High Vitamin Intakes during Pregnancy and Characteristics of Metabolic Syndrome in Wistar Rat Dams and their Offspring

by

Sandra Alicia Reza López

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Nutritional Sciences
University of Toronto

© Copyright by Sandra Alicia Reza López, 2011
High Vitamin Intakes during Pregnancy and Characteristics of Metabolic Syndrome in Wistar Rat Dams and their Offspring

Sandra Alicia Reza López

Doctor of Philosophy

Department of Nutritional Sciences
University of Toronto

2011

Abstract

High vitamin (HV), AIN-93G diet with ten-fold the regular amount of vitamins (RV), consumed by pregnant Wistar rats increases characteristics of metabolic syndrome (MetS) in their first litters. Therefore, the effects of the maternal HV-diet on tissue mechanisms regulating insulin resistance in offspring (Part 1) and on characteristics of MetS in the dams and their second litter (L2) offspring (Part 2) were examined. Part 1 (studies 1 and 2) hypothesis was that the maternal HV-diet alters tissue fatty acid (FA) concentrations, expression of peroxisome-proliferator-activated receptors (PPARs) genes, and their regulation of metabolism in the offspring, favoring insulin resistance. Part 2 (studies 3 and 4) hypothesis was that high-vitamin intakes during the first pregnancy increase weight gain, food intake and markers of MetS in both the dams and their litters. In all experiments, dams were fed the RV or HV-diet. In study 4, a high-folic-acid-diet (HFol, RV+10-fold folic acid) was added. In studies 1 and 2, the offspring were weaned to an obesogenic diet. The HV-diet affected tissue FA concentrations (study 1), increased muscle PPAR-α mRNA levels and
uncoupled relationships between hepatic PPAR-γ mRNA levels and insulin resistance (study 2) in male offspring. In study 3, dams fed the HV-diet during the first pregnancy were maintained on the RV-diet and then mated again after 12wk. Their litters were fed the RV-diet. The HV-diet increased weight gain and food intake of both dams and L2, and insulin resistance in their offspring. In study 4, both HV and HFol-diets increased post-weaning weight gain, but differed in their effects on biomarkers of food intake regulation. In conclusion, feeding the HV-diet during the first pregnancy increases post-weaning body weight and food intake in Wistar rat dams, uncouples tissue regulation of glucose metabolism and promotes characteristics of MetS in their litters. Folic acid is not the only vitamin involved.
Acknowledgments

All the way along the PhD program, I have received advice and encouragement from many people, faculty, staff, and students in the Department of Nutritional Sciences. Thank you all.

I am sincerely and heartily grateful to Dr. G. Harvey Anderson, for his thoughtful guidance and support in developing this thesis and the work behind it. My sincere thanks also go to Dr. David Ma for his advice and encouragement during all these years. I would also like to thank Dr. Richard Bazinet for his valuable advice and suggestions for improving this thesis and the studies it contains.

Thanks to all the members of Anderson and Ma Laboratories. My special gratitude to Dr. Bohdan Luhovyy for his help and encouragement, to Pedro Huot for sharing some of his knowledge, from analytical techniques to electronic devices, and to Ignatius Szeto for providing some data and samples from previous experiments. Special thanks to the research assistants and volunteers for their help in the laboratory work, in particular to Abraham Poon and Daniel Cho, for their assistance in gene expression analysis.

My gratitude extends to all the people from other laboratories and the departamental staff that enriched my experience along these years in the Department. Thanks to Dr. Thompson, Dr. Ward, Dr. Archer, Dr. El-Sohemy and Dr. Bazinet’s groups for their feedback and comments during lab meetings that helped me to improve seminar and other presentations. Also thanks to Dr. Elena Comelli for her advice on RT-PCR. My appreciation also extends to Louisa Kung and Vijay Chetty, and members of the staff, for their guidance and help in administrative matters and their kindness all along the program.

My sincere appreciation also to Nita Prayitno, Tina Akhavan, Rebecca Mollard, Maria Fernanda Nunez and Diana Sanchez, for their kindness, friendship and for sharing too many moments along these years.

I truly thank my husband Santiago for his unconditional help and support.
My gratitude extends to the Consejo Nacional de Ciencia y Tecnologia (CONACyT) in Mexico, for the scholarship provided during the first 4 years of the PhD program. Also special thanks to the Delta-Kappa-Gamma Society International for the World Fellowship, along with my profound respect and admiration for their contribution in improving opportunities for women in education, and to the University of Toronto for the Doctoral Thesis Completion Grant awarded during my last year of the program.

This research was supported by the Canadian Institute of Health Research, Institute of Nutrition, Metabolism and Diabetes Strategic Initiative (CIHR-INMD): “Excellence, Innovation and Advancement in the Study of Obesity and Healthy Body Weight” – Grant Number OOP-77980, and Institute of Nutrition, reference MOP-93624; and the Bristol-Myers Squibb / Mead Johnson Freedom to Discover Grant.
# Table of Contents

Abstract ............................................................................................................................................ ii

Acknowledgments ................................................................................................................................... iv

List of Tables ....................................................................................................................................... xii

List of Figures ....................................................................................................................................... xv

List of Abbreviations ............................................................................................................................ xvii

List of Appendices ............................................................................................................................... xxi

List of Publications Arising from this Thesis ....................................................................................... xxii

Chapter 1. Introduction ....................................................................................................................... 1

Chapter 2. Literature Review ............................................................................................................. 4

  2.1. Introduction ............................................................................................................................... 4

  2.2. Maternal Nutrition and Offspring Phenotype ......................................................................... 5

    2.2.1. The developmental origins of health and disease ............................................................... 5

      2.2.1.1. Maternal nutrition and overweight and adiposity in offspring .............................. 7

      2.2.1.2. Maternal nutrition and lipid metabolism in offspring ........................................... 10

      2.2.1.3. Maternal nutrition and glucose metabolism in offspring ..................................... 12

    2.2.2. Maternal characteristics and developmental origins of disease ................................... 15

    2.2.3. Diet during pregnancy and effects on the mother ........................................................... 17
2.2.3.1. Maternal physiology during pregnancy................................. 17
2.2.3.2. Diet components and maternal metabolism............................. 19
2.2.4. Animal models.................................................................................. 20
2.2.5. Potential mechanisms of developmental origins of disease ................. 20
  2.2.5.1. Alterations in organ/tissue structure ........................................ 21
  2.2.5.2. Hormone concentrations and early development ....................... 22
  2.2.5.3. Epigenetic regulation of gene expression .................................... 25
2.3. Maternal Vitamin Intake and Offspring Phenotype............................. 30
  2.3.1. General vitamin functions ............................................................... 30
  2.3.2. Consequences of vitamin deficiencies during pregnancy ............... 31
    2.3.2.1. Studies in humans ................................................................. 32
    2.3.2.2. Studies in animals ................................................................. 34
  2.3.3. Vitamin supplementation during pregnancy ............................... 36
    2.3.3.1. Birth weight .......................................................... 36
    2.3.3.2. Postnatal growth, lipid and glucose metabolism in offspring .... 39
  2.3.4. Consequences of excessive vitamin intakes ................................. 40
    2.3.4.1. Studies in humans ............................................................... 41
    2.3.4.2. Studies in animals ............................................................... 42
  2.3.5. Vitamin intake during pregnancy: effects on the mother ............... 44
4.2. Materials and Methods .................................................................64
4.3. Results..............................................................................................67
4.4. Discussion.........................................................................................69

Chapter 5. High Multivitamin Intakes during Pregnancy and Post-Weaning Obesogenic Diets Interact to Affect the Relationship between Expression of PPAR Genes and Glucose Regulation in the Offspring ..........................................................81
5.0. Abstract.........................................................................................81
5.1. Introduction...................................................................................82
5.2. Material and Methods ....................................................................83
5.3. Results.............................................................................................85
5.4. Discussion.........................................................................................87

Chapter 6. High Vitamin Intakes During the First Pregnancy Affect the Dams and their Offspring through Two Pregnancies.........................................................95
6.0. Abstract.........................................................................................95
6.1. Introduction..................................................................................96
6.2. Material and Methods .................................................................98
6.3. Results...........................................................................................101
6.4. Discussion.....................................................................................104

Chapter 7. Effects of High Multivitamin and Folic Acid Intakes during Pregnancy on Body Weight and Food Intake Regulation in Wistar Rat Dams..............................117
7.0. Abstract ................................................................. 117

7.1. Introduction ........................................................................ 118

7.2. Material and Methods ..................................................... 119

7.3. Results ............................................................................... 120

7.4. Discussion .......................................................................... 121

Chapter 8. General Discussion .................................................. 129

8.1. Part 1: The Effect of the HV-Diet on Tissue Fatty Acids and PPAR Gene Expression ...................................................... 129

8.1.1. The effect of the HV diet on tissue fatty acid concentrations ............... 130

8.1.2. Effects of HV-diet on PPAR gene expression ........................................ 132

8.1.3. Effects of the post-weaning obesogenic diet ......................................... 132

8.1.4. Effect of sex of the offspring .................................................... 133

8.1.5. Conclusion and significance ................................................ 135

8.2. Part 2: High Vitamin Diets during Pregnancy and Weight Gain, Food Intake and Insulin Resistance in the Dams and their Second Litters ......................... 135

8.2.1. Effects on the second litters ................................................. 136

8.2.2. Effects of the maternal HV-diet on the dams .................................. 137

8.2.3. Effects of parity ..................................................................... 138

8.2.4. Conclusion and significance ................................................ 139
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3</td>
<td>Strengths and Limitations of the Studies</td>
<td>139</td>
</tr>
<tr>
<td>8.6</td>
<td>Conclusions</td>
<td>144</td>
</tr>
<tr>
<td>8.6.1</td>
<td>Specific conclusions</td>
<td>144</td>
</tr>
<tr>
<td>8.6.2</td>
<td>Overall conclusion</td>
<td>144</td>
</tr>
<tr>
<td>8.7</td>
<td>Future Directions</td>
<td>145</td>
</tr>
</tbody>
</table>

References | 147 |

Appendices | 198 |

Copyright Acknowledgements | 213 |
### List of Tables

<table>
<thead>
<tr>
<th>TABLES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 4.1. Composition of experimental diets</td>
<td>74</td>
</tr>
<tr>
<td>Table 4.2. At birth, gestational diet influences liver FA concentrations</td>
<td>75</td>
</tr>
<tr>
<td>(milligrams per gram tissue) of the unsexed offspring</td>
<td></td>
</tr>
<tr>
<td>Table 4.3. At weaning, gestational diet and sex influence adipose tissue</td>
<td>76</td>
</tr>
<tr>
<td>FA concentration (milligrams per gram tissue) of the offspring</td>
<td></td>
</tr>
<tr>
<td>Table 4.4. At 12 weeks PW, gestational diet and sex influence muscle FA</td>
<td>77</td>
</tr>
<tr>
<td>concentration (milligrams per gram tissue) of the offspring</td>
<td></td>
</tr>
<tr>
<td>Table 4.5. At 48 weeks post-weaning, gestational diet and sex influence</td>
<td>78</td>
</tr>
<tr>
<td>FA concentrations (milligrams per gram tissue) of liver phospholipids</td>
<td></td>
</tr>
<tr>
<td>in the offspring</td>
<td></td>
</tr>
<tr>
<td>Table 4.6. At 48 weeks PW, gestational diet and sex influence FA</td>
<td>79</td>
</tr>
<tr>
<td>concentration (micrograms per gram tissue) of brain phospholipids</td>
<td></td>
</tr>
<tr>
<td>in the offspring</td>
<td></td>
</tr>
<tr>
<td>Table 5.1. Gene expression of PPARs on tissues from offspring at birth</td>
<td>91</td>
</tr>
<tr>
<td>(unsexed) and males at weaning</td>
<td></td>
</tr>
<tr>
<td>Table 5.2. Gene expression in male offspring 14 wk PW in selected tissues</td>
<td>92</td>
</tr>
<tr>
<td>Table 5.3. Correlation between PPARs gene expression and fat mass and</td>
<td>93</td>
</tr>
</tbody>
</table>
insulin resistance index at 14 wk PW, in all animals and by gestational diet group

Table 6.1. Food intake, glucose response and hypothalamic gene expression of the dams after weaning their second litters

Table 6.2. Effect of vitamin intake during the first pregnancy on litter size, body weight, food intake and abdominal fat of the male offspring from first and second litters

Table 6.3. Blood glucose concentration following a glucose load in first and second litters at 5, 9 and 13 wk post-weaning

Table 6.4. Glucose and hormone concentrations of the male offspring at weaning, 30 min following water or glucose gavage

Table 6.5. Blood glucose and plasma hormone concentrations of the second litters at 16 weeks post-weaning following glucose or water gavage

Table 6.6. Hypothalamic gene expression from the L1 and L2 offspring at weaning

Table 7.1. Body weight and food intake of the dams during pregnancy and lactation

Table 7.2. Glucose and hormone concentrations in blood from dams at
weaning and 20 wk PW

Table A.2.1. Weight gain and food intake of female offspring from first and second litters

Table A.2.2. Results from glucose tolerance tests of the female offspring from first and second litters
## List of Figures

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1. Epigenetic regulation of gene expression</td>
<td>27</td>
</tr>
<tr>
<td>Figure 2.2. Vitamins involved in one-carbon metabolism</td>
<td>29</td>
</tr>
<tr>
<td>Figure 2.3. Vitamins in intrauterine development</td>
<td>49</td>
</tr>
<tr>
<td>Figure 5.1. PPARs mRNA levels in the liver and adipose tissue and insulin resistance index and fat mass in male offspring at 14 weeks post-weaning, by gestational diet</td>
<td>94</td>
</tr>
<tr>
<td>Figure 6.1. Effect of HV diet during the first pregnancy on body weight gain of the dams from delivery to 12 weeks after weaning their first and second litters</td>
<td>115</td>
</tr>
<tr>
<td>Figure 6.2. Hypothalamic gene expression from the second litters at 16 wk post-weaning</td>
<td>116</td>
</tr>
<tr>
<td>Figure 7.1. Weight gain of dams after weaning their litters</td>
<td>127</td>
</tr>
<tr>
<td>Figure 7.2. Total food intake and weigh gain from weaning to 20 wk Post-weaning</td>
<td>128</td>
</tr>
<tr>
<td>Figure A.1.1. Protein abundance of PPARs in muscle from male offspring at weaning</td>
<td>200</td>
</tr>
</tbody>
</table>
Figure A.1.2. Protein abundance of PPARs in muscle from male offspring weaned to the RV-diet, at 14 wk post-weaning weaning

