methylated regions (DMR) in F0 sperm when comparing C and ON adult mice. When comparing F1 sperm of C and ON mice we obtained a list of 2976 differentially methylated probes. On the other hand, in F1 livers there were 4822 differentially methylated regions between C and ON mice. In F2 livers we found 4968 regions that were different between the two groups.

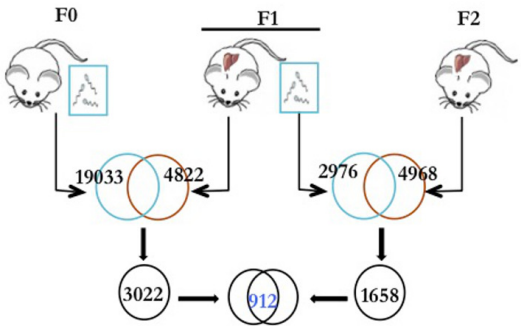


Fig 24. MedIP-microarrays. Differentially methylated regions comparing controls against over nutrition in sperm and liver of each generation, and finally all lists together, obtaining a list of 912 regions that are differentially methylated between CC and ON, and common in all generations and tissues.

We wanted to test whether in our model some methyl-marks are being transmitted from founder to offspring. Therefore, we first compared the list of more than 19000 regions in sperm from F0 mice to the 4800 regions found in F1 liver. Interestingly, 3022 differentially methylated regions were common in sperm of F0 and livers of the following generation, F1. At the same time, we compared the 2976 regions from F1 sperm against the 4968 regions in liver of his offspring, F2, obtaining a list of 1658 regions that maintained the difference of methylation between C and ON mice. In sum, a large proportion of marks present in the sperm were later detected in the liver of the following generations. This data suggest that a variable proportion of methyl-marks might be transmitted to the following generation (Figure 24).

Since we knew that the phenotype was initiated in F0 generation and maintained at least until the F2 generation, we were interested in knowing the regions of DNA that were differentially methylated between C and ON and were common in the three generations, in the two tissues analyzed. For this, we compared the two new lists described obtaining a list of 912 regions that are differentially methylated between controls and ON

in two tissues (sperm and liver), in response to nutritional challenges, and are subsequently transmitted to the following generations through the germ-line (Figure 24).

Next, we determined where the regions found on the methylation array fall in relation to the nearest gene: inside the gene body, within the promoter region or downstream (i.e. in intergenic regions). Of the total of regions found in the array, 64% of them corresponded to regions inside the gene body. Interestingly, when we analyzed the percentage of regions that fell inside a gene of the common list of 912 regions, this number increased to 81%. In second place the regions were grouped inside the promoter (Figure 25a, 25b). Since methylation patterns of these regions were either increased or decreased and found inside gene, we thought gene expression could also be affected.

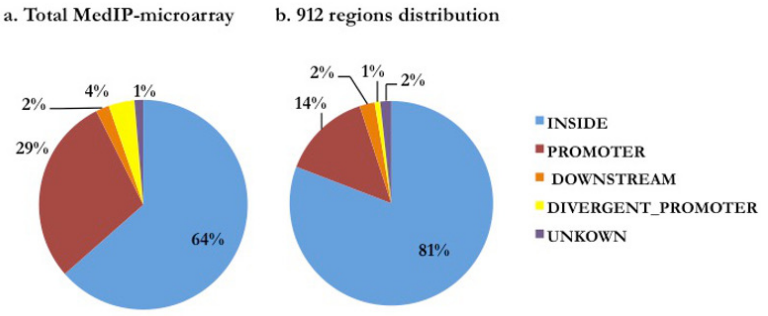


Fig 25. Location of differentially methylated regions (DMRs). a) DMRs of the whole of MedIP-microarray. b) List of 912 DMRs present in all generations in both tissues; sperm and liver.

We next clustered the 912 regions/probes using DAVID® Software, with the Functional Annotation Tool to determine which pathways were being affected. By two different analysis, Kegg pathways and Panther pathways, we found pathways involved in signaling, embryonic development and cancer processes (Figure 26). However, when analyzing these pathways by Bonferroni none of them where significantly enriched as opposed to when the analyses was done using t test, that signaling pathways were very significantly enriched ($p<0,05$). As Bonferroni is a conservative test and it assumes that

data analyzed is independent we may be observing false negatives, suggesting that selected pathways may be false rejections that are really significant between groups.

Kegg Pathways			
	p-value	FE	Bonferroni
MAPK signaling pathway	0,022	2,5	0,32
Calcium signaling pathway	0,002	3,1	0,22
Neuractive ligand-receptor interaction	0,002	2,7	0,13
Wnt signaling pathway	0,018	2,9	0,33
Colorectal cancer	0,072	3,1	0,99
Melanogenesis	0,009	3,7	0,64

CTNNB1
GNAS
MMP9
NFATC2
FZD1
FZD5
WNT3A

Panther pathways			
	p-value	FE	Bonferroni
P00012:Cadherin signaling pathway	0,013	2,4	0,58
P00057:Wnt signaling pathway	0,002	2,0	0,15
P00004:Alzheimer disease-presenilin pathway	0,004	2,8	0,24

CTNNB1
HOXC4
NFATC2
MYCN
DACT1
MMP9

Fig 26. Pathway enrichment using DAVID® Software. Pathways enriched are the ones involved in germ-cell development, imprinting and cancer

Each CpG island is represented by an average of 8 probes in the array. Thus, from the list of 912 probes we selected those genes that contained 4 or more probes differentially methylated (Figure 27). These are the genes that are probably keener to have been modified by nutrition in the F0 generation, as more probes are found to be different between controls and ON in all generations. Interestingly, a few genes were implicated in germ-development and imprinting processes. On the other hand there were also genes involved in glycoprotein biosynthesis. Therefore, we started validating the microarrays by AmpliconSeq in the sperm samples, and by PCR and Pyrosequencing in the liver samples.

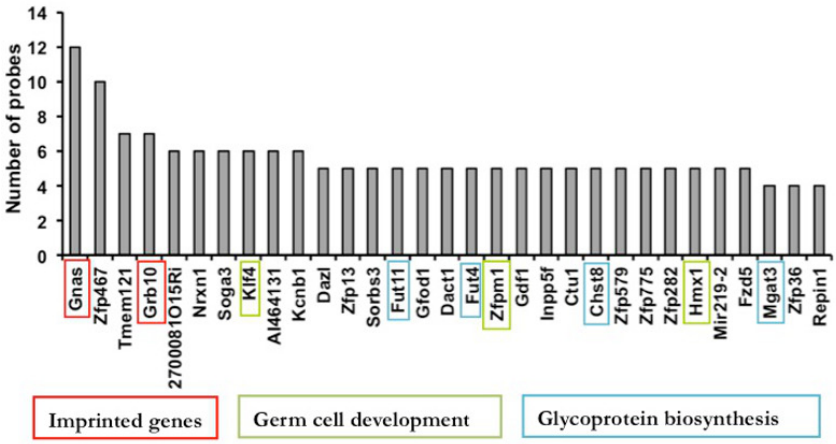


Fig 27. Genes represented by 4 or more probes. Each CpG island is represented by an average of 8 probes, we selected the genes that contained 4 or more probes,

4.2.3.1 Methylation in sperm samples

For the validation in sperm samples we selected 14 genes that were differentially methylated between controls and ON in the array, and were also previously described by Seisenberg and Reik to undergo a methylation reprogramming during embryogenesis. In their paper they performed a study of genome-wide DNA methylation and transcription across the different stages of primordial germ cells, providing a dynamic mapping of methylation and demethylation during embryonic development (Seisenberger et al., 2012). We did this validation in collaboration with Jorg Tost's laboratory (CNG, Evry, France). Amplicon Seq gave us the mean percentage (%) of methylation for each region (Figure 28, and Figure 1 Appendix). Of these 14 genes, *Hoxc4* (Homeobox C4) had lower mean percentage of methylation in ON mice in F0 generation, but was higher in F1 generation compared to controls. *Tbx1* (T-box 1) and *Hmx1* (H6 homeobox 1) had higher methylation in F0-ON and F1-ON sperm samples (Figure 28b, 28c, 28g).