Figure A.3.1. Caveolin 1 protein abundance in male offspring at weaning. A. Whole tissue homogenate; B. Plasma membrane fraction; C. Caveolae fraction

Figure A.3.2. Caveolin 3 protein abundance in male offspring at weaning. A. Whole tissue homogenate; B. Plasma membrane fraction; C. Caveolae fraction
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>11β-HSD</td>
<td>11β-hydroxy steroid dehydrogenase</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
</tr>
<tr>
<td>α-MSH</td>
<td>α-melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>ARH</td>
<td>Arcuate nucleous</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine-and amphetamine-regulated transcript</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitoyl transferase 1</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dnmt</td>
<td>DNA methyl transferase</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FAT</td>
<td>Fatty acid translocase</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational diet</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High density lipoprotein-cholesterol</td>
</tr>
<tr>
<td>HFol</td>
<td>High folic acid</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostasis model assessment</td>
</tr>
<tr>
<td>HV</td>
<td>High vitamin</td>
</tr>
<tr>
<td>IRI</td>
<td>Insulin resistance index</td>
</tr>
<tr>
<td>L1</td>
<td>Litter 1</td>
</tr>
<tr>
<td>L2</td>
<td>Litter 2</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low density lipoprotein-cholesterol</td>
</tr>
<tr>
<td>LepR</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>LGA</td>
<td>Large for gestational age</td>
</tr>
<tr>
<td>MD</td>
<td>Maternal diet</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple micronutrient</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>MR4</td>
<td>Melanocortin receptor 4</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PD</td>
<td>Pup diet</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal phosphate</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PW</td>
<td>Post-weaning</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended dietary allowances</td>
</tr>
<tr>
<td>RV</td>
<td>Regular vitamin</td>
</tr>
</tbody>
</table>

xix
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>RXR</td>
<td>Retinoic X receptor</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SGA</td>
<td>Small for gestational age</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element binding protein -1c</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>UL</td>
<td>Upper limit</td>
</tr>
<tr>
<td>UNIMMAP</td>
<td>UNICEF/WHO/UNU international multiple micronutrient preparation</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
</tbody>
</table>
List of Appendices

Appendix 1. Protein abundance of PPARs in muscle from offspring at weaning and 14 week post-weaning

Appendix 2. Effect of HV-diet on females from the first and second litters

Appendix 3. Effect of maternal HV-diet during pregnancy on caveolin protein abundance in cell fractions of muscle of the male offspring

Appendix 4. Glossary
List of Publications Arising from this Thesis

Published:


In preparation:


• Reza-López SA, Szeto IM, Huot PSP, Cho D, Ma DW, Anderson GH. High vitamin intake during the first pregnancy affects the dams and their offspring through two pregnancies.

• Reza-López SA, Poon AN, Szeto IM, Ma DW, Anderson GH. High multivitamin intakes during pregnancy and post-weaning obesogenic diets interact to affect the relationship between expression of PPAR genes and glucose regulation in the offspring.
Chapter 1. Introduction

Maternal malnutrition and overnutrition increase the risk of obesity and chronic diseases in the offspring later in life [1, 2]. Several terms such as “thrifty phenotype” [3] “programming” [4], “predictive adaptive response” [5] or “induced phenotype” [6] have been coined in the last three decades to describe the effects of the environmental factors during early life on the phenotype of offspring. Most of research on developmental origins of health and disease has focused on the consequences of inadequate energy and macronutrient intakes [7-9] in humans and animals. Some studies in animals have examined the effects of energy and macronutrients above the recommended intakes in maternal diets [10-14], but the effects of vitamin intakes, either below or above requirements on the risk of chronic diseases in later life have received little attention.

In humans, intakes of vitamins above recommendations occur in developed countries as a result of food fortification strategies of government, discretionary fortification by the food industry and readily available supplements [15-19]. Food fortification strategies, such as the addition of folic acid to flour, have been effective in preventing vitamin deficiencies and congenital malformations [20]. However, high intakes may also influence maternal metabolism and the normal development of their progeny [21-25]. Animal models have allowed exploration of the effects of restricted or increased maternal intake on development and phenotype of the offspring later in life. Rats and mice in particular have been useful in assessing long-term outcomes, because of their short gestational period and life span [2]. The focus of this thesis is on the effects of high vitamin intakes during the first pregnancy on characteristics of metabolic syndrome in Wistar rat dams and their offspring.

It is widely recognized that vitamins play fundamental roles in fetal development in both human and animals [26-28], but their role when consumed above requirements in modulating the phenotype of the offspring later in life has been addressed only recently [29-31]. Previous studies by our group show that high multivitamin intake during pregnancy by
Wistar rats results in higher body weight and fat mass, food intake, and insulin resistance in offspring [32] that were exacerbated by feeding an obesogenic diet post-weaning [33]. However investigations of either the mechanisms by which these metabolic changes occur or of the effect of the HV-diet on the dams and their later progeny have not been reported. These topics are the focus of Part 1 and Part 2, respectively, in this thesis.

Metabolic syndrome is characterized by a combination of obesity, hypertension and altered glucose and lipid metabolism [34]. Because alterations in tissue fatty acid composition in muscle have been related to adiposity and insulin resistance [35] and peroxisome-proliferator activated receptors (PPARs) play a substantial role in regulating lipid metabolism and insulin resistance in several tissues [36], the objective of Part 1 of the thesis was to explore the effects of the HV-diet on these parameters in the tissues of offspring fed an obesogenic diet. Tissue fatty acid concentration and composition of tissues were examined in study 1 (chapter 4). In study 2 (chapter 5), gene expression of PPARs was measured and related to measures of abdominal fat mass and insulin resistance.

In addition to exploring mechanisms at the tissue level, physiologic and metabolic effects of the HV maternal diet on the dams and their second offspring were explored in Part 2 of the thesis. First, the effects of a single exposure to the HV-diet during the first pregnancy on the body weight, food intake, fat mass and insulin resistance on both dams and their offspring after the first and second pregnancies were investigated and are presented in study 3 (chapter 6). The rationale for this study was that studies testing the effects of nutritional components of the maternal diet often report outcomes in offspring from a first pregnancy only or do not specify the parity status of the mothers [29, 32, 37]. Furthermore, reports of the effects of dietary interventions during pregnancy on the mothers are few and limited to immediate outcomes, such as maternal mortality, anemia or preeclampsia [38, 39] or short-term follow up [23, 40-42]. Second, in study 4, a 10-fold increase of folic acid alone was compared with the high multivitamin diet for effects on body weight, biomarkers of food
intake regulation and insulin resistance of Wistar rat dams after their first pregnancy (chapter 7). Multivitamin mix was selected in the four studies as the main dietary intervention because multivitamin supplements are commonly consumed by pregnant women [43, 44]. However, high intakes of folic acid [45] are also common, due to the consumption of fortified food [18, 19], and the recommendations for it as a prenatal supplement [46] that in some cases reaches 1-5-fold the tolerable upper limit [47] (1-5 mg, according to the product manufacturers information) [48, 49]. Because of the role of folic acid in epigenetic regulation of gene expression, not only during early development but also in later stages of life [50, 51], folic acid was hypothesized to be a significant component of the high multivitamin diet affecting outcomes in the dams.
Chapter 2. Literature Review

2.1. Introduction

Maternal nutrition is a fundamental factor influencing fetal development and the health of the offspring later in life [52]. The effects of nutrient deficiency in maternal diets (e.g. energy, protein, or global restriction) on the offspring have been extensively documented in human and animal studies (Reviewed in [7, 53-55]). More recent research in humans and animal models has provided evidence that maternal overnutrition (e.g. fat, protein) during pregnancy also affects the phenotype of the offspring [56-59], but the effects of high intakes of vitamins during pregnancy have received little attention.

To provide a framework for the work conducted, the first theme of the review addresses the effects of maternal diet on components of the metabolic syndrome, with emphasis in body weight, adiposity, glucose and lipid metabolism of the offspring. This is followed by a brief discussion on adaptations of maternal physiology and the effects of diets during pregnancy on the dams, and mechanisms by which maternal diets affect fetal development and metabolism of the offspring.

The second theme section provides an overview of vitamin functions followed by an examination of the effects of consumption of single or vitamin combinations during pregnancy on birth weight and long-term outcomes of the offspring and the dams. Finally, the potential mechanisms by which maternal vitamins regulate gene expression in the offspring and the expression of phenotype are discussed.
2.2. Maternal Nutrition and Offspring Phenotype

Maternal nutrition plays a fundamental role in fetal development influencing short-term (i.e. birth weight) and long-term (chronic diseases) outcomes in the offspring. In the following sections, some of the hypotheses that capture the idea of the developmental origins of health disease are described. Then, the effects of maternal nutrition on the metabolism of the offspring and mothers are described. Finally, potential mechanisms underlying the developmental origins of disease are discussed.

2.2.1. The developmental origins of health and disease

Currently, the idea that maternal nutrition influences fetal development with consequences far beyond the first year of life is widely accepted. This is a core concept that led to the formulation of terms such as the “thrifty phenotype hypothesis” [60], the “fetal programming” of disease, the “predictive adaptive response hypothesis” [61]. They have framed the hypotheses of the developmental origins of health and disease.

An association between events occurring in childhood and mortality was first suggested by Kermack et. al. in 1934. They found a decline in the mortality rates of Great Britain from 1841-50 and in Sweden from 1751-60 and suggested that the “improved child environment” of the examined cohorts during their first 15 years of life and improved maternal health could be reasons for this decrease in mortality [62]. Almost 50 years later, an epidemiological study by Barker and Osmond in Great Britain reported a strong correlation between the mortality rates for ischemic heart disease in 1960-70 and the rates of infant mortality around 1920, and suggested that variations in the distribution of ischaemic heart disease reflected variation in prenatal and early life nutrition [63]. In 1992, Hales and Barker proposed the “thrifty phenotype hypothesis” postulating that “poor fetal and early-postnatal nutrition imposes mechanisms of nutritional thrift upon the growing individual”. According
to this hypothesis, the fetus would respond to an adverse environment by limiting its growth to increase its chances of postnatal survival, but at greater risk of chronic diseases. Then a poor fetal nutrition, maybe due to maternal undernutrition, would be detrimental for the development of structure and function of the pancreas, for instance, thus increasing the risk of type 2 diabetes in adulthood [60].

Later studies showed associations between birth weight and cardiovascular disease. A delay in fetal growth and development associates with increased risk of chronic diseases during adulthood [64-66], suggesting that fetal programming occurred. The concept of “programming”, originally stated by Dörner in 1974, was that events occurring during sensitive periods of development have long-term consequences in the offspring. Because “fetal programming” or “fetal origins of disease” are restricted to intrauterine life, “early programming”, “early origins” or “developmental origins” of health and disease have been also used to include other sensitive periods of development, such as lactation.

Epidemiological studies in many populations around the world have confirmed an association between birth weight and chronic diseases during adulthood. The main measure of the exposure used in these studies was birth weight or derived measures (e.g. low birth weight, small for gestational age (SGA), ponderal index), but did not clarify the association between maternal nutrition and disease of the offspring during adulthood, since birth weight is influenced by many maternal factors, including age, size, parity and hormonal status [67, 68]. However, the importance of the maternal diet during pregnancy and the timing of exposure were highlighted by retrospective cohort studies of men and women exposed during gestation to the Dutch famine during the World War II. A deficient maternal diet during early pregnancy resulted in higher body mass index (BMI), a more atherogenic lipid profile and coronary heart disease in progeny by 50-years of age [9, 69, 70]. In contrast, men and women whose mothers were exposed to the famine during late pregnancy showed altered glucose metabolism (lower glucose tolerance and insulin sensitivity) [71].
Environmental stimuli may induce structural and physiological changes *in utero* to increase the chances of survival in the external environment. An example commonly cited in the literature is the observation that offspring of daphnia, a freshwater crustacean, develops a protective “helmet” when the mother is exposed to chemicals produced by predators [72]. This kind of adaptation is often referred to as “developmental plasticity” which allows different phenotypes to appear from a given genotype, due to environmental factors [73]. Plasticity allows the organism to adapt to its environment, thus increasing survival by matching its characteristics to its environment. Because the fetus adapts its physiology to the intrauterine environment the changes induced may also modify its response to postnatal environments, expected to remain similar across the life span. The “predictive adaptive response” hypothesis proposes that if the later environment does not correspond to the predicted *in utero*, this mismatch would increase the risk of chronic disease [61].

These hypotheses provide a framework for deriving explanations to the core concept that there are sensitive periods in development characterized by rapid growth and/or differentiation in which environmental stimuli exert a strong influence modulating the organism’s further adaptation and the risk for chronic diseases later in life. Under this concept, metabolic changes may be induced in early life, modulating the risk of obesity and chronic diseases during adulthood. Studies in animals have provided some explanation of the mechanisms of the effects of maternal diets on metabolic response in the offspring, as discussed in the following sections.

### 2.2.1.1. Maternal nutrition and overweight and adiposity in offspring

In humans, epidemiological studies show that birth weight is associated with higher body mass index and adiposity later in life [66, 74-76]. This relationship seems to have a “U” shape. Lower birth weight was associated with greater adiposity and fat distribution during adolescence in a cohort of girls born in Southampton [66], small for gestational age with obesity at the age of 31 in a cohort of men and women from Finland [77], and lower birth
weight with higher risk of adult adiposity in a study from The Netherlands [78]. At the other extreme, high birth weight was associated with higher BMI. Women from the Nurses Health Study and men from the Health Professionals Follow Up Study weighing >10 lb at birth had higher (62% or more than double, respectively) risk of being in the highest quintile of BMI in adulthood, respectively [79, 80]. Being either in the lowest or the highest birth weight were also reported to be associated with higher BMI in a British cohort [81] and increased risk for abdominal obesity in Chinese adults [82].

Maternal nutrient restriction during pregnancy is also associated with increased risk of overweight and central obesity during adulthood in the offspring. Women whose mothers were exposed to the Dutch famine in early gestation had 7.4% higher BMI and increased waist circumference at ~50 years of age [69]. Similarly, results from the Chinese famine (1959-1962) showed that the probability of being overweight was higher in the provinces that experienced more severe famine. The effects of famine exposure during pregnancy in these cohorts were primarily observed in women, but not in men [83]. However, in another cohort in Nigeria, both men and women exposed to a famine period in utero had higher waist circumference and BMI>25 [84]. These associations between birth weight and maternal nutrition with the risk of overweight and obesity during adulthood provide support for the concept of developmental origins of obesity.

Experiments in sheep and rodents also find that both under and overnutrition during pregnancy are related to the risk of higher body weight and adiposity later in life. Global or protein deficits in maternal diets of rats or mice during pregnancy increase body weight and fat in adult offspring, and this effect is enhanced when followed by overnutrition during lactation [85-88]. In rats, overnutrition caused by feeding high-fat or palatable obesogenic diets before and during pregnancy and lactation also increases body weight gain and adiposity in offspring at 15 weeks post-weaning [89] or at 12 months of age [90], respectively. Similar results have been observed as early as 20 days of age in offspring from dams fed a cafeteria diet (standard chow+condensed milk+saturated animal fat+milk powder, cakes and biscuits) before and during pregnancy [59].
The cause of overweight/obesity in the offspring may be related to the effects of the maternal diets on the fetus, affecting the development of mechanisms regulating body weight homeostasis. Body weight homeostasis is maintained by a series of complex mechanisms that regulate energy balance and requires the integration of peripheral signals by specialized areas of the brain that sense the nutritional status of the rest of the body. The excess of energy is stored as fat, primarily in adipose tissue [91]. Peripheral tissues (e.g. gut, pancreas, adipose tissue) release hormones related to short and long-term food intake regulation that have their targets in the brain, mainly in the hypothalamus. Ghrelin, or the “hunger hormone”, is released by the stomach and it is known for its effects in stimulating food intake in rats, mice and humans [92-94], in particular in its acetylated or active form [93]. Among other hormones, peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) are released from the gut and act to reduce food intake [95, 96]. Insulin, secreted by the pancreas in response to circulating nutrients, and leptin, produced by adipocytes in proportion to fat content, also serve as peripheral signals of body fuel stores and stimulate or inhibit regulatory neuropeptides in the hypothalamus [97]. These hormones have receptors in the brain and serve as signals to the central nervous system [98].

In the hypothalamus, two subpopulations of neurons from the arcuate nucleus (ARH) expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY) or proopiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART) integrate peripheral signals to regulate energy balance [98]. AgRP and NPY are orexigenic, increase fat storage and decrease thermogenesis and energy expenditure. In contrast, CART is mainly known for showing the opposite effects, decreasing food intake and increasing thermogenesis. However, CART effects may result also in increased food intake depending on the hypothalamic nuclei stimulated [99, 100]. Similarly, α-melanocyte-stimulating hormone (α-MSH) derived from POMC decreases food intake, fat storage and increases thermogenesis and energy expenditure, but β-endorphin, also cleaved from POMC increases food intake [101]. Furthermore, effects of POMC also depend on the stimulated nuclei in the
hypothalamus leading to either decrease or no effect on food intake or even increase the risk of weight gain in animals fed a high fat diet [102].

The development of the hypothalamic circuitry in utero is sensitive to maternal nutrition influences. ARH neurons are detected around embryonic day 12 in the rat and during the second trimester in humans [103] when the fetus is nourished by maternal nutrient circulation only. In mice, global restriction during pregnancy (50%) has shown to increase the gene expression of leptin receptor in the hypothalamus in the 90 day old offspring [87]. Maternal high-fat diet fed to rats before and during pregnancy also increased gene expression of leptin receptor, NPY and AgRP, POMC and melanocortin receptor 4 (MR4) in the offspring hypothalami [104] and maternal high-carbohydrate diets increased release of NPY in adult offspring 100 d old, resulting in higher 1 and 2 hour food intake [105].

In rodents, development of hypothalamic neurocircuitry continues during lactation and it is not fully established until the third postnatal week [106]. Thus, nutrition manipulation during this period also affects the development of food intake regulation, as has been demonstrated by raising rats in small litters, as a model of overnutrition during lactation. Overnourished rats show increased gene expression of CART, NPY and AgRP and decreased expression of leptin receptor in the hypothalamus compared to controls [107]. Furthermore, the effects of maternal high-fat diets provided during pre and postnatal periods of hypothalamic development in rats result in increased expression of orexigenic neuropeptides in offspring detected as early as postnatal day 15 [108], providing evidence for early programming of food intake and a potential explanation for increases in body weight and fat.

2.2.1.2 Maternal nutrition and lipid metabolism in offspring

Epidemiological studies have shown that poor fetal growth is associated with a lipid profile that confers higher risk of cardiovascular disease in adulthood. In a cohort from
Herfordshire, U.K women in the lower categories of birth weight had higher triglycerides and lower HDL-C concentrations than women with adequate birth weight [109]. However, the relationship between birth weight and triglycerides concentrations shows a U-shape. Men and women, born with either low or high weight exhibit higher concentrations of triglycerides during adulthood [110]. This association between serum lipids and birth weight is evident in early life. Newborns small or large for their gestational age have higher triglyceride concentrations in cord blood, compared to those born with a weight considered adequate for their gestational age [111].

Maternal fat intake influences cholesterol and fatty acid concentrations in blood of offspring in early life because cord blood total cholesterol, HDL-C and LDL-C concentrations are correlated with those in maternal blood [112]. Factors that alter maternal lipid status, such as the amount [112] and composition of fat [113] in the diet, influence cholesterol, triglycerides and LDL-C concentrations in cord blood. As well, the proportions of saturated, mono- and polyunsaturated fatty acids in maternal diets are correlated with fatty acid composition of cord plasma phospholipids [114, 115]. However, maternal plasma concentrations do not necessarily predict the rate of transfer from the mother to placenta and fetus, due to placental metabolism and preferential transfer for LC-PUFAs [116, 117]. Higher concentrations of selected fatty acids [118] and of labeled fatty acids [119] are found in cord plasma compared to those in maternal plasma. Whether these associations persist over time and relate to the risk of disease during adulthood is currently unknown.

In animal models, maternal malnutrition induced by feeding energy or protein restricted diets also affects plasma and tissue concentration of lipids. Global restriction (50% from the ad libitum) of nutrients during pregnancy results in higher cholesterol in rat offspring at 1 month of age [120], whereas a moderate global restriction (25%), has no effect on plasma lipid, but increased triglycerides and cholesterol concentrations in the liver of male offspring at 3 weeks of age [121]. Protein restriction during pregnancy affects fetal body weight, and lipid composition in tissues of rat offspring. Low protein diets result in reduction of arachidonic (AA) and docosahexaenoic acid (DHA) concentrations in fetal brain [122] and
in reduced concentration of DHA in fetal brain phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [123], but has no effect on fatty acid composition of the liver [124] and the brain [125]. Differences in fatty acid composition of muscle have been also reported in offspring from low-protein fed dams [126].

Maternal overnutrition, by means of feeding obesogenic diets, such as the “cafeteria diet” also influences the lipid profile of rat offspring. In this experiment, the “cafeteria diet” consisted on a mix of pate, cheese, bacon, chips, cookies, and chocolate, providing 420 kJ/100g, 23% protein, 35% carbohydrate and 42% lipids, compared to the control diet that provided 330 kJ/100g, 25% protein, 65% carbohydrates and 10% lipids. Feeding rodents the “cafeteria diet” before and during pregnancy and lactation induced a fatty acid profile characterized by higher saturates and monounsaturates and decreased polyunsaturate concentration in VLDL and the liver, leading to a lower polyunsaturates/saturates ratio in 90 d old offspring [127].

2.2.1.3 Maternal nutrition and glucose metabolism in offspring

The regulation of glucose metabolism in adulthood is also influenced by early life nutrition as shown by both epidemiological and experimental studies. Insulin resistance, a dysregulation of glucose metabolism, is an important component of metabolic syndrome [128]. A relationship between birth weight and glucose intolerance has been established by several epidemiological studies. A cohort of men born between 1920-30 in Herfordshire, England had higher glucose concentrations during a glucose tolerance test if they had lower birth weight and weight at 1 year of age, suggesting an increased risk of non-insulin dependent diabetes at the age of 64 years [129]. Other cohort studies have confirmed this association [130-134].

The role of maternal diets during pregnancy on long-term glucose regulation in adult progeny is clear from studies of the effects of famine. Detailed records kept during the
Dutch famine showed that glucose and insulin concentrations after an oral glucose tolerance test were higher in adults whose mothers were exposed to the famine during mid and late gestation, and were the highest among those that become obese [71]. However, not only total caloric intake, but also composition of the maternal diets is important. A retrospective study from people born in Aberdeen, Scotland during 1948-54 found that plasma insulin was 7% lower for every 10 g increase in protein intake, and ~17% lower plasma insulin concentrations 30 min after a glucose challenge in those that consumed 80 or more g/d of protein compared with those that consumed 60-80 g/d. Similarly, ~5% lower for every 10 g of increase in fat intake in the maternal diet during late pregnancy and 11-29% lower plasma insulin 30 min after the glucose challenge was observed when maternal diet contained more than 115 g of fat/d compared to <85-115 g/d [135].

Experimental studies in sheep and rats have confirmed the effects of maternal diets on glucose regulation in later life of the offspring. Global restriction alters glucose metabolism. In sheep, periconceptional global dietary restriction (70% vs 110% in control) decreased plasma glucose in male lambs at birth, and increased their insulin response to a glucose challenge at 10 wk [136]. In guinea pigs, global dietary restriction to 85% during pregnancy or 70% during the first half of pregnancy and followed by 90% until delivery increased fasting insulin concentrations in plasma and glucose response to a glucose tolerance test [137]. In rats, a global restriction of 50% during the last 11 days of gestation also resulted in higher glucose response to glucose gavage at 8 and 22 wks of age suggesting insulin resistance [138].

Protein restricted diets also affect glucose metabolism in the offspring, but the results vary with the period of exposure. Offspring from Wistar rats were fed either control (20%) or low-protein (10%) diets during pregnancy and/or lactation. The protein restriction during pregnancy resulted in higher fasting glucose and insulin concentrations and higher 30 min glucose but not insulin. In contrast, those animals restricted during lactation only had lower fasting and 30 min glucose concentrations and lower insulin only 60 min after the glucose challenge. Those restricted during both pregnancy and lactation did not differ from controls.
in these measures. Sex influenced the results with reduced effects in females compared with males [139].

Macronutrient content of maternal diets interacts with pup diets and age in determining effects in the offspring suggesting that maternal diet effects are modifiable. For example, in a low protein diet (7% vs 25%), offspring at 20 weeks of age had lower glucose concentrations in a tolerance test. However, after 8 weeks exposure to a high fat diet those from protein-restricted dams, but not from control dams, showed impaired glucose response compared to offspring from dams fed the control diet [140]. Similar impaired glucose response has also been observed in offspring at older ages (17 months) from dams fed low-protein diet (8 vs. 20%) during pregnancy and lactation but not in younger animals (i.e. 16 weeks old) [141], suggesting that age is another factor playing a fundamental role in the regulation of glucose metabolism.

Although most experiments have shown that protein restriction during pregnancy and/or lactation affects glucose metabolism in an age-dependent manner, whether those effects are due to the low protein or the high carbohydrate content of the diets is not completely clear, since the protein deficit is fulfilled by higher amount or cornstarch or dextrose, in order to provide isocaloric diets. Therefore, the effects of low-protein diets could be seen as effect of nutrient imbalance or even as of carbohydrate excess. This simple contrast is often missed in the discussion of effects of protein deficient diets.

Micronutrient content of maternal diets also affects glucose metabolism. Selenium, zinc, and magnesium, but not iron amounts in maternal diets affect glucose and/or insulin level of offspring at several ages. Adequate or high levels (about 9-fold of selenium), combined with deficient (60%) or high energy (140%) diets altered glucose response in sheep [142]. A zinc-deficient diet fed before during and after pregnancy and lactation to rats resulted in higher glucose concentrations in offspring 15 wk old and affected glucose response after insulin and glucose tolerance tests in a sex-dependent manner and affected insulin signaling in muscle [143]. Offspring of dams fed a magnesium deficient diet (70%) also have a lower
insulin response to a glucose challenge in rats 180 d old, but not in early stages, with no effect in body weight [144]. Despite the well known deleterious effects of maternal iron deficiency, iron restriction in rats resulted in better glucose tolerance, without change in insulin levels in the offspring [145]. The restriction of about half of the mineral mix (iron, zinc, magnesium and calcium) resulted in lighter pups at birth and weaning, but no difference in glucose and insulin concentrations at the ages of 40, 70, 100 and 180 days when compared to adequate fed animals [146].

Increased amount of nutrients in maternal diets have been also found to have deleterious effects on glucose metabolism in the offspring. Pups from dams fed high-fat diet during pregnancy, lactation of both periods showed higher glucose concentrations after a glucose tolerance test at 3 weeks of age [147] and a diet high in saturated fat during pregnancy results in altered development and physiology of the pancreas in rats at birth and weaning [148]. Furthermore, offspring from dams fed saturated fat diets have reduced pancreatic islets and a higher insulin response after glucose load, despite having similar hexosamine expression [149].

2.2.2. Maternal characteristics and developmental origins of disease

Although it is clear that maternal nutrition during pregnancy plays a fundamental role in early development of the offspring, other maternal factors affect the intrauterine environment and influence outcomes in the offspring. These include maternal obesity, gestational diabetes, and parity.