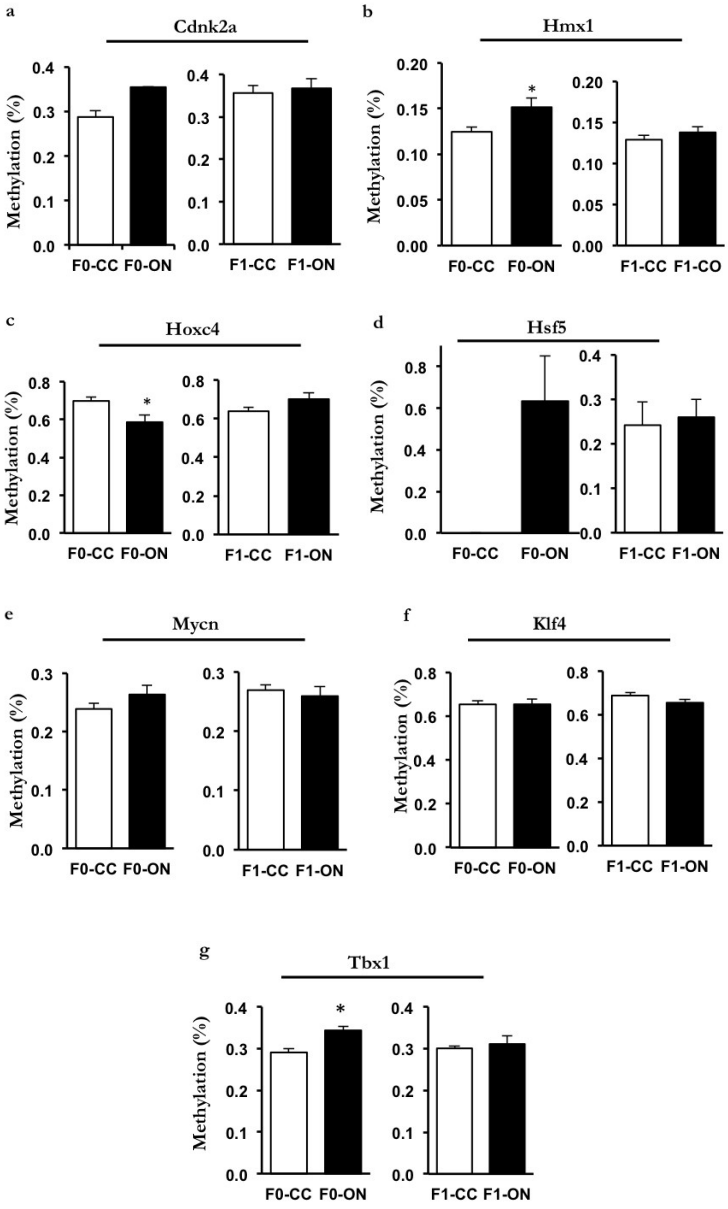
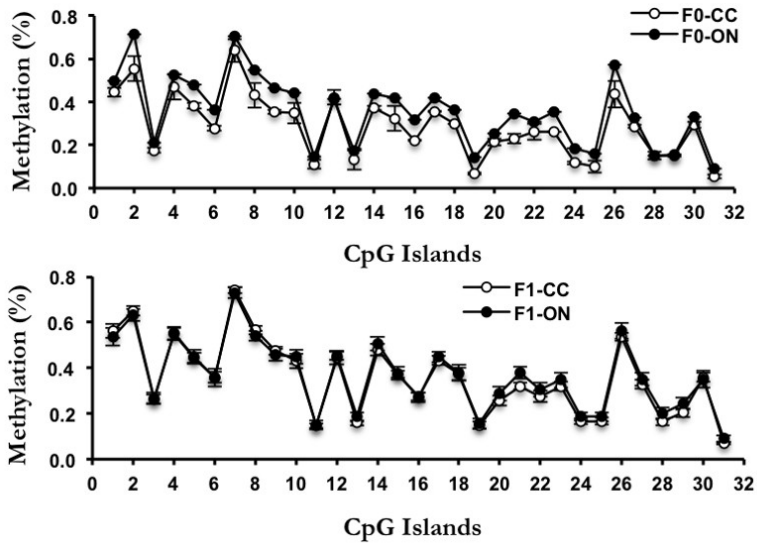


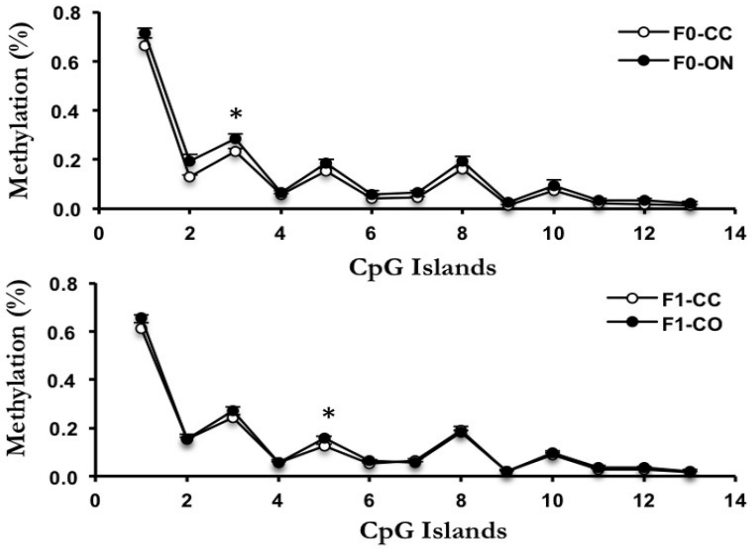
Fig 28. Mean methylation % in sperm samples. Sperm samples were analyzed by Amplicon Seq obtaining % of methylation of the gene in each generation. *p<0,05

By Amplicon Seq we can also obtain the percentage of methylation of each individual CpG site. Of the remaining analyzed genes, even though the mean methylation was not different between C and ON mice of each generation, when each CpG island is analyzed separately some of them have higher or lower % methylation even if the mean is not significant (Figure 29 and Figure 2 in Appendix). It is questionable whether these individual changes might have an impact in regulating gene expression (this possibility deserves further investigation).

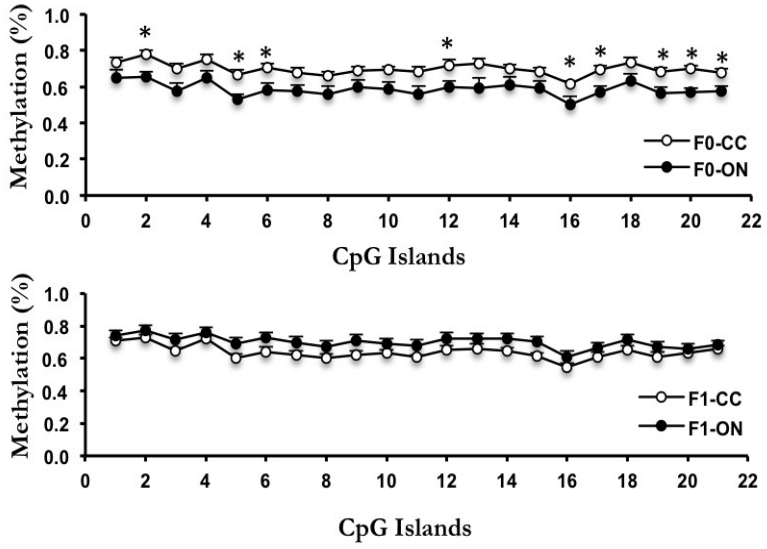
a. Cdkn2a



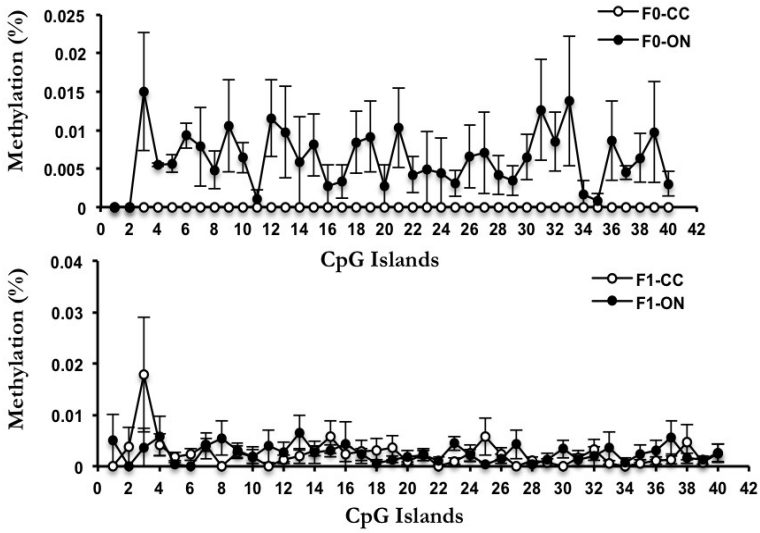
b. Hmx1



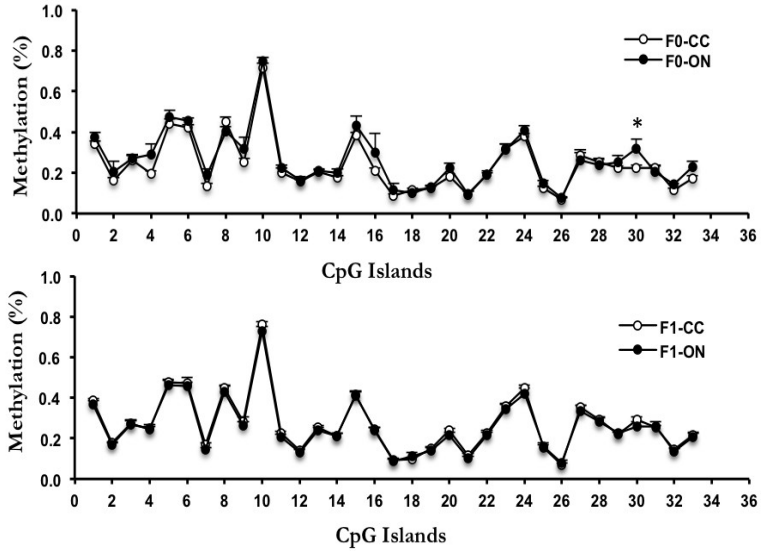
c. Hoxc4



d. Hsf5


F0-CC
F0-ON
Methylation (%)
0.025
0.02
0.015
0.01
0.005
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
CpG Islands
F1-CC
F1-ON
Methylation (%)
0.04
0.03
0.02
0.01
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
CpG Islands

e. Mycn


F0-CC
F0-ON
Methylation (%)
1.0
0.8
0.6
0.4
0.2
0.0
*
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
CpG Islands
F1-CC
F1-ON
Methylation (%)
1.0
0.8
0.6
0.4
0.2
0.0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
CpG Islands