Maternal preconceptional overweight and obesity have early effects on offspring growth and metabolism. Newborns large for gestational age (LGA) [150] or with higher fat mass [151] are more often born to overweight or obese women. Moreover, in addition to higher fat mass, they have higher homeostatic model assessment index (HOMA-IR, ~50% higher), cord blood leptin (~75% higher) [152] and twice the risk of components of metabolic syndrome
during adolescence [153]. Similarly, in rats and mice, preconceptional maternal obesity leads to higher food intake and body weight in offspring at 6 months or 1 year of age and also showed alterations in glucose metabolism [90, 154]. These effects are further enhanced by a high fat diet after weaning [155].

Maternal diabetes mellitus has been associated with an increased risk of adiposity and metabolic disturbances in progeny. Neonates from mothers with gestational diabetes mellitus have increased fat mass and lower fat-free mass when compared in the same category of birth weight (adequate-for–gestational-age) [156]. They also have higher BMI and HOMA index at 4-9 year old [157] and increased risk of overweight and central obesity at 9-11 year old [158]. Offspring of women with type 1 diabetes also have higher fasting serum insulin, insulin response and HOMA-B index values than in those from gestational diabetic mothers or controls [159]. Later in life they have higher risk of overweight, as seen in 7-year old children from women with type 1 diabetes that had higher body weight, BMI, waist circumference and adiposity, although children from the diabetic and non-diabetic mothers did not differ in glucose tolerance [160]. Similarly, in rats, gestational diabetes resulted in overweight and increased NPY and AgRP, and decreased MSH, without changes in POMC, in the hypothalamus [161].

Parity is also a factor related to the future development of offspring. In epidemiological studies, increasing maternal parity has been associated with higher birth weight, abdominal circumference, skinfold thickness and fat mass [162, 163]. Although first-borns are usually lighter at birth than their siblings, maternal nutrition during early pregnancy can modify this relationship, as was observed in women exposed to the Dutch famine [164]. Later in life, the risk of rapid weight gain during childhood, a factor known to increase the risk of later obesity, was found to be 2-fold higher in first-born than in later-born children from the German Multicenter Allergy Study [165]. Similarly, first-born girls from Japan showed higher risk of being overweight at ~12 years of age than their siblings [166]. In contrast, an epidemiological study in Sweden, the mortality risk was lower in first-born girls than in their later-born siblings during adulthood [167]. Few studies have been done in animals, but
studies in sheep have found higher amount of body fat in lambs from primiparous than from multiparous mothers [168].

Altogether, a number of maternal factors interact with nutrient imbalances during pregnancy to affect the offspring, but the mechanisms mediating these effects are unclear.

2.2.3. Diet during pregnancy and effects on the mother

Pregnancy induces a series of changes in maternal physiology in the brain and peripheral tissues [169-171]. Almost all organs adapt to allow growth in maternal, placental and fetal tissues, and respond to the increased energy and nutrient demands. However, these physiological responses may be modulated by dietary factors.

2.2.3.1. Maternal physiology during pregnancy

Maternal physiology during pregnancy adapts to ensure fetal growth and development as well as to meet the increased demands of mothers during pregnancy, parturition and lactation. Maternal systems are modified in response to signals coming from the corpus luteus, embryo and as gestation progresses, placenta and fetus [172]. These signals include hormones, such as chorionic gonadotropin, progesterone, estrogens, and placental lactogen that will trigger different organs to allow the gestation to continue [173]. At early stage of pregnancy human chorionic gonadotropin is needed to maintain the corpus luteus and therefore, production of progesterone [174] and estrogens (17β-oestradiol) [175]. High levels of progesterone are important to preserve the pregnancy state, since this hormone acts on the uterus to facilitate embryonic development and implantation during early pregnancy [176]. Progesterone also stimulates fat deposition [177] and estrogens are related to the adaptations observed in food intake and glucose metabolism during pregnancy [178].
Maternal metabolism adapts to ensure the adequate nutrient supply for embryonic and extraembryonic tissues. First, maternal adaptations assure enough energy and nutrients to allow growth and development of maternal (e.g. mammary gland, gravid uterus), placental and fetal tissues; and second, maternal metabolism adapts to redistribute these nutrients. Therefore, mechanisms that regulate food intake are altered during pregnancy, as well as hormones related with nutrient sensing/metabolism [68]. Increased food intake starts early in pregnancy in preparation for the future maternal and fetal energy and nutrient demands and continues during lactation [179]. Although the mechanisms are not completely clear, it is likely that the multiple hormonal changes (e.g. in estrogens, progesterone, placental lactogen, prolactin, growth hormone, glucocorticoids) may mediate the central response of food intake. During pregnancy the hypothalamic mechanisms that regulate food intake are modified. For example, increased leptin serum concentrations in the non-pregnant state, down-regulate the release of orexigenic neuropeptides and up-regulate those anorexigenic, thus inducing a decrease in food intake. During pregnancy, despite the increase in serum leptin concentrations, food intake is increased due decreased gene expression of the leptin receptor resulting in leptin resistance [180]. Increased expression of other regulatory genes, such as NPY, have been also found in some [181] but not all studies [182] and an increase in the expression of AgRP in the hypothalamus has been also found in pregnant rats [182]. The down-stream signals of the primarily anorexigenic neuropeptide POMC also adapt during pregnancy. Pregnant rats do not decrease their food intake in response to α-MSH, a product of the cleavage of POMC [171], whereas POMC expression appears to be unchanged [181, 182]. These adaptations may result in increased food intake, since NPY and AgRP are orexigenic and the anorexigenic signals (i.e. α-MSH) seem to be attenuated.

Redistribution of nutrients towards the fetal-placental unit involves adaptation of maternal peripheral tissues. Glucose metabolism changes during the course of pregnancy [183]. In early pregnancy, there is an increase in insulin response to food consumption, but a decrease in insulin sensitivity, without changes in fasting blood glucose concentrations. In contrast, during late pregnancy fasting blood glucose is slightly decreased despite increased hepatic
gluconeogenesis and decreased insulin sensitivity [184]. Glucose is the major source of energy to the fetus, thus altered maternal insulin sensitivity in the liver and muscle may serve to redirect glucose towards the feto-placental unit, instead of maternal tissues. Another alternative is that glucose could be redirected to maternal fat storage, as suggested for the increase of insulin receptors in adipose tissue during the first half of pregnancy [185].

Maternal lipid and protein metabolism adapt to pregnancy demands [186-188]. Higher concentrations of triglycerides and increased adipocyte size are observed during the first half of pregnancy. This stage is also characterized by an increased lipogenesis and reduced lipolysis, mediated by the actions of insulin, progesterone and glucocorticoids [189]. This metabolic response is consistent with the fat accretion observed during this stage. However, lipid metabolism shifts from this anabolic to a catabolic state during late pregnancy, when part of the fat storage is mobilized, probably to sustain the increased rate of fetal growth [190]. Pregnancy is also associated with decreased plasma concentrations of amino acids and a state of positive nitrogen balance [191], following tissue accretion in both the mother and the fetus.

2.2.3.2. Diet components and maternal metabolism

Composition of diets during pregnancy modifies the mother’s glucose, lipid and hormonal metabolism. Total fat intake in early pregnancy increases the risk of glucose intolerance and gestational diabetes mellitus, although this response is dependent on the source [192]. When fat intake is primarily polyunsaturated fatty acids, the incidence of glucose intolerance is reduced in women. In contrast, a higher saturated to polyunsaturated fatty acids ratio was a predictor of glucose intolerance [193]. High sucrose and total fat intakes are associated with higher serum leptin concentration during the third trimester of pregnancy in overweight women [68, 194]. In rodents, a low-protein diet during pregnancy decreased body fat, reduced liver AA and DHA, and increased fasting insulin and leptin [122], whereas diets high in saturated fat or in sucrose increased plasma insulin and glucose [195, 196]. The high
sucrose diet also altered lipid metabolism in the liver of the dams [196]. Similarly, a high fructose diet increased glucose and triglycerides by mid pregnancy in rats [197]. Many adaptations of maternal metabolism occur during pregnancy, as previously discussed. However, the diet consumed during pregnancy has the potential to affect later life outcomes for not only offspring but also the mother. However, whether these adaptations during pregnancy affect maternal metabolism in the long-term has not been reported.

2.2.4. Animal models

Animal models have been useful to investigate the potential mechanisms underlying the developmental origins of health of disease. Results from experiments in sheep or in rodents (rat and mice) are frequently reported in this research field.

Sheep have the advantage of having a reduced number of offspring and similarities in the timing of adipose tissue and brain development with the human fetus. However, being ruminants, the digestion of food components is an issue to consider in nutritional interventions [198]. On the other hand, in rodents the development of certain organs, such as the brain continues during lactation [199]. Unlike humans and sheep, rodents give birth to several pups per litters. This characteristic is advantageous in follow up studies, when analyses at different time points are required. Small animals, such as mice and rats, are particularly useful for the study of long-term outcomes, due to their short life span and gestation and to their lower cost of maintenance compared to bigger animals [198].

2.2.5. Potential mechanisms of developmental origins of disease

Several potential mechanisms have been proposed to explain the effects of maternal diets on the developmental origins of disease. Suggested first was the disruption in organ growth and
development, with the consequent changes in their structure [60]. However, although structural changes would in turn alter the organ/tissue function, they can also be considered as an outcome of developmental disruptions. More recently, attention has focused on early alteration in the fetal hormone environment, in particular glucocorticoids, insulin and insulin-like growth factors [200-202] and in altered gene expression due to epigenetic regulation [203-205]. However, rather than being isolated, these mechanisms interrelate and modify offspring phenotype.

2.2.5.1. Alterations in organ/tissue structure

Embryo and fetus organ development is sensitive to nutrient imbalances in maternal diets, and structural changes in key metabolic organs contribute to the development of chronic diseases later in life.

Because of their role in glucose and lipid metabolism, structural changes in pancreas, liver, muscle and adipose tissue have been investigated in offspring from under and overnourished dams. Maternal diets, restricted in either energy or protein, decrease the number of pancreatic cells, their insulin content and insulin secretion [206]. In addition, altered liver structure [207], decreased hepatocyte number [208] and reduced muscle mass and fiber number and composition have been found in the offspring of rats and/or sheep [209-211] and the rate of proliferation in rat preadipocytes is increased in offspring of protein-restricted dams [212, 213].

Maternal obesity and overnutrition also result in developmental impairment of pancreatic cells in sheep and rat offspring [148, 214] and increase hepatic steatosis, predisposing the offspring to non-alcoholic fatty liver disease [215]. Similarly, maternal overnutrition during pregnancy and lactation increases the number of adipocytes and fibrosis in muscle, impairing insulin signaling in lambs [216]. This model of maternal overnutrition also
increased gene expression of lipogenic factors, such as PPAR-\(\gamma\), a transcriptional factor involved in adipogenesis, lipid and glucose metabolism [13].

Experiments in animals show that the structure and development of the brain are altered by maternal undernutrition or overnutrition. Low-protein diets during pregnancy and lactation decreased the number of efferent AgRP and \(\alpha\)-MSH fibers in rat hypothalami [217]. Maternal nutrient restriction also affects neuronal differentiation and synaptogenesis in the offspring [218]. Similarly, maternal high-fat diets and obesity alter neuronal differentiation and decrease neurotrophic factors in the hippocampus of the offspring [219, 220]. However, when provided only during the second week of development, high-fat diets augmented cell proliferation and differentiation in the hypothalamus increasing the number of neurons that express orexigenic neuropeptides in the paraventricular nucleus [221].

At cellular level, maternal diets have the potential to alter the function of tissues by inducing changes in the expression of nutrient transporters, such as glucose transporters (GLUT 2 and GLUT 4 in the liver and muscle, respectively) and hormone receptors, such as glucocorticoid, insulin and leptin receptors [51, 87, 222] among many others, thus modulating cell signaling. Maternal diets also affect cellular functions by inducing changes in enzymatic activity. In rats, maternal overnutrition from a cafeteria diet increased adiposity and activity of enzymes involved in fatty acid synthesis in offspring at weaning and 3 months of age [223], while those related to beta-oxidation decreased [127]. However, the expression of these molecules may be partially regulated by circulating hormone concentrations that during intrauterine life originated in maternal, placental or fetal tissues [224].

### 2.2.5.2. Hormone concentrations and early development

Hormones regulate intrauterine growth by controlling cell proliferation, differentiation and apoptosis in embryonic tissues [189] and by their actions on fetal tissues and the placenta
Insulin, leptin, insulin-like growth factors, thyroid hormones and glucocorticoids play a fundamental role in fetal growth and development [225, 227]. Although these hormones are in general related to nutrient sensing, glucocorticoids have been the most favoured mechanism for fetal programming, particularly in models of undernutrition.

Glucocorticoids are steroid hormones with widespread metabolic effects in nutrient metabolism. Cortisol in humans and corticosterone in rodents, are the main glucocorticoids. They participate in the response to stress and in energy metabolism, by binding to glucocorticoid receptors and initiating transcription of target genes [229]. Genes up-regulated by glucocorticoids are related to amino acid metabolism, gluconeogenesis (glucose-6-phosphatase, G6Pase), energy (leptin) and lipid metabolism (lipoprotein lipase), glucose transport (glucose transporter 4, GLUT 4), among many others. Genes down-regulated by glucocorticoids include POMC, prolactin and tumor necrosis factor α (TNF-α) [230]. Because glucocorticoids participate in the regulation of metabolism, chronic glucocorticoid excess has deleterious metabolic effects [231].

In utero, a number of cell functions are affected by glucocorticoids. These include the regulation of receptors for glucocorticoids, growth hormone, insulin-like growth factor, prolactin and leptin in many tissues. They also regulate the action of enzymes such as the 11β-hydroxysteroid dehydrogenase (11β-HSD) types 1 and 2, a number of enzymes involved in metabolism of glucose (G6Pase, fructose diphosphatase, phosphoenolpyruvate carboxykinase,), lipids (e.g. fatty acid synthase) and proteins (e.g. argininosuccinate synthase and lyase, aspartame transaminase, aminopeptidase) (reviewed by [224]).