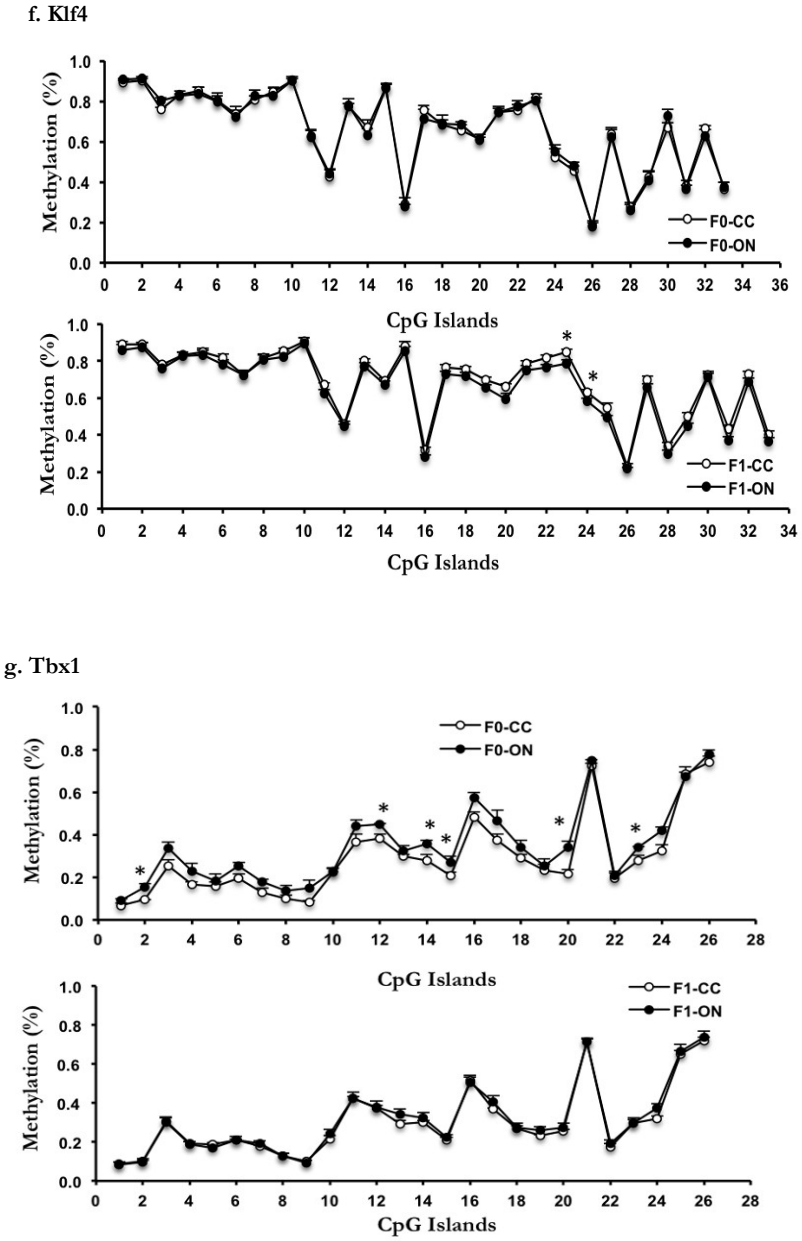


Fig. 29. Methylation of CpG Islands in sperm samples. Methylation percentage of each CpG Island was measured by Amplicon Seq in sperm samples. Differences in specific CpG Islands were observed (*p<0,05)

4.2.3.2 Methylation in liver samples

Cytosine methylation of liver samples was validated by bisulfite pyrosequencing. This was done in collaboration with David Monk's lab (Genomic and Imprint Cancer Group, IDIBELL, L'Hospitalet de Llobregat, Barcelona). At the moment of writing this thesis these analyses are still on going and therefore we can only present some of the results. First genes that we could analyze include *Hoxc4*, *Tbx1, Hmx1, Nfatc2* (Nuclear factor of activated T-cells 2) and *Stk11* (Serine/threonine kinase 11) (Figure 30a, 30b, 30c, 30d, 30e). Of these genes we saw no differences in percentage of methylation in total of the sites. Only *Stk11* presented higher methylation in F1-ON mice (Figure 30e).

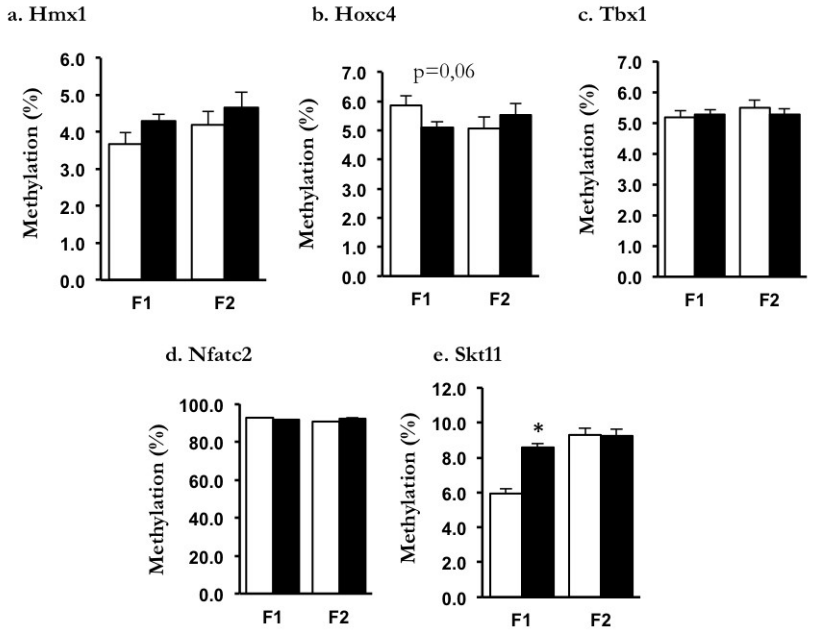


Fig 30. Methylation in liver samples. Liver samples retrieved from 8 days-old mice and analyzed by pyrosequencing (*p<0,05, white bars CC, black bars ON)

4.2.4. Gene expression in obese phenotype inheritance.

DNA methylation is associated to gene expression, being hypermethylation correlated with low expression and hypermethylation with higher gene expression in the promoter region. Since the majority of the probes that were differentially methylated between controls and ON were found in the promoter region, we then analyzed the liver samples by RT-qPCR. We started with the genes that were represented by 4 or more probes in the array (Figure 31a, 31b, and Figure 3 in Appendix). Expression of these genes did not necessarily correlate with DNA methylation in the sperm or in the liver. In fact, gene expression was largely unaltered, suggesting that in our mouse model changes in methylation do not primarily influence gene expression.

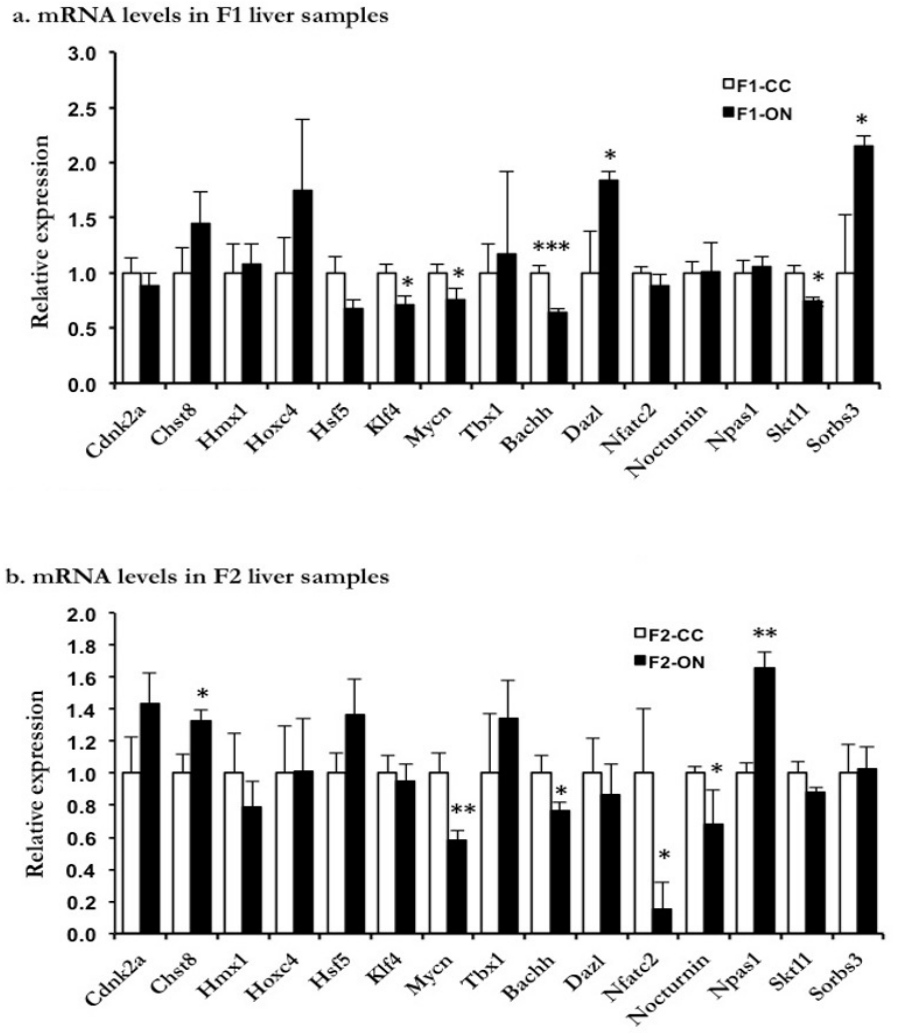


Fig 31. Gene expression in liver samples. Liver samples retrieved from 8 days-old mice and analyzed by qPCR. a) mRNA levels in F1 liver samples. b) mRNA levels in F2 liver samples *p<0,05; **p<0,01; ***p<0,001; Data are mean ±sem CC, white bars (F1 n=8, F2 n=12), ON black bars (F1 n=8, F2 n=12)

5. Discussion

The prevalence of obesity in adult subjects has been increasing since the early 80s, and in 2014 more than 600 million people over 18 years old were obese (WHO, 2015). According to WHO data, 41 million children under 5 years of age were overweight or obese in 2014 (WHO, 2015). This is a situation that deserves further attention, since childhood obesity is a risk factor for adult obesity. Overweight and obesity have been linked to higher risk for cardiovascular disease, hypertension, diabetes, dyslipidemia, osteoarthritis, sleep apnea and certain cancers, all of these considered non-communicable diseases (Fahed et al., 2016; l'Allemand-Jander, 2010; Lloyd et al., 2012; Manios et al., 2017; Mesarwi et al., 2013). Also, obesity has been linked to mental health and depression (Williams et al., 2015).

A number of early-life factors including BMI of both parents, birth weight, postnatal nutrition, breastfeeding duration, antibiotic use during infancy, levels of physical activity during childhood, and maternal diet are key in determining childhood obesity risk (Azad et al., 2014; Bailey et al., 2014; Carling et al., 2015; Harder et al., 2005). Therefore, modulating these factors might help reduce the number of overweight and obese subjects. Some of these early-life risk factors are linked to accelerated weight gain during infancy (Isganaitis et al., 2009), itself a major risk factor for developing obesity during adulthood, along with elevated cholesterol levels, impaired insulin response, and higher fat percentage (Kerkhof et al., 2012). Childhood obesity or overweight is predictive of overweight in adults; even more, growth trajectories during early years are associated with greater BMI during adulthood (Péneau et al., 2017). Therefore, early nutrition, which strongly influences growth trajectories, plays a crucial role in long-term disease risk. Breastfeeding has protective effects against childhood obesity and also offers an excellent window of opportunity for preventive interventions in the offspring (Weber et al., 2014; Yan et al., 2014).

In this doctoral thesis, we have explored the mechanisms by which early-life nutrition modulates long-term obesity and metabolic health. Specifically, we have focused on weight gain in early stages of life and its effect on long-term metabolic health. For this purpose, we have taken two approaches:

Firstly, compared to formula feeding, breastfeeding duration has been correlated to slower early weight gain and reduced childhood obesity risk (Harder et al., 2005; Horta and Victora, 2013; Horta Bernardo, 2013). We analyzed components in breast milk finding betaine as the one-carbon metabolite that correlated most significantly with infant growth at one month. Betaine has been positively correlated with lower diabetes incident, lower BMI, percent body fat and ameliorated glucose homeostasis in adult subjects, both in humans and mouse studies (Ejaz et al., 2016; Konstantinova et al., 2008; Walford et al., 2016). As low milk betaine levels correlated to higher early growth of infants, we analyzed if betaine administered during lactation in a mouse model by supplementing the dams diet could have long-term effects on metabolic health of the offspring.

Secondly, excessive caloric intake in early-stages of life is a major risk factor for an accelerated weight gain during infancy increasing childhood obesity risk and long-term metabolic disease risk (Isganaitis et al., 2009; Lloyd et al., 2012; Parikh et al., 2017; Taveras et al., 2009). These metabolic complications in the adult can be transmitted to the next generation (Joss-Moore and Lane, 2016; Pentinat et al., 2010; Youngson and Whitelaw, 2011). Transmission of obesity and obesity-related metabolic complications cannot be fully attributed to genetic factors. Hence, it is proposed that epigenetic factors might have an additional role in this process (Symonds et al., 2009; Vickers, 2014). Indeed, nutrition can induce epigenetic changes, including altering DNA methylation patterns (Johnson and Belshaw, 2014; Mathers et al., 2010; Mckay and Mathers, 2011; Plagemann et al., 2009). If these epigenetic modifications occur in the gametes they can be passed on to future generations (Fullston et al., 2013; Yauk et al., 2008). Here, we studied the role of DNA methylation in mediating the transmission of phenotypes in our model of over-nourished mice. For this, we analyzed sperm and liver from 3 successive generations, comparing patterns of methylation.

5.1. Maternal betaine supplementation during lactation improves offspring long-term metabolic health.

During pregnancy, low maternal intake of one-carbon metabolites has been correlated to impaired development of the infant and poor health outcomes (Zeisel, 2006). Low levels of folate in overweight or obese pregnant women correlate with higher BMI and increased risk of adverse metabolic outcome among their offspring (Wang et al., 2016).

Different breast milk components including leptin, adiponectin, glucose and IL6 have been associated with infant growth rate and adiposity (Alderete et al., 2015; Fields and Demerath, 2012; Fields et al., 2016), indicating a relationship between the mothers' nutritional state and the nutrients that infants are receiving through breast milk. For long there has been evidence that maternal metabolite plasma levels composition of metabolites and vitamins in breast milk (Englich et al., 2017; Guven et al., 2009).

As mentioned before, lower plasma betaine concentrations in adults correlate with higher BMI, early insulin resistance, and type 2 diabetes risk (Ejaz et al., 2016; Konstantinova et al., 2008; Walford et al., 2016). In our analyses of one-carbon metabolites in human milk samples, we did not find a correlation between milk betaine levels and maternal obesity, therefore other variables, including insulin sensitivity or diet, may contribute to milk betaine content. We did find a correlation between higher betaine levels and slower infant growth at 1 month, suggesting it may modulate infants growth rate.

In our study with mice, we found higher betaine levels in milk from betaine-supplemented normal weight dams, and higher plasma betaine concentrations in lactating pups. This suggests that betaine supplementation in dams' drinking water was increasing directly or helping to increase betaine levels in milk, and the pups were receiving this increase. However, to confirm that dietary betaine can be directly transferred to milk other studies should be conducted, for instance supplementing water with labeled betaine and monitoring its presence in milk.

Growth during lactation in offspring from supplemented dams was slightly slower than non-supplemented, indicating that betaine was reducing early growth rate. Accelerated

early-growth rate has been previously associated with adult disease (Jimenez-Chillaron et al., 2006). When moderating growth rate during lactation, mid- and long-term metabolic health of the supplemented mice was improved, indicating that growth in early stages of life is key to determining future metabolic health. In addition, supplementation was done during lactation, indicating that breastfeeding is a window of opportunity for ameliorating health in later stages of life, in accordance with other studies (Azad et al., 2014; Cordero et al., 2014; Le Huërou-Luron et al., 2010). Our data suggest that exposure to higher levels of betaine in early stages of life might be sufficient to reduce adiposity. Male and female offspring from normal weight dams responded differently to betaine treatment. Body weight was similar between groups and glucose metabolism was not ameliorated in female offspring. These differences between female and male offspring suggest that long-term betaine's beneficial effects are sex-dependent when fed a normal diet. When challenged with a high fat diet to induce obesity, female mice also had improved metabolic health during adulthood. Therefore betaine's effects may be more prominent under obese phenotype. Males and females respond differentially to diets, treatments and stress (Argente-Arizón et al., 2016; Bengoetxea et al., 2017; Ward et al., 2017) highlighting the importance of performing sex-specific studies.

Lifestyle and nutrition during adulthood is also crucial for optimal metabolic health. An unhealthy diet, like one with high fat content, will induce obesity in the adult. In adult mice fed a high fat diet (HFD), betaine improves glucose homeostasis, reduces hepatic lipid, and increases energy expenditure, ameliorating overall metabolic health (Ejaz et al., 2016). In farm animals betaine has been used as food supplementation, and for example in chickens and pigs it reduces adiposity (Williams et al., 2015). Changes observed in our studies on young mice induced by maternal betaine supplementation also protected against diet-induced obesity in male and female offspring. When fed a high fat diet during adulthood, we did observe a small decrease in caloric intake in betaine-treated offspring. Even though it was not statistical significant we cannot discard it as small differences can lead to body weight differences over time.

However other mechanisms may be contributing to betaine's beneficial effects.

Areas that we did not explore are, for instance, betaine's role as a methyl donor in the methionine cycle, increasing SAM and glutathione availability. DNA/protein methylation mediated by betaine could be contributing in improving metabolic health, and would be interesting to analyze liver methylation patterns from these mice in the future.

Adding these findings to the ones already in the literature, lactation is a window of opportunity to reduce risk of developing long-term diseases (Gruszfeld and Socha, 2013). In a simple and safe way one can supplement the maternal diet with vitamins and micronutrients so that offspring can benefit from these. It would be interesting to increment betaine levels in the mothers diet when obesity or overweight is presented. Also if when the infant is gaining weight rapidly, a nutritional action implicating the mothers diet can help reduce risk of childhood obesity. When infants are formula fed, modifying the maternal diet will not influence child's development; in these cases formula milks that are substituting breast milk should be reviewed and when necessary betaine could be added, as now some contain choline (precursor of trimethylglicine, betaine) but not all of them. Betaine is rapidly absorbed when orally administered and it is found in a high diversity of foods present in a healthy diet (Ueland et al., 2005), therefore adding it to formula milk could be a more efficient way to reaching the infant. Future studies to determine the amount and concentration would be necessary, and a more personalized direction in this field could be approached.