At present, glucocorticoids and leptin have received the most focus for their role in fetal growth and development. Glucocorticoids may contribute to the programming effects of nutrient restricted diets but their role in mediating effects of overnutrition is less clear. Despite higher levels of glucocorticoids in maternal circulation, the fetus is protected by the activity of the placental enzyme 11β-HSD 2, in the placenta. This enzyme inactivates glucocorticoids, thus reducing the amount of active glucocorticoids in fetal circulation.
Its activity is modified by several environmental stimuli (e.g. stress, restraining, nutrient deprivation). For example, feeding a low protein diet during pregnancy results in the reduction of the expression of the 11β-HSD 2, thus increasing the placental transfer of active glucocorticoids to the fetus, associated with the increased risk of chronic diseases observed in the offspring [233]. Restriction of micronutrients such as vitamin E, zinc and copper (restricted 50%) in mice also lowers expression of 11β HSD 2, and has been associated with altered glucose and lipid metabolism [234].

Leptin also regulates fetal growth and may modulate some effects of maternal diets on development. In humans, leptin is produced in maternal adipose tissue and in the placenta [235]. Leptin concentrations are reduced in infants born small for gestation age [236]. In rodents, the contribution of placental [237, 238], and maternal leptin to the fetus is unclear [227]. However, the expression of leptin is detected in placental and embryonic tissues (cartilage, heart, hair follicles) by midgestation in mice [238, 239] suggesting a role for fetal development. Leptin administration opposes the programming effects of glucocorticoids when low-protein diets are fed to rodents [240]. Similarly, leptin administration to pregnant rats prevented the effects of a high-fat diet on body weight, fat mass and glucose metabolism of the offspring [241].

Alteration in leptin concentrations may mediate the effects of overnutrition in early programming of disease, in particular, those related with energy homeostasis in the central nervous system. Actions of leptin in neurodevelopment have been documented. Leptin seems to be involved in neurogenesis and synaptogenesis [106, 227], affecting proliferation and differentiation of neural cells. Leptin also affects the development of the food intake regulation neurocircuitries, which may account for higher food intake and obesity in the offspring [106, 242].
2.2.5.3. Epigenetic regulation of gene expression

Maternal diets alter gene expression in the offspring tissues, including those regulating food intake, adipose tissue development, glucose and lipid metabolism [243, 244]. These effects have been associated with epigenetic changes in gene expression that persist through life. Experimental data have provided evidence that epigenetic changes in regulatory genes play a fundamental role in the developmental origins of disease.

*Epigenetics* has been defined as ‘the study of mitotically (and potentially meiotically) heritable alterations in gene expression that are not caused by changes in DNA sequence’[245]. Cell genetic and epigenetic profiles confer specific phenotypic characteristics to organs and the whole organism. After fertilization, germ cells undergo a series of modifications in parental epigenetic marks, establishing an epigenomic pattern during embryonic and fetal development [246]. However, environmental influences in critical periods of development modify this pattern by several mechanisms, namely chromatin remodelation, histone acetylation and methylation and DNA methylation [247]. Far from referring to one specific process, epigenetic regulation of gene expression is a series of complex interactions between the genome and environmental factors.

Epigenetic regulation of gene expression occurs at several levels within chromatin structures (Figure 2.1). The basic units of chromatin are the nucleosomes. These are complexes formed by DNA that wraps around proteins, namely histones H2A, H2B, H3 and H4, and linked by H1. Chromatin can be remodeled by the action of enzymes, thus altering the accessibility of genomic regulatory regions to transcription factors and other proteins [248]. Also, histones can be post-translationally modified by addition or removal of other molecules, most commonly acetyl or methyl groups, generally to the lysine of arginine residues of these proteins [249]. Similarly, cytosine bases in some regions of DNA (e.g. promoter region) can be modified by a DNA methyltransferase at the C5 position, thus regulating its transcription. Histone methylation can influence DNA methylation, and vice versa, and can induce or repress transcription [250]. Histones and DNA methylation
patterns, in addition to the enzymes that modify them, influence signaling pathways, ultimately regulating gene expression and biological functions [251].

Methylation of DNA and histones depends on the availability of S-adenosylmethionine (SAM) and the activity of methylation enzyme (e.g. DNA methyl transferases, or Dnmt), both of them partially regulated by vitamins and other nutrients. Therefore, nutrients influence methylation processes by two mechanisms. First, by regulating the availability of SAM, since as shown in Figure 2.2, methionine, zinc and several vitamins participate in the synthesis of this methyl donor; and second, by influencing the expression and activity of methyl transferases. However, these mechanisms may be tissue-specific as suggested by experiments in rats, in which methyl-deficient diets (methionine, folate, choline) affected histone and DNA methylation by lowering the availability of SAM in the liver [252] or by influencing the expression of DNA methyl transferases (Dnmt, lower Dnmt3 and lower Dnmt1), without differences in SAM levels, in the brain. These differences in methylation patterns were further reflected in differences expression of more than 30 genes, from which 21 were up-regulated and 12 down-regulated [253]. The importance of changes in histone and DNA methylation patterns has been stressed by their association with ageing and chronic diseases, particularly cancer, and currently, there is growing evidence that nutrient availability modulates epigenetic profile during development [254].

**Evidence for the role of epigenetics in the developmental origins of disease**

Research on epigenetics and its role in the context of nutritional programming, has focused primarily on the effects of maternal diets on DNA methylation. Nutrient restriction during pregnancy, as the imposed by the Dutch famine, alter the methylation status of imprinted and non-imprinted genes related to a variety of biological functions [255, 256], and is dependent on the period of exposure (periconceptional and late gestation) and gender of the progeny. Animal studies have confirmed that both deficit and excess of energy and
macronutrients in maternal diets alter DNA methylation patterns and gene expression in different organs of the offspring [50, 51, 257].

Figure 2. 1. Epigenetic regulation of gene expression

Restricted diets and overnutrition alter DNA methylation patterns of genes involved in energy balance in several brain regions. Low-protein diet during pregnancy increased the expression of AgRP and POMC in the hypothalamus and altered the methylation level, either decreased or increased, of specific regions of the POMC gene [217]. A maternal high-fat diet was found to alter DNA methylation of dopamine and opioid receptors in hypothalamus, prefrontal cortex and other brain regions and the offspring showed a phenotype characterized by preference for palatable foods [258].

DNA methylation not only affects gene expression by regulating DNA transcription, but also modulates gene expression by affecting the expression of nuclear factors (i.e. transcriptional factors), and therefore, the expression of down-stream genes.

PPAR-α, PPAR-β or δ, and PPAR-γ, are the three main isotypes from the PPAR superfamily of nuclear receptors expressed in most tissues, including liver, muscle and adipose tissue. They serve as nutrient sensors and transcriptionally regulate the expression of enzymes related to glucose and fatty acid metabolism and adipogenesis [36, 259]. A common
characteristic of animal models with altered expression of PPARs is their increased adiposity and/or body weight, and in some of them altered glucose metabolism [13], stressing the role of these transcriptional factors in the regulation of energy balance in peripheral tissues, particularly in nutrient utilization and storage.

The expression of PPARs in the offspring is sensitive to maternal diets and has been suggested to be one of the mechanisms explaining the developmental origins of disease [260]. In sheep, global nutrient restriction (50%) during pregnancy increased the expression of peroxisome-proliferator-activated receptor α (PPAR-α) in adipose tissue from term feti [261] and maternal overnutrition increased mRNA levels of PPAR-γ in adipose tissue and positively correlated with insulin concentrations in plasma of the offspring [13].

Gene expression of PPARs is epigenetically modulated. Studies in vitro show that in preadipocytes, the promoter region of PPAR-γ gene is hypermethylated and it is gradually demethylated during differentiation resulting in increased gene expression and chemical inhibition of DNA methylation resulted in lower PPAR-γ expression [262]. Maternal diets low in protein also resulted in hypomethylation of PPAR-α and increased mRNA levels in the liver of the offspring. Acetyl Co-A oxidase mRNA, a gene transcriptionally regulated by PPAR-α, were also increased by the maternal diet [51].
Vitamins showed in grey shadow are involved in the one-carbon cycle as methyl-donors, reducing agents and as cofactors/coenzymes of enzymes catalyzing the reactions of the cycle.

DNA-methylation: DNA-methyltransferases (Dnmt) target the 5-carbon position of the cytosine residues, as one of the main epigenetic mechanisms regulating gene expression.

Other methyl-transferases include the family of phosphoethanolamine N-methyltransferases (PEMT) that catalyze the methylation of phosphoethanolamine to phosphocholine. SAM is also the methyl donor for protein (e.g. histones), guanidinoacetate (to form creatine) or niacin (to urinary excretion) methylation reactions.

[1] S-adenosyl-L-methionine-dependent methyltransferases catalyze methylation reactions of molecules (X in the diagram) including DNA and phospholipids. The products of the methylation reaction are the methylated substrate (CH$_3$-X in the diagram) and S-adenosylhomocysteine.

SAM:S-adenosylmethionine; SAH: S-adenosylhomocysteine; BHMT: betaine-homocysteine methyltransferase; MS: methionine synthase; GNMT: glycine N-methyltransferase; CBS: cystathionine $\beta$-synthase; MTHFR: methylenetetrahydrofolate reductase; SHMT: Serine hydroxymethyltransferase; MAT: methionine adenosyltransferase; 5-CH$_3$-THF: 5-methyltetrahydrofolate; 5, 10-CH$_3$-THF: 5,10- methyltetrahydrofolate; THF: tetrahydrofolate; DHF dehydrofolate. References [263-266].
2.3. Maternal Vitamin Intake and Offspring Phenotype

The previous sections have provided background establishing the effects of energy and macronutrient content of the maternal diets on health and later life risk of disease in the offspring. In contrast, there is relatively little evidence documenting the role of micronutrients in the maternal diet on later life consequences in the offspring beyond birth defects and birth weight. In the next sections, the role of vitamin content of the maternal diet on later life outcomes of the offspring is reviewed. General vitamin functions, followed by the consequences of vitamin deficiencies and the beneficial effects of vitamin supplementation on birth weight, postnatal growth and metabolism are included. Finally, studies addressing consequences of excessive intakes during pregnancy will be reviewed. Although other outcomes have been investigated, including birth defects [267], still birth [268], preeclampsia [269], mortality and morbidity [270], only studies relating maternal vitamin intake or status and birth weight, postnatal growth and glucose/lipid metabolism are addressed, for being relevant to this thesis work.

2.3.1. General vitamin functions

Vitamins are essential nutrients with regulatory functions, mainly as coenzymes in metabolic pathways, and the regulation of cell cycle and gene expression. Several coenzymes required for energy transformation processes are derived from vitamins. For instance, thiamine diphosphate, flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD) and pyridoxal phosphate (PLP) are coenzymes derived from thiamine, riboflavin, niacin and pyridoxine, respectively [271].

Vitamins are also involved in the regulation of cell cycle and gene expression. For example, vitamin A has a dual role in apoptosis, either promoting or repressing it, depending on the cell type and the dose [272, 273] and studies in vitro have shown that niacin inhibits proliferation and induces apoptosis in hepatic cells [274]. Although vitamin E is well known
for its antioxidant properties, it also participates in cell signaling and gene expression, by interacting with transcription factors regulating the expression of tocopherol-transfer proteins and several genes in the liver, muscle and the brain [275]. Vitamins are also involved in one-carbon metabolism that provides the methyl groups needed for methylation reactions in the organism, including the synthesis of phospholipids and regulation of gene expression by DNA and histone methylation [276-278] as shown Figure 2.2.

Vitamins play a fundamental role in fetal development by altering tissue structure and function, epigenetic regulation of gene expression and mediating the effects of glucocorticoids. The role of single vitamins or combinations during intrauterine development has been demonstrated by studies in animals, most frequently related to vitamin deficiency or high toxic doses. Although most experiments have been related to immediate but not long-term outcomes, they provide evidence that highlights the importance of vitamins during early development [279]. For instance, due to their role in one-carbon metabolism, vitamins are known to be involved in epigenetic regulation of gene expression, one of the mechanisms proposed to explain the programming effects of maternal diets [6]. However, there are very few studies investigating the programming effects of the vitamin content of maternal diets and very little is known about the effect of vitamins on maternal hormone status during pregnancy.

### 2.3.2. Consequences of vitamin deficiencies during pregnancy

Micronutrient deficiencies during pregnancy are prevalent in developing countries and are associated with decreased birth weight, prematurity and birth defects [280, 281]. Studies in humans have focused on the effects on the offspring and have primarily investigated the effects of vitamin deficiencies, vitamin supplementation to malnourished population, or on short term pregnancy outcomes. Therefore, an understanding of the role of vitamins during pregnancy comes primarily from observational and vitamin supplementation intervention
studies in which birth weight has been used as a surrogate of fetal growth [282] and very few studies on postnatal development. Similarly, studies in animals have investigated the effects of vitamin deficiency during fetal life, but few studies have addressed the effects of maternal vitamin intake on postnatal growth and lipid and glucose metabolism postnatally.

2.3.2.1. Studies in humans

Studies in humans have reported associations between the nutritional status of single vitamins and low birth weight, small-for-gestational-age, birth weight, and only two studies have evaluated the effects of vitamin deficiencies on post-natal growth, adiposity and glucose metabolism. Although multiple deficiencies are commonly concurrent, the studies have addressed the effects of single vitamin deficiencies.

A) Vitamin deficiencies and birth weight

Results from epidemiological studies suggest that deficiencies of both fat and water-soluble vitamins are associated with lower birth weight. Women with low serum retinol (0.7 micromol/l) are more likely to have a newborn weighing 2.5-2.9 kg, compared to women with normal retinol [283]. Vitamin D deficiency (<37.5 nmol/L of 25(OH) D) associates with 7.5 times the risk of having small-for-gestational-age newborns [284] and a cohort study from The Netherlands, suggested that deficiency of vitamin D (serum level <30 mmol/L) between 12-14 weeks of pregnancy was associated with lower birth weights (-114 g) [285]. Maternal vitamin E deficiency has also been associated with intrauterine growth retardation. In low-birth-weight infants the serum levels of vitamin E were reported to be 50% lower than in normal weight infants in a cross-sectional study. Other studies have found that higher homocysteine, a biomarker of folate deficiency, is a predictor of lower birth weight and intrauterine growth retardation and may be related to a deficient B-group
vitamin status of the mother [286-288]. A correlation between low serum B12 concentrations in serum and low birth weight [289] also supports a role of this vitamin in fetal growth.