More important than to reduce an accelerated weight gain during lactation is to maintain what is considered a normal weight gain for height and age (Wu and Chen, 2009). Therefore, a slower weight gain is also associated to undernourishment, cognitive diseases, psychomotricity (Kang et al., 2017) and cases of under nutrition during lactation should also be addressed.

Early postnatal feeding is also important in influencing the infants' gut microbiome (Bokulich et al., 2016). Breastfeeding is a protective factor against transmission of obesity risk to the newborn (Yan et al., 2014), and microbiota from breastfed infants differ largely from formula fed infants (Le Huërou-Luron et al., 2010; Urbaniak et al.,

2014). In addition to all the nutrients and growth factors that breast milk provides to the infant, some hormones like leptin and insulin can still have biological activity in the infants' gastrointestinal tract as well as other factors that can contribute to the intestinal barrier function and composition of the intestinal microbiome (Bokulich et al., 2016; Cox et al., 2014; Soderborg et al., 2016). Microbial composition is also found to be different between breastfed infants born to normal weight or obese mothers (Lemas et al., 2016).

The gastrointestinal microbiome has an important role in metabolism and immunological function, helping to maintain the intestinal barrier and participating in the communication between organs (Geurts et al., 2014). In adults, shifts in intestinal microbiome composition contribute to obesity, type 2 diabetes and metabolic syndrome (Hartstra et al., 2015; Miles et al., 2006; Tilg and Kaser, 2011). Changes in the gut microbiota occur by different mechanisms that involve stress, diet and antibiotic administration (Diao et al., 2015; Tellez et al., 2015). As the gut microbiome is established during early life, it is likely to influence the development of the child. Indeed, the early-life microbiome has an important role in long-term metabolic disease risk (Cox et al., 2014). In our studies we found that offspring gut bacterial communities were modulated by maternal betaine supplementation. Surprisingly, direct supplementation of betaine to the dams' drinking water did not modify maternal microbiota. We only detected differences in *Akkermansia* spp at 2 weeks-old mice, suggesting that in some way betaine is increasing this population. Recent studies have correlated increased *Akkermansia muciniphila* to improved metabolic health (Everard et al., 2013; Ganesh et al., 2013; Greer et al., 2016; Shin et al., 2014). Also in a mouse model of postnatal under-nutrition, higher *Akkermansia* spp has been associated with slower weight gain (Preidis et al., 2016). Furthermore, *Akkermansia spp* in adult mice increased goblet cell number in the ileum and improved the gut barrier function, decreasing systemic inflammation and having beneficial consequences in glucose tolerance (Everard et al., 2013; Ganesh et al., 2013; Greer et al., 2016; Shin et al., 2014). In addition to increased *Akkermansia spp* population, and in agreement with these data, we observed longer villi in 2 week-old mice indicating that ilea morphology was improved in betaine supplemented young offspring. We also detected increased goblet cell number in 6

week-old mice compared to controls suggesting that the mucous layer in these animals was different, either it was being renewed more rapidly or was more abundant. As overall inflammation was also reduced in betaine treated mice, thickness of mucous barrier could be increased as this helps reduce systemic inflammation by ameliorating permeability (Everard et al., 2013). We were not able to determine the thickness of the mucous barrier, as ilea should be fixed in a Carnoy solution to preserve the layer for better measurement (Gouyer et al., 2011; Matsuo et al., 1997).

A. muciniphila degrades mucins secreted from goblet cells in the intestinal barrier (Derrien et al., 2004). Levels of *A. muciniphila* are increased in low inflammation intestines inducing a protective effect on the organism that has yet to be determined. However, with these results we cannot confirm that an increase in early-life stages of *A. muciniphila* is sufficient to improve long-term metabolic health. Additional experiments during early life, such as direct bacterial transfer to the offspring during lactation, would be helpful in this question.

How betaine increases *A. muciniphila* abundance is unclear. As an osmoprotectant, in the intestine betaine may be increasing epithelial cell volume by retaining more water, and therefore inducing cell proliferation. Betaine in the gut is not only interacting with epithelial intestinal cells but also with microbiota (Metzler-Zebeli et al., 2009; Ratriyanto et al., 2009), protecting degradation of certain strains like E. Coli under high adverse conditions. Therefore, we cannot confirm if betaine is directly shifting the composition of microbiota or if this occurs as a consequence of the increased number of goblet cell in ilea and this is occurring as a consequence of the osmoprotectant function of betaine.

Use of antibiotics during infancy has been associated with childhood obesity in diverse studies, indicating that early-life gut microbiome possibly has a role in determining obesity risk (Azad et al., 2014; Cho et al., 2012). When administering antibiotics to lactating mice, gut microbiota was altered along with ileal structure, leading to overweight adult mice (Cox et al., 2014). Antibiotic administration disrupts the intestinal epithelium causing bacteria to "leak" into the organism and cause inflammation (Knoop et al., 2017).

Antibiotics are a very aggressive insult to the gut, in addition of decreasing gut microbiota also changing the intestinal barrier. On poultry antibiotics increase absorptive surface, decrease cell proliferation, induce a thinner mucosae layer and less lamina propria (Miles et al., 2006).

When we co-administered antibiotics with betaine we could no longer observe the beneficial effects on offspring metabolic health, suggesting that early-life microbiome contributes to betaine actions. For this it would be interesting in future work to further analyze gut microbiota and ilea structure at short and long-term of offspring treated with antibiotics during lactation.

It is clear rapid weight gain during early stages of life is a risk factor for rapid weight gain during childhood (Taveras et al., 2009). This risk is increased when parents are overweight or obese (Kim et al., 2015; Li et al., 2013). And furthermore, childhood obesity induces impaired metabolic health during adulthood (Davies et al., 2016; Lloyd et al., 2012). Therefore the necessity to monitor and modulate growth during lactation is crucial. Learning how to take advantage of this window of opportunity will help reduce risk of obesity during childhood. Using treatments that are basically diet supplementation is a better choice than administering drugs at early ages or having to treat overweight or obese adults, that as proven to be costly and not always effective. With the results presented in this thesis we have shown that betaine administration in normal weight and even obese dams during lactation moderated offspring growth after weaning, suggesting that betaine was preventing an accelerated growth during this period and in a long-term manner. Therefore betaine can be a safe and easy supplement to administer through diet when obesity risk is detected.

5.2 Neonatal overfeeding induces impaired metabolic phenotype and is transmitted to future generations.

The DOHaD theory was firstly described as adaptations *in utero* that affect health outcomes during adulthood (Barker DJ, 1990). Nowadays, this theory is extending over different life phases including gestation, infancy, childhood, juvenile and pubertal growth phases. Many studies have suggested that environmental factors including stress and nutrition, during early life periods can play a role in health outcomes in adult life. On this regard, an increased intake of specific nutrients such as proteins (Weber et al., 2014) or global calories during breastfeeding (Pentinat et al., 2010) causes accelerated weight gain during infancy (Ekelund et al., 2007). In consequence, there is an increased risk for childhood obesity and impaired metabolic health later in life (Taveras et al., 2009) including obesity and type 2 diabetes (Lloyd et al., 2012; Ong et al., 2000; Parikh et al., 2017).

A genetic component for obesity and diabetes has been determined by twin studies and family studies (Mcmillen and Robinson, 2005; Singhal, 2017; Wong et al., 2010). Genetic factors can explain 40-80% of variance in BMI and risk in diabetes in twin studies. However, heritability of these diseases showed lower values: genome-wide association studies (GWAS) identified specific genetic variants but they explain less than 10% of inheritance of obesity and diabetes (Burgio et al., 2015). Recently, several authors have suggested that other mechanisms might be involved in the inheritance for non-communicable diseases. It has been proposed that these mechanisms could be epigenetic (Hanson and Gluckman, 2015)

Epigenetics can be defined as the study of heritable changes in gene expression that are not mediated by variations of DNA sequence (Whitelaw and Whitelaw, 2008). There are different known epigenetic modifications, including DNA methylation, histone modifications, and noncoding-RNA that might be involved in the inheritance of diseases and in particular diabetes risk (Lillycrop, 2011). Importantly, many epigenetic marks, such as DNA methylation, are primarily set during critical windows of development, including infancy and childhood (Hochberg et al., 2011). Once they are established their effects on the phenotype may be strong and have long-term effects (English et al., 2016).

Furthermore, evidence shows that these epigenetic features acquired during early growth can be transmitted to following generations (Carone et al., 2010; Gluckman et al., 2005; Hochberg et al., 2011). For example, exposure to air-pollutants changed sperm methylation patterns in adult mice (Yauk et al., 2008) and other studies showed that neonatal nutrition altered methylation patterns of specific genes (Jiménez-Chillarón et al., 2012; Plagemann et al., 2009).

In the experimental model from our lab, it was shown that overfeeding during lactation period induced overweight mice. When these overweight mice were bred, the following generations also had impaired metabolic health even though weight gain was normal during early-life (Pentinat et al., 2010). With these results it was suggested that some epigenetic mechanisms like methylation modifications might be involved in this process. Specifically, in our model, where transmission of metabolic dysfunction occurs through the paternal line, the epigenetic modifications should occur in the sperm. In agreement, accumulating evidence suggests that sperm and testis methylation patterns are modified by paternal nutrition. For example, rats exposed to high fat diet resulted in global hypomethylation in sperm (Fullston et al., 2013). Others have shown minor global cytosine methylation patterns when rodents were fed low protein diets, but modest changes were detected in offspring livers (Carone et al., 2010).

During germ-cell maturation, DNA methylation is extensively remodeled genome-wide. Some elements (i.e. Intracisternal A particles, IAPs) remain methylated along all stages of the embryo, but other sites are reprogrammed almost in total (Carone et al., 2010; Fullston et al., 2013). During spermatogenesis changes in methylation occur in CpG and non-CpG sequences either inside genes or intergenic sequences (Walford et al., 2016). As mouse spermatogenesis already begins during the first week after birth (Yoshida, 2006), it is reasonable to think over-nutrition might cause changes in DNA methylation as compared to control, and also in their offspring. In the same way as Walford et al, we were able to detect 10-20% individual CpG changes in the differentially methylated regions found in our sperm samples. These changes were also found in offspring liver samples suggesting that some methylation is indeed potentially inherited from fathers to offspring

and also grand-offspring. Finally, it is important to note that we have measured patterns of methylation in the sperm of adult mice (F0-ON) These alterations might be due to the direct effects rapid weight gain during early life or, alternatively, to the signals derived to the progressive development of obesity and insulin resistance. In order to confirm which of the situations are responsible for the epigenetic changes it would be necessary to determine methylation patterns in developing gonads, and other tissues of these mice at early ages

We cannot minimize maternal contribution, even though all dams had no environmental factor influencing their "normal" epigenome and supposedly their contribution should be the same or very alike. Therefore it would be interesting to analyze methylation and gene expression at blastocyst level knowing that during fertilization sperm and eggs undergo methylation-programming processes (Arand et al., 2015; Saitou et al., 2012), to determine on which level male gametes contribute to epigenetic inheritance (Li et al., 2016; Seisenberger et al., 2012).

Our results are in accordance with Fullston et al, and Carone et al, as we found a great number of differentially methylated regions in sperm of over-fed mice and sperm and liver of the offspring. When same tissues of the following generations were analyzed we also observed differentially methylated regions between groups. Interestingly, we found that some of these regions were differentially methylated along the three generations (the founder generation that was the only one overfed and the two following generations that had normal nutrition). These regions were present both in sperm of the F0 and sperm and liver of following generations. This data suggests that changes in methylation patterns were at some degree being transmitted to the next generation. ON-F1 and ON-F2 mice did not undergo any additional environmental exposure, and sperm methylation was still different between groups, suggesting that these marks were inherited from their grandfathers. Alternatively, they could be "re-stablished" as F1 and F2 mice undergo some metabolic alteration.

DNA methylation might influence gene expression; therefore our next step was to

analyze gene expression in our mouse model. For years, a simplistic association between methylation and gene expression was accepted. Hypermethylation (extra number of methyl groups on CpG islands) of the promoter will impede gene transcription, whereas hypomethylation (less methyl groups on CpG islands) will leave the gene free to be transcribed (Bird, 1986, 2002). This dogma is now reviewed and gene expression may be more linked to differences in individual CpG sites rather than totally methylated or totally de-methylated regions (Siegfried and Simon, 2010). Furthermore, location of methylation patterns in relation to gene body alters gene expression; hypermethylation inside gene body will allow gene expression and hypomethylation will result in gene silencing as opposed to when found inside the promoter region (Ball et al., 2009). In our samples we could only detect 2-10% of changes in methylation between the two groups, but in some regions they were significant or very significant according to Wilcox test ($p<0,03$). This is in accordance with other studies that also could detect very low percentage of methylation differences (Carone et al., 2010; Radford et al., 2014; Youngson et al., 2015). Sperm methylation is already starting at very low rates, and when looking at CpG islands it is very important to determine the extent of the region. We found significant differences in specific CpG sites between ON and control mice, suggesting that even few methylation marks can affect gene expression and contribute to their inheritability.

Therefore, we analyzed the genes contained in the regions that appeared to have altered methylation. It was not surprising that some genes were upregulated and others downregulated regardless of the methylation status of CpGs.

Genes analyzed in sperm are all implicated in developmental processes. Of these; *Cdkn2a* has been described to have a role in glucose homeostasis in humans and induces hepatic gluconeogenesis after fasting period (Bantubungi et al., 2014; Pal et al., 2016). *Hoxc4* expression is directly correlated with fat accumulation and when highly methylated expression is decreased (Mansego et al., 2015; Singh et al., 2016). In this specific gene we found higher levels of methylation in the founder generation, but similar methylation levels in ON-F1 mice compared to controls. We observed a tendency to higher mRNA levels of *Hoxc4* in ON-F1 ($p=0,06$) liver, suggesting in this case that methylation was lower as

described by Mansego et al.

Hsf5 and *Tbx1* where significantly hypermethylated in ON-F0, and had similar methylation % in ON-F1. When analyzing expression in liver, *Tbx1* showed similar mRNA levels between groups in both F1 and F2 mice. This was also expected as *Tbx1* is mainly found in developmental processes and expressed in the testis. *Nfatc2* was not analyzed in sperm and only gene expression in liver was detected. In ON-F1 generation mRNA levels did not change, but in ON-F2 mice they decreased. This gene induces gene transcription during an immune response, and in pancreatic islets it promotes βcell proliferation and insulin secretion. When knockout in mice they are protected against diet induced obesity, are leaner and have increased insulin sensitivity. Based on these studies *Nfatc2* is an interesting target to further analyze methylation status in sperm and liver. In sum, here we have identified a group of genes that might be directly involved in the development of metabolic disturbances, whereas a few other are potentially involved in developmental processes. We propose that developmental genes might influence normal embryonic development, which might lead secondary metabolic abnormalities as individuals grow and age. This is a hypothesis that deserves being tested in the future.

Considering that the epigenetic signature is present in the sperm, and it is being transmitted to the offspring, all tissues in the embryo may carry it. Therefore, it would have been interesting to also analyze other metabolic tissues such as eWAT, skeletal muscle or hypothalamus. Indeed, adipose tissue deserves special interest. First, ON-F0 mice weighed more and had had increased adiposity as compared to the control group (Pentinat et al., 2010). On the other hand, ON-F1 and ON-F2 mice had similar body weight. Yet, we cannot completely rule out that the adipose tissue might additionally contribute to impaired glucose metabolism in ON-F0-F1-F2 mice. Consequently, eWAT might also have epigenetic changes worth analyzing. Weight of this tissue was also significantly increased in F0 and F1 generations in previous work of this laboratory (Pentinat et al., 2010). When adipose tissue weighs more due to an obese phenotype compared to leaner phenotype, this can be due to inflammation causing a major productivity of inflammatory markers that can have a role in metabolic health (Ouchi et al., 2011). Therefore in our mouse model WAT is

an interesting target to analyze further, as well as other development stages such as blastocyst.

In this thesis we have focused our epigenetic studies to DNA methylation and gene expression. But we cannot underestimate other epigenetic traits including histone modifications and small non-coding RNAs (Hochberg et al., 2011). Recently it is proposed that small non-coding RNAs (sncRNAs) might play an earlier role in transgenerational inheritance because they regulate DNA methylation, histone modifications and mRNA transcription; and some species of sncRNAs (piRNAs and tRFs) are particularly enriched in male gametes (Carone et al., 2010; Corella and Ordovas, 2017; Hutcheon et al., 2017; Reilly et al., 2016). In future work, it would be interesting to analyze sperm samples for small non-coding RNAs to detect differences between groups and then compare them with the following generations. In parallel analyzing histone methylation of the described genes would be helpful to better understand how the phenotype is being inherited along the generations.

6. Conclusions

1. Breast milk betaine levels correlate to infant weight-for-length z-score at one month of age; this correlation is especially strong in overweight and obese mothers.
2. Maternal betaine supplementation during breastfeeding in mice increases milk betaine content.
3. Maternal betaine supplementation slows down offspring weight gain during lactation in mice.
4. Maternal betaine supplementation during lactation decreases adiposity in young mice.
5. Maternal betaine supplementation during the lactation period has beneficial long-term metabolic effects in the offspring.
6. Maternal betaine supplementation improves offspring long-term metabolic effects in a maternal obesity mouse model.
7. Maternal betaine supplementation increases intestinal *Akkermansia muciniphila* abundance in the offspring during lactation.
8. Maternal betaine supplementation increases intestinal goblet cells in the offspring in a short and long-term manner.
9. Co-administering antibiotics with betaine blunts its long-term beneficial effects, suggesting a role for the gut microbiome in mediating betaine's effects.
10. Over nutrition during the lactation period alters adult metabolism not only in exposed mice (F0) but also offspring (F1) and grand-offspring (F2).
11. Transmission of metabolic dysfunction through the male lineage strongly suggest epigenetic inheritance via the germ-line
12. Neonatal over nutrition altered global patterns of cytosine methylation in sperm of F0 mice.
13. Some methyl-marks present in the sperm of neonatally overfed mice were subsequently observed in the sperm and liver of the following generations.
14. Transmission of epigenetic information is possibly occurring across generations through the male germ-line.
15. DNA hyper or hypo-methylation does not always correlate with gene silencing or expression.