Vitamin deficiencies during pregnancy are less common in developed countries, except for vitamin D, which has received more attention in the last few years for the high prevalence of deficiency/insufficiency at the population level. In U.S., 29% of African-American and 5% of white women were vitamin D deficient (25-(OH)D₃<37.5 nmol/L) at delivery. Similarly, in The Netherlands, the prevalence of vitamin D deficiency during pregnancy (25-(OH)D₃<25 nmol/L) was 50% of non-Western and 8% of Western women (<25 nmol/L) [290] and in Canada, 53% of pregnant women had 25-(OH)D₃ below 50 nmol/L [291]. In these countries, both inadequate intake and low plasma concentrations of vitamin D have been associated with low birth weight. The difference in birth weight in infants from women with adequate vs. inadequate (<200 IU) intake of vitamin D from dietary and supplemental sources has been reported in about 60 g [292]. Vitamin D deficiency (<30 nmol/L of 25-(OH)D₃) accounted for a decrease of 114 g in birth weight in a cohort study [293] and concentrations below 37.5 nmol/L were associated with 7-times higher risk of having a neonate born small for gestational age compared to those with 25-(OH)D₃ concentrations above this cutoff [284]. In contrast, a longitudinal study in UK found no significant association between maternal vitamin D status and birth weight [294].

**B) Vitamin deficiencies and postnatal outcomes**

The relationship between vitamin deficiency during pregnancy and postnatal adiposity, glucose and lipid metabolism in the offspring has been evaluated by very few cohort studies. One study in The Netherlands reported that children born to vitamin D deficient women (<30 nmol/L) had accelerated growth during their first year of life compared to those born to women with serum vitamin D levels >50 nmol/L [293] and a study in India found that children from vitamin D deficient mothers during pregnancy had higher body fat at 5 years
of age, and higher fasting insulin and HOMA-IR at 9.5 years of age, but no difference was found in serum lipid concentrations [295]. Also in India, low maternal B12 concentrations during mid-pregnancy predicted higher HOMA-IR in their 6-year old children [31]. There are no studies examining vitamin intake or status during pregnancy on obesity and metabolic measures during adulthood.

2.3.2.2. Studies in animals

Experiments in animals have been useful to characterize the effects of vitamin deficits during pregnancy on fetal development, but as in studies in humans, very few have addressed the long-term effects of vitamin deficient diets.

A) Vitamin deficiencies and birth weight

Feeding the mothers vitamin restricted diets has shown mixed results regarding fetal and birth weight in rats. A multivitamin deficient diet (50% from the regular amount of multivitamins) given to rat dams from weaning to the end of pregnancy had no effect on birth weight [29]. Similarly, vitamin A deficiency did not affect fetal weight (at several time points in later pregnancy) [296], but it resulted in decreased body weight at postnatal day 1 in another experiment [297]. Maternal diets restricted in vitamins involved in one-carbon metabolism also resulted in 10% lower fetal weight (day 21) in rats [298] and decreased the offspring weaning weight by 57% [299].

B) Vitamin deficiencies and postnatal outcomes

Experiments in animals also provide support for the role of vitamins during pregnancy in the glucose and lipid metabolism of the offspring. In rats, restricting vitamin intake to 50%
before, during pregnancy and lactation increased fasting glucose in 180 day old offspring weaned to a control diet, without difference in insulin concentrations. However, in response to a glucose load, higher insulin area under the curve (AUC) was observed in offspring from dams that received the vitamin restricted diet either during lactation or during lactation and post-weaning [29]. A folic acid deficient diet fed before and during pregnancy also resulted in increased insulin content in the pancreas of fetus and offspring at 4 wk. Despite having higher insulin, these offspring also showed higher glucose AUC after a glucose challenge, which indicates that maternal folic acid deficiency may result in decreased insulin sensitivity in the offspring [300]. Similarly, a diet deficient in methyl donors (folate 0, choline 0.05% and methionine 50%) before and during the first 5 days of pregnancy resulted in higher HOMA index and higher insulin after a glucose load at 6 and 12 months of age in males [301]. A diet restricted in vitamin E (50%) given to female mice from weaning to lactation also resulted in impaired glucose tolerance in the offspring at 6 months of age [234].

As observed with other nutrients, maternal vitamin status may also affect lipid profiles in plasma and tissues of the offspring. However, only few animal experiments have assessed these outcomes. Earlier experiments found that B12 deficient diets increased concentrations of palmitic and stearic acids and decreased concentrations of AA and DHA in the liver phospholipids [302]. Maternal vitamin intake during pregnancy has also been shown to alter the expression and/or activity of enzymes related to lipid metabolism in early life. Restriction of folate or a combination of folate+choline+methionine during pregnancy led to altered expression of enzymes involved in lipid metabolism in fetal livers of rats at 21d of gestation. Gene expression of carnitine palmitoyl transferase 1 (CPT1) was up-regulated and the fatty acid translocase (FAT/CD36) was down-regulated in feti from folate-restricted dams. When choline and methionine were also decreased the expression of acetyl CoA-carboxylase, but no effect on the expression of SREBP-1c and PPAR-α in fetal livers was found [303].
2.3.3. Vitamin supplementation during pregnancy

Vitamin supplementation shows benefits if used appropriately in at risk groups. Birth weight and derived measures (i.e. low birth weight, small or large-for-gestational-age) have been widely used as a proxy of fetal growth in studying the association between maternal nutrition and the risk of obesity and chronic diseases later in life in humans. Therefore, human studies reporting the effects of multiple micronutrient (MM) supplementation on birth weight and postnatal growth are included in the following section.

2.3.3.1. Birth weight

Vitamin supplementation trials have been conducted in developing countries, in which vitamin deficiencies are prevalent. In the following section, results of intervention studies on birth weight are included. Multiple micronutrient supplementation is discussed first and then the effects of single vitamins are described. A description of the few studies relating maternal vitamin intake to postnatal growth, glucose and lipid metabolism in the offspring is also included.

A) Multiple micronutrient supplement intakes

The importance of micronutrients for fetal growth has been demonstrated in intervention studies when intakes are inadequate. An earlier review of the effects of intervention studies concluded that there was no additional benefit of supplementing with many micronutrients to pregnant women when compared with iron+folate supplementation alone [304]. However, more recent studies providing multiple micronutrients have shown that newborns from supplemented women are heavier than those born to unsupplemented or those receiving an iron+folate supplement. Supplementation of pregnant women in Pakistan with multiple micronutrients resulted in an increase of 70 g in birth weight [305]. Similarly,
supplementing women with a MM preparation (UNICEF/WHO/UNU international multiple micronutrient preparation, UNIMMAP mix consisting of: vitamin A 800 µg, vitamin E 10 mg, vitamin D 5 µg, vitamin B1 1.4 mg, vitamin B2 1.4 mg, niacin 18 mg, vitamin B6 1.9 mg, vitamin B12 2.6 µg, folic acid 400 µg, vitamin C 70 mg, iron 30 mg, zinc 15 mg, copper 2 mg, selenium 65 µg, and iodine 150 µg) also resulted in increases in birth weight ranging from 22 to 95 g in developing countries [306-308]. Two recent meta-analyses calculated the pooled effect of micronutrient supplementation to be an increase of 22 g to 54 g in birth weight compared with iron+folate supplements alone [309]. Clearly, the results of these interventions indicate that micronutrients are involved in fetal growth as evaluated by birth weight.

The response to supplements varies according to baseline nutritional and economic status. For example, in a study conducted in China, the MM supplement increased birth weight (68 g) compared with folic acid supplementation, in only the poorest households, whereas in wealthier households no effect was observed [310]. The differences in baseline nutritional and socioeconomic status may explain the lack of association between multivitamins use and birth weight in developed countries. In a prospective birth cohort of British women, the use of multivitamin supplements during pregnancy was not associated with birth weight [311]. However, in a retrospective cohort study in the U.S.A., multivitamins supplementation was not associated with birth weight in a non-hispanic white population, but it was increased in non-hispanic African-American and the direction of these associations did not change after taking into account socioeconomic variables [312].

B) Single vitamin supplement intake

Except for vitamin A, D, and folic acid, few studies have provided a single vitamin supplement.
**Vitamin A.** Supplementation with vitamin A during pregnancy may have a beneficial role of vitamin A in increasing birth weight in malnourished population. A recent systematic review of randomized controlled trials reported that supplementation with this vitamin in HIV-infected women resulted in an increase of ~90 g in birth weight [313].

**Vitamin D.** Observational studies suggest a role for vitamin D intakes on birth weight, studies in low-income women in U.S.A. and in a cohort from New Zealand found an positive association between maternal vitamin D intake from dietary and supplement sources and birth weight [292, 314]. Similarly, in a group of women with low milk consumption in Canada, an increase in 1 μg of vitamin D in maternal diets was associated with 11 g increase in birth weight [315]. However, most intervention studies supplementing vitamin D have shown no effect on birth weight (recently reviewed by [316]).

**Vitamin E.** There are no studies on vitamin E supplementation alone on birth weight in humans. However, observational studies suggest a role of this vitamin in birth weight. Higher birth weights have been found in neonates from women with higher vitamin E intake [45] or vitamin E plasma concentrations [317, 318] in prospective studies in U.S.A. and South Korea.

**Folic acid.** An effect of folate (the natural occurring form) or folic acid (the synthetic form) intakes during pregnancy on birth size have been reported for few studies, whereas others have found no association. In women from Norway or Japan, neither intakes of folate and folic acid, nor folate plasma concentrations during the second trimester of pregnancy, were associated with birth weight [287, 319, 320] and a re-analysis of trials using folic acid supplements concluded that supplementation was not associated with birth weight. High doses of folic acid (5mg) were associated with a reduced risk of low birth weight (<2500g) [321]. Then, it seems that folic acid supplementation increases birth weight only in newborns from folate-deficient women, since folate deficiency results in lower weight and length at birth [322].
Other vitamins. Little is known about the effects of other vitamins on birth weight. Maternal plasma levels of vitamin C have been associated with higher birth and post-natal weight [322]. However, plasma concentrations of retinol, tocopherol, thiamine, riboflavin, pyridoxal-5'-phosphate, cobalamin and folate in early pregnancy were not associated with birth weight in an observational study [323].

Further analyses are warranted to clarify whether the variation in the results of these epidemiological studies is due to the baseline values, the stage of pregnancy, the kind of measurements taken, the form of the vitamin evaluated, or the adjustment by confounding variables.

2.3.3.2. Postnatal growth, lipid and glucose metabolism in offspring

Very few studies have been conducted to investigate the effects of supplementing women dietary intake during pregnancy with vitamin, alone or in combination on their children body weight and fat and results varied among studies. In Nepal, children from women supplemented with UNNIMAP mix during pregnancy had higher body weight, greater chest, head and arm circumferences and triceps skinfold thickness at 2.5 years of age compared with controls [306]. Similarly, multivitamin (B, C, and E) supplementation to HIV-infected women during pregnancy resulted in higher postnatal (until 24 months) absolute weight as well as in weight-for-age and weight-for-length z-scores in an intervention study in Tanzania [324]. Children born to women supplemented with iron-only during pregnancy and with iron+vitamin A (i.e. never received MM consisting of 1-1.5-fold the RDA of 10 vitamins +iron, zinc and magnesium) from 3-24 months had higher BMI than those either born to women supplemented with MM during pregnancy or that were born to women supplemented with iron-only, but received MM post-natally. In the same study, children that consumed the MM supplement on regular basis (>79% compliance) were taller than those that received iron+vitamin A in both maternal groups [40].
Only one study in Nepal reported the effects of maternal vitamin supplementation and adiposity and glucose metabolism in 6-8 year old children. The combination of vitamin A+folic acid+iron+zinc increased height and decreased adiposity (skinfold and subscapular thickness and arm fat area), but no effect of maternal supplementation with any of the 5 different micronutrient combinations utilized in this study (vitamin A, vitamin A+folic acid, vitamin A+folic acid+iron, vitamin A+folic acid+iron+zinc or multiple micronutrients) was related to body weight, BMI or waist circumference of these children [325]. Children from women supplemented with folic acid+vitamin A had reduced the risk of metabolic syndrome (OR=0.63) than control (only vitamin A supplement), but this decreased risk was not observed with the multiple micronutrient or with folic acid + iron supplementation [325].

Although these studies highlight the importance of evaluating long-term outcomes of multivitamin supplementation during pregnancy, currently there is a lack of evidence from long-term prospective studies in humans that could confirm the relationship between maternal vitamin intakes and metabolic outcomes in their adult progeny.

### 2.3.4. Consequences of excessive vitamin intakes

Undesirable side effects may also occur with vitamin intakes above the recommendations. In observational studies, the consumption of supplements in the third trimester was associated with higher risk of preterm birth [311] and high intakes of vitamin E during pregnancy through diet and supplements associated with 9-fold increased risk of congenital heart defects in the offspring [21]. Furthermore, increased rate of twinning has been observed since food fortification [326]; and folic acid supplements are associated with higher risk of wheeze and respiratory infections in children [327]. In addition, the dual role of folic acid in cancer development [328-331] has raised concerns. The effects of increasing vitamin intake during pregnancy on birth weight, adiposity and other metabolic diseases have been investigated by few studies in humans and animals, as discussed in the following.
2.3.4.1. Studies in humans

As previously discussed many studies have evaluated the relationship between maternal vitamin intake or status and perinatal outcomes, particularly with birth weight, but only one has addressed the effects of high vitamin status during pregnancy on long-term outcomes.

A) Birth weight

According to the limited number of studies, excess of fat-soluble vitamins during pregnancy is related to lower birth weight. Higher blood concentrations of retinol at 28 weeks of pregnancy were associated with lower birth weight in a cohort of women in the U.K. [332]. Similarly, in a prospective cohort in New Zealand women, birth weight was reduced in those with high beta-carotene intakes in the 4th month and in those infants whose mothers had high retinol and beta-carotene intakes in month 4th and 7th of pregnancy [314].

Maternal serum concentrations above 75 nmol/L of 25(OH)D3 have been associated with 2-times the risk for SGA [284]. Women enrolled by the Motherrisk program in Canada with high intake of vitamin E (400-1200 IU/day) during the first trimester of pregnancy gave birth to neonates of lower weight (~240 g) compared to matched controls [333].

B) Postnatal outcomes

Only one study in humans evaluated the effects of vitamins during pregnancy on adiposity and glucose metabolism of the offspring. A study conducted in India found that adiposity and HOMA-IR index was higher in 6-year old children if the mother had increased folate concentrations during pregnancy, and even higher in those that in addition to high folate had low B12 status during pregnancy [31]. The results of this study indicate that the excess of
one vitamin affects outcomes in the offspring, but also that vitamin deficiencies and excess can be concurrent in the population and in the same individual.

2.3.4.2. Studies in animals

As observed in human studies, there are few studies in animals assessing intakes of vitamins above the recommended levels, and the results have not been consistent.

A) Birth weight

Few animal studies have examined the effects of multivitamins on birth weight. Results from an experiment providing high intakes of a multivitamin mix to Sprague-Dawley rats during pregnancy and lactation showed that the high intake of vitamins, ranging from 1 to 10-fold (Vitamin A 8.7-fold), D 7.5-fold, E 5.9-fold, K 2.2-fold, B1 9.9-fold, B2 10-fold, B6 4.7-fold, B12 1.0-fold), C (990 mg), biotin (0.51 mg), folic acid 7.9-fold, niacin 9.7-fold and pantothenate 11.3-fold the requirements per kg of diet), did not affect the length of gestation, the number of pups alive after birth, or weight at birth and weaning [179]. Similarly, previous experiments in our laboratory have shown that a 10-fold increase in multivitamin intake by Wistar rats does not have an effect on the number and weight of pups at birth [32].

B) Postnatal outcomes

Experiments in animals addressing the role of vitamins on postnatal growth and adiposity have shown mixed results. In earlier experiments in rats, maternal diets supplemented with vitamin B12 (50,000 μg/kg diet vs. 50 μg/kg diet in the control group) increased weight of the offspring at birth and at 1 year of age. This difference in body weight was due to higher body protein and lower total lipid amounts [334]. More recently, a vitamin mix containing
~7-fold and ~4-fold the recommended amounts of vitamins A and E, respectively, fed to rats before and during pregnancy and lactation prevented the increase in adiposity observed in the offspring fed a “Western diet” (high energy, high fat) at 2 weeks and at 2 months of age [335]. Conversely, studies in our laboratory have shown that maternal intake of multivitamins (10-fold the recommended amounts of vitamin mix) during pregnancy resulted in higher body weight, abdominal fat and food intake in the adult offspring, effects that were exacerbated by weaning the offspring to a liquid obesogenic diet [32, 33].

The results of experiments in animals also suggest that maternal vitamin intake programs lipid metabolism. Folic acid supplementation (4-fold) decreased DHA concentrations and increased n-6/n-3 ratio in the brain of male offspring at 11 months of age [30]. Similarly, DHA concentration in the fetal brain (20 d gestation) was found to be lower in offspring born to a normal B12 with high folate (4-fold) diets [336].

Few studies have investigated the effects of vitamin supplementation above the requirements on glucose metabolism in the offspring. In our previous experiments increasing the multivitamin mix (10-fold) in the maternal diet during pregnancy, adult offspring from the high multivitamin diet shows a phenotype that resembles the metabolic syndrome, including higher glucose AUC after a glucose tolerance test at 28 wk post-weaning and a transient elevation in insulin concentrations at 14 wk post-weaning in the male offspring weaned to a AIN-96G control diet [32]. In offspring weaned to a liquid obesogenic diet the increase in glucose AUC was observed at earlier age (i.e. 13 wk post-weaning) and in both males and females [33]. Other studies have found that increasing vitamin content of the maternal diet may improve glucose metabolism. Increasing vitamin A (~7-fold) and vitamin E (~4-fold) content of the diet provided to Sprague Dawley rats before and during pregnancy and lactation prevented the alterations in glucose metabolism in the offspring fed a “Western diet” [335]. Supplementation during pregnancy with a mix of folic acid, B12, choline, zinc, methionine and betaine in higher doses than the control diet (Folic acid (~9-fold), B12 (~60-fold), choline (~9-fold), zinc (~4.7-fold), methionine (~3.1-fold) and betaine (not determined), as compared with the content of these nutrients in the NIH-31 diets) shifted the
phenotype of agouti mice offspring from being fatter and hyperinsulinemic to a leaner normoinsulinemic phenotype [205]. These contrasting results may be explained by the interaction between genetic background and vitamin supplementation since the expression of the agouti gene (viable yellow) was prevented by increased DNA methylation due to the abundance of methyl-donors and coenzymes involved in one-carbon metabolism [337].

2.3.5. Vitamin intake during pregnancy: effects on the mother

Despite the number of vitamin supplementation trials during pregnancy, very few studies have evaluated outcomes in the mothers. Similarly, the evaluation of maternal outcomes in animal experiments has been limited to the pregnancy and perinatal period, as discussed in the next section.

2.3.5.1. Studies in humans

Vitamin intakes are associated with physiological adaptation during pregnancy, but the studies are few and the outcomes are variable. Vitamin D is involved in the regulation of glucose metabolism during pregnancy that in turn, may influence fetal development [338]. Maternal plasma concentrations of vitamin D during pregnancy were positively correlated with insulin sensitivity in a cross-sectional study [24]. Vitamin D deficiency has been also associated with poor glycemic control in women with gestational diabetes [339]. Similarly, 5-11% higher incidence of gestational diabetes was observed in women with higher folate and B12 deficient concentrations [340].

Few studies have related the maternal nutritional status with outcomes beyond the perinatal period. A recent study in India showed that the deficiency of vitamin B12 during pregnancy was associated with higher adiposity, insulin resistance and prevalence of diabetes in women after 5 years post-partum [340]. Vitamin A supplementation during pregnancy resulted in
higher 3-6 months post-partum weight retention in South African HIV-infected women [23]. Similarly, supplementing MM during pregnancy also resulted in higher weight retention one month after delivery in overweight women [40]. However, the combined effect of vitamin intakes above the recommendations on long-term maternal outcomes has not been reported.

2.3.5.2. Studies in animals

Studies in animals have also shown that vitamin intake affects maternal metabolism during pregnancy. An experiment in mice shows that chronic restriction of vitamin E (50%, from weaning to lactation) in maternal diet did not affect food intake or weight gain until mid-gestation, but these measures decreased after day 10 of pregnancy. Chronic vitamin E deficiency also led to higher glucose and insulin plasma concentrations before mating in the dams, but their concentrations were not measured during pregnancy [234]. Studies in rats fed a folate-deficient or a methyl-deficient (folate, choline, methionine) diet reported increased amino acid concentrations in plasma, a 30-60% decrease in phosphatidylcholine and altered expression of 18 (folate-deficient diet) and 26 (methyl-deficient diet) proteins in the liver, many of them related to lipid and glucose metabolism in the dams. In this experiment, hepatic gene expression of acetyl-CoA-carboxilase and SREBP-1c were down-regulated and CPT1, PPAR-γ, up-regulated. mRNA levels of PPAR-α were decreased in the folate-deficient animals and the effects were enhanced in those methyl-deficient, compared to control [341].

These examples provide some evidence of the effects of vitamin deficiency on maternal metabolism during pregnancy. Whether they affect the maternal metabolism after the post-partum period is unclear as is the effect of excess of vitamin intake.

The effects of vitamin intake above the recommendations require investigation. The current environment in developed countries is characterized for highly available fortified food and
vitamin supplements that have the potential to result in intakes above those recommended as is discussed in the next section.

2.3.6. Vitamin intake during pregnancy

Preventing deleterious effects of vitamin deficiencies during pregnancy on fetal development has motivated public health and clinical strategies. However, these strategies may lead to intakes above the recommended amounts, but the long-term consequences of these high intakes are still unknown.

Food fortification programs have been successful in preventing vitamin deficiencies. Fortification of flour, sugar, or milk has been used to prevent deficiencies of vitamin A, D, and B-group vitamins in the population [342]. In addition, many countries have mandatory food fortification with folic acid to prevent neural tube defects [343]. On the other hand, the use of supplements has increased in the last decades and constitutes an additional source of vitamins. For instance, in the U.S.A., the use of any vitamin/mineral supplements was reported by 23.2 and 23.7% in 1987 and 1992, respectively in National surveys. By 2000, this percentage increased to 33.9%; and specifically for multivitamins, these percentages were 17.4, 19.3 and 27.9 in 1987, 1992 and 2000, respectively. The use of single vitamin A supplements doubled and the use of vitamin E increased almost 3-fold between 1987 and 2000 [44]. More recent data indicate that this trend continued in the last decade. By 2003-2006, the percentage of adults that reported the use of multivitamin supplements was 54% [344]. The use of supplements has been reported by 44% of women (non-smokers, 18-65 year old) in a Canadian survey [345].

Pregnant women and those that could become pregnant are encouraged to take multivitamins [346, 347], thus the use of supplements during pregnancy is a common practice. For instance, in a study in low-income women from U.S.A. 86%, reported the consumption of prenatal vitamins more than 4 times/week, and another 5% 2-3 times/week [348]. A study in
Portugal reported that 97% of pregnant women took a folic acid supplement [349] and in Australia, 79% of pregnant women reported the use of folic acid supplements and 35% reported the use of multivitamins [350].

Both food fortification and the use of supplements have been successful in preventing congenital malformations and in improving the nutritional status of both the mother and their infants. However, unintended high intakes of vitamins may occur. Data from a cohort study in the U.S.A. in adults 45-75 years (men and non-pregnant women) showed that 50% of the population used a multivitamin/mineral supplement. In this study, the 90th percentile of vitamin intakes from supplements exceeded the UL of vitamin A, niacin and folate [351]. Other studies in the U.S.A. have reported that 20% of women who used supplements exceeded the UL for niacin and 1.5% the UL for folate [352]. In pregnant women, a study from Boston, found the vitamin intake to be 2-7 times the recommended values for 9 vitamins in 25% of the participants (e.g. third quartile of intake) and the median of intake of folate and niacin exceeded their respective UL [353]. In pregnant women from Norway 36% reported the use of single folic acid supplements, 31% informed the use of MM supplements and 16% the use of multivitamins. Despite the small proportion of women using supplements, some exceeded the UL of vitamin A (4%), folic acid (0.8%) and vitamin D (0.35%) [43].

Biomarkers of folate intake, such as the folate concentrations in blood, have been found to be high. 40% of participants evaluated in the Canadian Health Measures Survey showed high folate concentrations in erythrocytes (>1360 nmol/L) and the 75th percentile of this biomarker was higher than 1360 nmol/L in women in childbearing age [354]. In a study in pregnant women, 97% reported the use of a supplement and their average intake of folic acid at the end of pregnancy was 900 μg/d (UL=1000 μg/d) with erythrocyte concentrations of ~3000 nmol/L [355].

As discussed in previous sections, evidence from previous studies suggests that vitamins are involved in the developmental origins of disease. First, early and current investigation has
demonstrated their role in cell proliferation, migration and differentiation, affecting fetal
growth and development during all stages of intrauterine development [356, 357]; and second, many vitamins participate in one-carbon metabolism reactions, involved in the epigenetic regulation of gene expression [358] one of the mechanisms underlying the effects of maternal nutrition during pregnancy on offspring outcomes.

2.3.6. Vitamins in the developmental origins of disease: Potential mechanisms

A summary of the role of vitamins in different stages fetal development is shown in Figure 2.3. Potential mechanisms by which both deficit and excess of vitamins may be involved in the developmental origins of disease are discussed in the following. These include alteration in organ/tissue structure, hormone status and epigenetic modification of gene expression.

2.3.6.1. Alterations in organ/tissue structure.

Normal intrauterine organ growth and development depend on cell proliferation and differentiation, processes in which vitamins are involved. Therefore, vitamin imbalances in maternal diets during pregnancy have the potential to alter organ structures depending on the embryonic/fetal stage. As discussed in the following, several vitamins are involved in cell proliferation, migration, differentiation and apoptosis, thus inducing changes in the structure (e.g. cell number, organ size) and function of embryonic and extra-embryonic tissues.

*Vitamin A.* Both deficiency and excess of retinol and other derivatives have deleterious effects in embryonic and fetal development. Early research demonstrated that rats accumulate vitamin A at different stages during gestation, almost paralleled with organ differentiation, suggesting that vitamin A is required during organogenesis [359]. This was later confirmed by experiments showing that vitamin A deficiency before and during pregnancy results in lower fetal organ weight (liver, kidney and lung) [360], decreases beta
cell area and number in the pancreas, and alters lung, liver and muscle development affecting the structure and/or protein expression in these organs [296, 297, 361, 362].

Vitamin A has the potential to alter the development of most if not all organs, depending on the dose and the stage of development [363, 364], with outcomes varying from death to malformations in different organs at similar high dose, but administered at different stages during pregnancy [365-367]. The effects of high, but not teratogenic doses, of vitamin A during development have not been defined.

Vitamin D. Current evidence suggests that vitamin D is important for growth and development of several tissues including bone, muscle, heart and brain [316]. Vitamin D
deficiency during pregnancy leads to bone deformation and muscle weakness [368], cardiac hypertrophy [369], and alters the brain structure (thinner and longer brain cortex) [370] in rodent offspring. Similar to vitamin A, the effects of vitamin D depend on the dose and stage of development. Vitamin D participates in cell differentiation (reviewed by [371], regulates growth factors, such as neutrophins [372-374] and affects the balance between cell proliferation and apoptosis in the brain [375]. Vitamin D receptor (VDR) is present in the rat brain by embryonic day 12th to 21th, being the highest at day 18th [376], which is an important stage for the actions of this vitamin in brain tissue. Although the effects of vitamin D insufficiency or deficiency have been investigated, few have reported the effects of high dose of vitamin D. For example, higher concentrations of vitamin D in follicle fluid, which correlate with serum vitamin D concentrations, are related with lower pregnancy rate and embryo quality in in vitro fertilization studies in humans [377] and administration of high dose of vitamin D (20,000 IU) at day 8th or 10th of pregnancy reduced fetal weight and affected bone development in rat fetus at 22 d [378].