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Appendix

Table 1. qPCR primers

Gene	Forward (5'-3')	Reverse (5'-3')
Ccl2	GTCCCTGTCATGCTTCTGG	GCTCTCCAGCCTACTCATTG
Tlr4	GCCTCCCTGGCTCCTGGCTA	CAGGGACTTTGCTGAGTTTCTGATCCA
Hprt	GCCCCAAAATGGTTAAGGTTG	GTCAAGGGCATATCCAACAAC
Ocl	ATGTCCGGCCGATGCTCTC	CTTTGGCTGCTCTTGGGTCTGTAT
Zo1	TTTTGACAGGGGGAGTGG	TGCTGCAGAGGTCAAAGTTCAAG
Zo2	CTAGACCCCCAGAGCCCCAGAAA	TCGCAGGAGTCCACGCATACAAG
Nos2	CATCAACCAGTATTATGGCTC	TTTCCTTTGTTACAGCTTCC
Cd11c	ACAGTTCTGTTATGACATGC	AGTCTGTTGGGTTCTGTAAG
2700081015 Rik	CCTCAGGCCCAGTTCCCTA	GGGAGTTGGGAGTTGAGATTTGA
Al464131	AACCCAAGCCCACTAAAGA	GTTCAGCTTTGCGTAGGGAG
Arvcf	AAGAAAGCAAAAGAGGAGT	GTAAGGAGTGSGAGGTAGA
Bachh	AACCGTCAACGTATCTACTC	AACTCTACCAACACTGAGTC
Ccno	CAGAAAGCAGACAAGTCAA	AGACCACTCTGACTTCTAAC
Chst8	TTTCGGACGAGTTGTTGCC	GCACACGAGATTCACCAG
Ctnnb1	CAAGATCTGCAGTCTCATTC	TCCTATTCCGAATGTCTGAG
Dact1	GAGTCGCTCTTCCACTCCAC	CAGATTAAACCCCCGCTCTC
Dazl	CTGGTGTGTCGAAGGGCTA	GTGGCTGCACATGATAAGT
Fut11	AGGAGTCGCCGCTCAATAAC	GGGACTGCAAATAGAGCAA
Gdf1	TCGAAGAAGAGCACGGAGA	ATGTGAGCTTCCGTGAGGTG

Table 1 (cont). qPCR primers

Gene	Forward (5'-3')	Reverse (5'-3')
Gdf1	TCGAAGAAGAGCACGGAGA	ATGTGAGCTTCCGTGAGGTG
Gnas	GGAGAAGGCGCAGCGCGAG	CTCCGTTAAACCCATTAAC
Grb10	TGCACCACTTCTTGAGGATG	GCCGGAAGAGATGCAGTT
Hmx1	GCTACGGAGGTGGTCTAAG	GCGCTTCAGATCGAAAGTG
Hoxc4	GATTTACGAGCGAGAATGGT	CTCTAATTCCAGGACCTGCT
Hsf5	AATCCCTCGGAGAATATCT	ATGAAGATCAGGGGACTTTC
Kcnb1	GAGGACAGGAACCTCAGCA	CTGGAGAAGCCCAACTCATC
Klf4	CCCCTCTCTCCATTATCAAG	CTCTTGGTATAGGTTTTGCC
Mael	TGCAAACAGCTAAAGCACAGA	GAGCGGGCATCAGAAATAAG
Mgat3	ATGAAGATGAGACGCTACA	GGCCAGTTCTCTCGGGAAG
Mmp9	CTTCCAGTACCAAGACAAAG	ACCTTGTTCACCTCATTTTG
Mybl	CTAATTGAATCGGATCCTG	CTTCAAGTACAAGACTGACG
Mycn	ACTCAGATGATGAGGATGAC	GTGATCGTGAAAGTGGTTAC
Nfatc2	TGGATGACGAGTTGATAGAC	GATTTCTCGGATCAAAGACC
Nocturnin	ATCCAGGGGAGCTATCTGTG	CTCACGTCATTGGGAGTGG
Npas1	AAGGATAGAGCAAAGAGAG	CATTTTCCGAGTGTTACCA
Nrxn1	AATCTGCGTCAGGTGACAATATC	GCCACCACACCGTGAATCTT
Per1	TGAAGCAAGACCGGGAGAG	CACACACGCCGTCACATCA
Phospho1	CTGAAGATGTGTTGAAGACC	TAGGTGATCCTAACCAATCC
Skt11	TCACACTTTACAACATCACC	CTCTCCCAATGTTCTCAAAG
Soga3	AGATGGAGAAGCTGAGGGAAGAG	AGTTGACAGGCATCCTCCTCGA
Sorbs3	TCAGGCTTTGTGATGATGGT	ACTCTGGGTTTGGGATCTGG
Srcin1	CCTGATAAAGGCAAACATGG	CTCTTTAGATGGTTTAGTGGC
Sycp2	TAAGGAGATGCATGAAGAGG	CACAAGAATAGAGGTGCTTAG
Sycp2a_1	TAAGGAGATGCATGAAGAGG	CACAAGAATAGAGGTGCTTAG
Sycp2b_2	GAATTTTTGCATTCAGCAGG	CTGTGGTCATTCTACACAAAG
Tbx1	GGCCATATTATTCTCAACTCC	GTTCTCCTCTGCATATTTCTC
Tmem 223	CGGCGTTCTACTTCCTTTT	TGGGCACCTTTTTATTGTCC
Tmem 121	GCCACCACGTTCTTGCCTAC	GCCTCACCTTTTTCTATTGCT
Zfp467	TCCTGCTCAGGGCATGAGA	TCCGAATCATCCATTCCTCCC
Zfp775	GGGATATAGGGGAGGACTGG	ACAGGTAAGGCCACAGAAGG
Zfpm1	TCTGGTGGACTGCTATATGTGC	CCTTGTTGATGACTGCGGTAG

Appendix Fig 1. Mean methylation % in sperm samples. Sperm samples were analyzed by Amplicon Seq obtaining % of methylation of the gene in each generation. *p<0,05. Data are mean ±sem

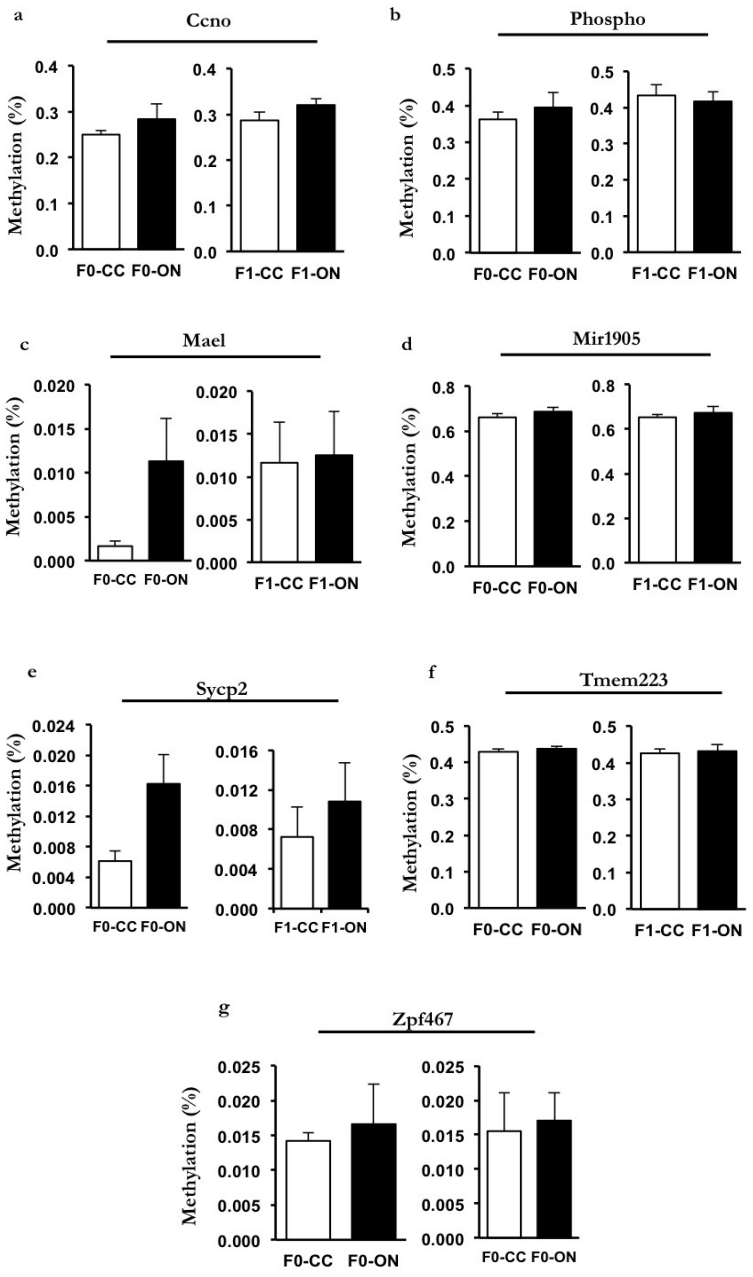
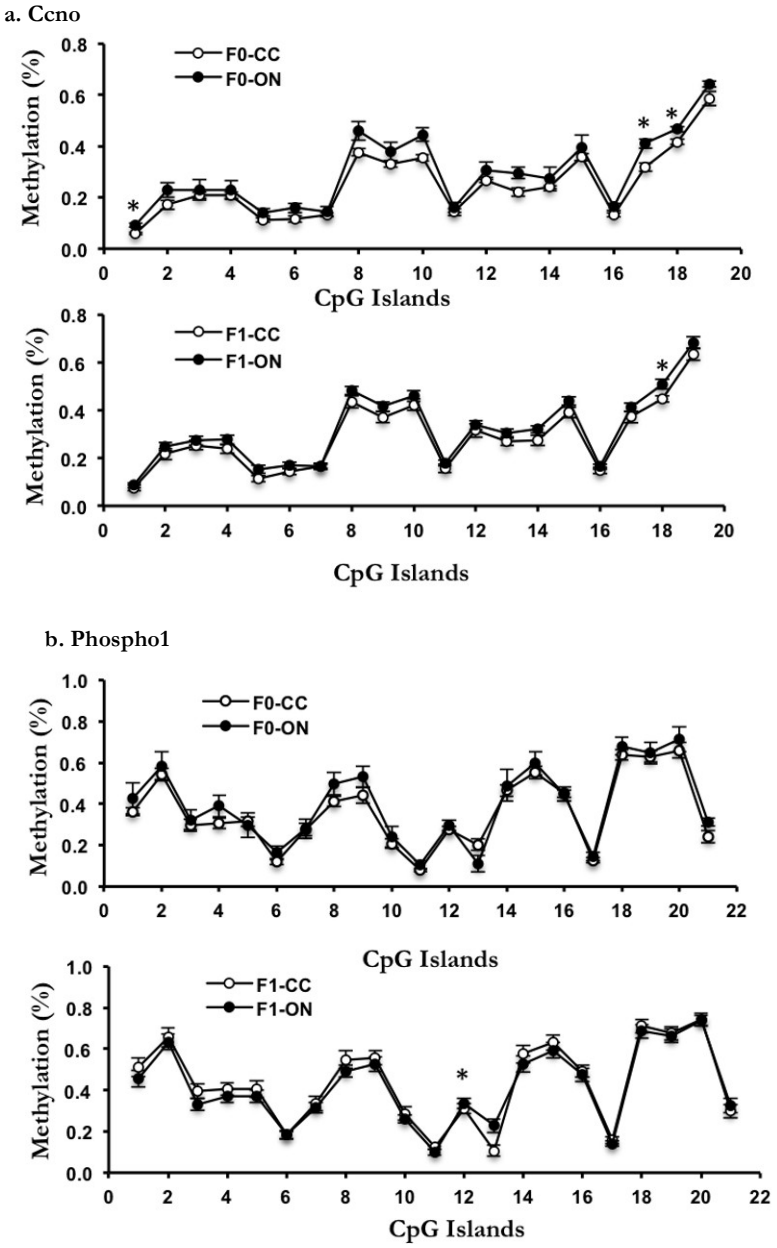
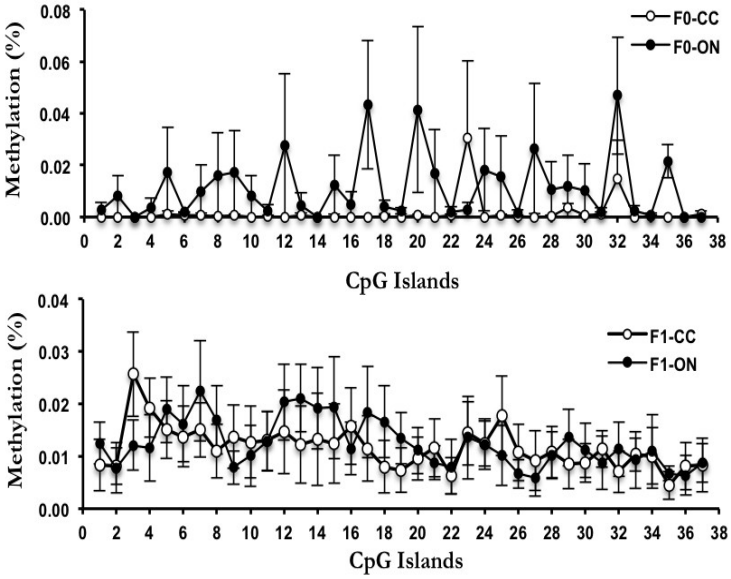


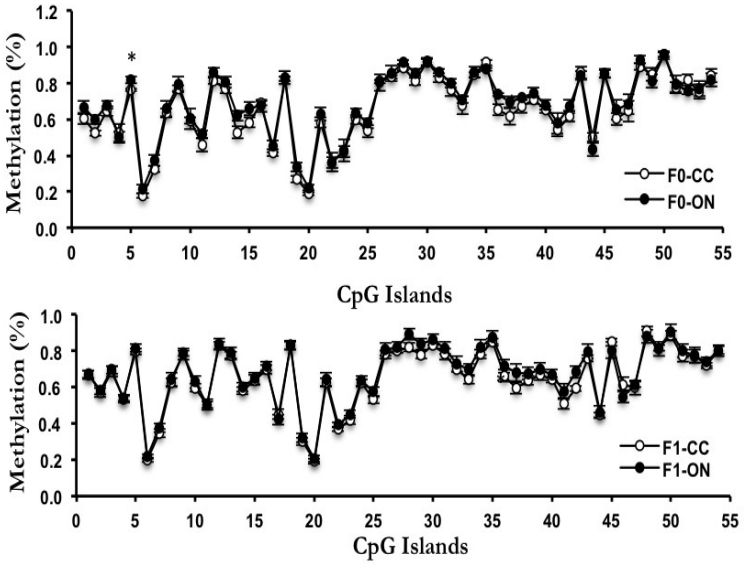
Fig 2. Methylation of CpG Islands in sperm samples. Methylation percentage of each CpG Island was measured by Amplicon Seq in sperm samples. Differences in specific CpG Islands were observed (*p<0,05)



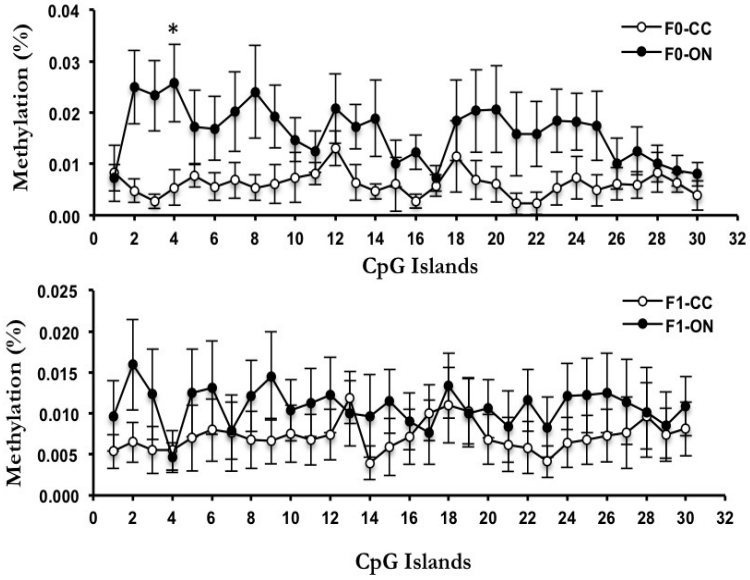
c. Mael



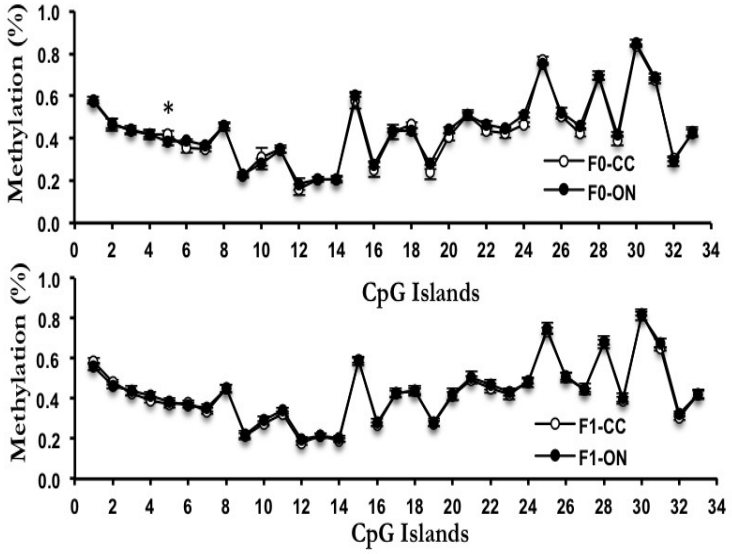
d. Mir1905



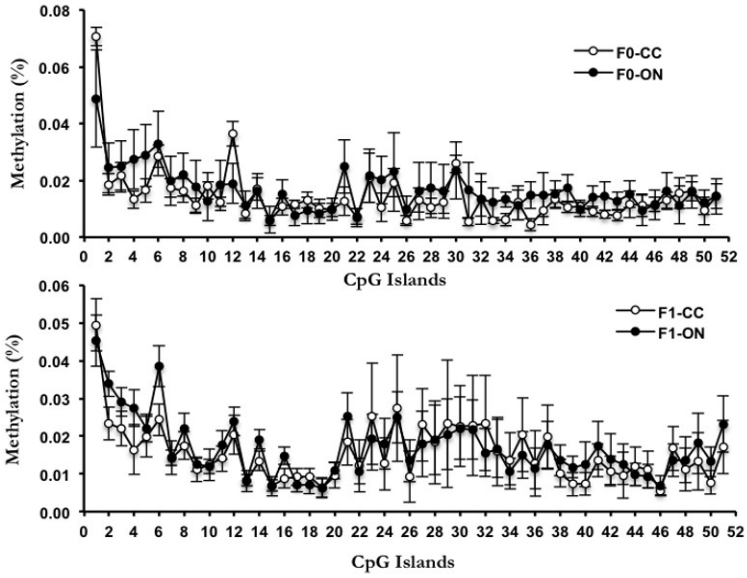
e. Sycp2



f. Tmem223



g. Zfp467


F0-CC
F0-ON
Methylation (%)
CpG Islands
F1-CC
F1-ON
Methylation (%)
CpG Islands

Appendix Fig 3. Gene expression in liver samples. Liver samples retrieved from 8 days-old mice and analyzed by qPCR. a) Gene expression in F1 liver samples. b) Gene expression in F2 liver samples (white bars CC, black bars ON)