Vitamin E. Tocopherols are well known for their ability as antioxidant agents. They may also modulate gene expression and be involved in fetal growth through their role in placental development. Oxidative stress is related to decreased neural tube closure by inhibiting the expression of an essential gene in this process (Pax-3, involved in cell migration) and indeed, vitamin E has shown to prevent the effects of oxidative stress in mice cultured embryos [379] and to improve the development of mice blastocysts treated with reactive oxygen species [380]. However, the protective effect of vitamin E is more effective earlier during development [381-383]. Vitamin E may also modulate gene expression in the brain. This is suggested by one experiment in rats providing vitamin E deficient diet from day 3 to 17 of pregnancy. The deficient diet lowered the vitamin E concentrations of the fetal brain and resulted in changes in expression of genes related to defense antioxidant mechanisms and to lipoprotein metabolism [384]. Vitamin E is also involved in placental development, thus influencing fetal growth [385].
There is little knowledge of the effects of high dose of vitamin E. However, a study reported that high doses (500 mg/d) of vitamin E have detrimental effects during pregnancy, increasing embryo reabsorption [386].

Vitamin K. Vitamin K may play a role in the development of the central nervous system, as suggested by the increased survival in neural cells from rat embryos at day 19th of development when vitamin K is added to the media [387] and by the effects of the vitamin K dependent protein Gas6 (growth arrest specific gene 6) in cell cycle regulation, including neurotrophic effects [388]. However there is a lack of information of the effects of high intake of this vitamin during pregnancy from experiments in animals.

Folic acid. Clinical observations initially suggested a role of the deficiency of folate intake on birth defects [389]. Further research reported that the recurrence and prevalence of congenital malformations involving defects in the neural tube closure was decreased after maternal supplementation with folic acid [390, 391]. Although it is clear that folate has important effects on fetal development, the mechanisms are not totally clear.

Folate is involved in cell proliferation and apoptosis [392] as demonstrated by studies in vitro in which folate deficient media results in increased apoptosis in fetal neural stem cells [393]. Increasing media folate concentrations results in the opposite effect not only decreasing apoptosis, but also increasing cell proliferation [393]. Experiments in mice fed high (10-fold), regular or deficient folic acid (0 folic acid) diets during gestational days 11th to 17th showed that folic acid deficiency decreased cell replication in the brain (~50% depending on the brain region) and increased apoptosis (106%) but the high-folic acid diet had no effect on these measures [394]. However, higher doses of folic acid in maternal diets (20-fold) have resulted in embryonic development delay [22] and reduced fetal weight and length [395].

Vitamins B1, B2, B6 and choline deficiency in gestational diets affect fetal organ growth and development, particularly in the central nervous system. For instance, a thiamin deficient diet during pregnancy results in lower fetal weight brain weight and neuronal death.
in the cerebellum of offspring in rats [396, 397]. Similarly, riboflavin deficiency results in decreased growth in rat embryos \textit{in vitro} [398] and \textit{in vivo} conditions [399]. In the case of pyridoxine and choline, both deficiency and excess have shown to alter fetal development. Pyridoxine deficiency in maternal diets alters the development of central nervous system affecting neurogenesis, neuron differentiation and synaptogenesis in the rat brain [400] and deficiency or excess result in lower body and organ weight [400-402]. Some of the deleterious effects of vitamin deficiencies on the brain have been prevented by choline supplementation [394, 403].

Choline derivatives (i.e. acetylcholine) are involved not only in neural transmission, but also in cell differentiation in neurons [404] and in processes such as mitosis and apoptosis in neural cells during mouse embryonic development. This has been observed by feeding choline deficient diets during pregnancy that resulted in lower mitosis and increased apoptosis in the hippocampus of feti at 18-20\textsuperscript{th} day. A higher dose of choline (~3-fold) has resulted in higher proliferation, but no change in apoptosis rate in neural cells compared with control [405].

The effects of other vitamins such as niacin, panthothenic acid, or vitamin C, on organ development remain elusive. In addition, most experiments have been focused on the effects of deficiency of single vitamin, but the combined effects and the effects of high non-toxic dose on tissue structure and function are still unknown.

\subsection{2.3.6.2. Hormones during pregnancy}

The effects of vitamins on the concentrations of glucocorticoids, leptin and insulin have not been completely described. It has been suggested that vitamins might influence the action of glucocorticoids on fetal development by several ways. First, it is possible that vitamins induce changes in glucocorticoid circulating levels; second, vitamins may modify glucocorticoid placental transport; and third, vitamins may influence the expression of
glucocorticoid receptors. However, there are only few experiments evaluating these outcomes, and the direction of the observed changes varies by vitamin, as discussed below.

Vitamins D and E and folic acid have been found to be related to glucocorticoid plasma concentration in pregnant animals. A study in rats showed that vitamin D deficiency increases corticosterone response (AUC 0-120min) to stress due to restraining. Although the stress test also increased corticosterone concentrations in control animals, the corticosterone AUC values were more than two-fold higher in the deficient dams but were not affected in the male offspring [406]. In water buffaloes, providing an injection of α-tocopherol 1 or 4 times during pregnancy decreased the rise in cortisol observed at the end of pregnancy and during the 2 weeks post-partum. However, cortisol was not measured in the offspring [407]. In rats, increasing folic acid amount (4-fold) of marginal protein-restricted diets during pregnancy increased corticosterone plasma concentrations in male adult offspring [30]. However, it is unclear whether the rise in corticosterone was due to the increased folic acid or to the moderate protein restriction. This distinction is important, since increased glucocorticoid levels have been found in other models of protein restriction [233], but not in previous studies in our laboratory feeding a 10-fold multivitamin diet to pregnant rats [32].

Vitamins A and E may indirectly regulate fetal exposure to glucocorticoids by modulating the expression of the enzyme 11β-HSD 2 in the placenta. In a cell line that simulates placental tissue, the treatment with all-trans or with 9-cis retinoic acids increased the expression 11-βHSD 2 activity in a dose-response manner [408]. Restricting 50% vitamin E content in maternal diet of Swiss albino mice decreased the protein expression and activity of this enzyme [234]. Changes in the activity of 11-βHSD 2 could have resulted in changes in the levels of glucocorticoids of maternal origin in the offspring. Based only on these two studies it seems that vitamin deficiency might decrease the activity of 11-βHSD 2, but supplementation could have the opposite effect. However, further evidence is required to elucidate the role of vitamins on the expression of this placental enzyme.
Gene expression of glucocorticoid receptor also seems to be modulated by folic acid content of maternal diet. When pregnant rats were fed a protein restricted diet, the offspring had 200% higher expression of glucocorticoid receptor (GR) in the liver. Increasing the content of folic acid (5-fold the control) the expression of these receptors was similar to that from offspring from non-restricted dams [51]. Similarly, intrauterine-growth-restricted piglets had higher GR expression in the liver at birth and the addition of folic acid to maternal diet (~24-fold the control diet) rescued the GR gene expression to the values observed in non-restricted animals [409]. Whether increasing folic acid or other vitamins amount in maternal adequate diets has the same effect is currently unknown. However, some evidence from non-pregnant animals suggest a role for B6 in regulating the expression of GR, but its effects on pregnant dams and their offspring have not been investigated [410].

Leptin concentrations are also decreased by vitamin A, but no study has shown its effects during pregnancy. Studies in vitro showed that addition of all-trans retinoic acid (ATRA) to the culture media significantly decreased leptin mRNA expression and secretion of adipocytes from human and murine origin [411]. Retinoic acid and retinol have shown similar effects in animal experiments. Administration of retinoic acid to 6 month-old rats resulted in decreased mRNA levels of leptin in white adipose tissue [412]; whereas feeding rats a diet with 5-fold the amount of retinol than control resulted in a 44% decrease in leptin mRNA levels and 65% in serum concentrations [413]. During pregnancy, an alternative source of leptin is provided by the placenta. A study in vitro showed that ATRA decreased the secretion of leptin in a placental cell line (BeWe), but no effect on mRNA levels were observed [411]. The effect of dietary vitamin A on placental leptin production in vivo has not been reported.

2.3.6.3. Vitamin intake and epigenetic regulation of gene expression

Despite the demonstrated role of vitamins in modulating methylation processes in the development of chronic diseases [254], few studies have addressed their role in setting
epigenetic marks during fetal development. In humans, lower concentration of cobalamin has been associated with decreased SAM:SAH ratio and higher homocysteine serum concentrations, suggesting that methylation pathways are impaired by vitamin deficiency [414]. Experiments in animals feeding sheep a restricted B12/folate/methionine diet from periconception until adulthood resulted in altered methylation of the offspring liver, being more than half of the altered gene, specific to males [415]. Similarly, feeding mice a choline deficient diet during middle pregnancy decreased global and gene-specific DNA methylation in fetal brains [416].

Vitamins in maternal diets above the recommended intake also affect DNA methylation and gene expression. In humans, periconceptional maternal folic acid use increase has been associated with DNA methylation in children. In a cross-sectional study in The Netherlands, the insulin-like-growth factor 2 gene differentially methylated region (IGF2 DMR) was hypermethylated (4.5% higher DNA methylation) in children 12-18 month old from mothers that reported the use of folate supplements during pregnancy. Higher IGF-2 methylation was associated with lower birth weight, suggesting that methylation could have silenced IGF2 expression, resulting in reduced growth. This methylation pattern was associated with SAM level in maternal blood [417].

Early work in animals demonstrated that supplementation with high amount of nutrients involved in one-carbon metabolism (choline, betaine, folic acid, B12, methionine and zinc) modifies the epigenotype and phenotype of the offspring. When female agouti mice were fed this diet from 2 weeks before mating and during through pregnancy and then fed a lower dose diet for 1-2 weeks followed by the control NIH-31 diet, the proportion of offspring expressing the “viable yellow” phenotype characterized by being large, obese, hyperinsulinemic, and more susceptible to cancer, was lower in the methyl supplemented groups, showing instead a greater proportion of the black and healthier phenotype. The authors suggested that DNA methylation was the mechanism underlying the observed changes in the expression of the agouti gene [205]. This hypothesis was tested and confirmed by measuring the degree of DNA methylation in tissues from dams fed methyl-
supplemented diets 2 weeks before and during pregnancy and lactation. Because the pattern of DNA methylation was consistent across tissues derived from the three different germ layers (tail, liver, kidney, brain), the authors suggested that the methylation occurred during early embryonic development [204]. Further work showed that altered methylation and expression of other genes (e.g. Axin(Fu)) by methyl-donor supplemented diets also occur during mid-gestation [203].

Nutrients also regulate the activity and/or expression of the Dnmt and although few studies have confirmed it, information is limited to the effects of choline and folic acid in maternal diets. For example, choline deficient diets have shown to increase the expression of Dnmt1 and Dnmt3 in rat fetal liver and brain [418] and folate deficient diets have been also associated with increased expression of these enzymes [419]. As discussed above, Dnmt have been found to be up-regulated in nutrient deficiency. In contrast, one study supplementing folic acid (2.5-fold the control) before mating and during pregnancy and lactation reported no effect of the maternal diet in the activity of Dnmt in the offspring. Conversely, the increased folate content of the post-weaning diet decreased Dnmt activity [420]. Whether supplementation with higher dose or with other vitamins involved in one-carbon metabolism influence the expression or activity of Dnmts is currently unknown. However, these studies suggest that vitamins affect fetal and postnatal development by different mechanisms.

2.3.6.4. Regulation of PPARs by vitamins

Vitamin A and folic acid have been shown to be involved in the regulation of activity and gene expression of PPARs. PPARs are activated by several nutrients, including derivatives of vitamin A, and are involved in regulating glucose and lipid metabolism. They form heterodimers with the retinoic acid receptor and retinoic X receptors (RAR, RXR) and are activated by a ligand, such as fatty acids, eicosanoids and retinoids. These heterodimers bind to the promoter region of several genes, thus regulating their transcription [421].
expression of a great number of genes related to glucose and fatty acid metabolism is regulated by these transcriptional factors in several tissues, including liver, muscle and adipose, as well as in the placenta [36]. Furthermore, the expression of PPARs has been also related to tissue differentiation during fetal development and regulation of adipogenesis [422, 423].

Gene expression of PPARs seems to be epigenetically regulated. For example, PPAR-α was hypomethylated and its mRNA increased in the offspring of protein-restricted dams. Acetyl Co-A oxidase mRNA, a gene transcriptionally regulated by PPAR-α, was also increased by the maternal diet [51]. The addition of folate to the protein restricted diet, prevented the hypomethylation of PPAR-α, and the mRNA levels of both PPAR-α and Acetyl-CoA oxidase were similar to those found in the control group [51]. Gene expression of PPARs has been also found to be regulated by vitamin A. Addition of ATRA to culture media of human and rat adipocytes increased mRNA levels of PPAR-γ [411], but there are no studies assessing the effects of vitamin A during pregnancy on the expression of these transcriptional factors.

2.4. Summary

Both human and animal studies provide evidence supporting the hypothesis that chronic diseases have their origins in early development. Both under and overnutrition affect birth anthropometry, and widespread effects have been demonstrated in many outcomes including growth measures, hormone concentrations, glucose and lipid metabolism, and gene expression in the brain and peripheral tissues. Other maternal factors, including pregestational obesity, gestational diabetes and parity status, also affect the future health status of the mothers and their progeny. However, the interaction between these factors and components of maternal diets has received little attention.
Despite their fundamental role during all stages of embryonic and fetal development, the effects of vitamins on long-term outcomes have just started to be explored in the last decade. Yet it is clear that vitamins alter the metabolism and gene expression of the offspring by epigenetic mechanisms in mice and rats. Recent studies in humans have correlated differences in global and gene specific DNA methylation with maternal and newborn vitamin status.

The research on the effects of diets during pregnancy has focused on outcomes of the offspring and little is known about their effects on the mother. Dietary components, including vitamins, have the potential to modify maternal hormone concentrations, glucose and lipid metabolism during pregnancy, but whether they have long-term effects for the mother is still unknown.
Chapter 3. Rationale, Hypotheses and Objectives

3.1. Rationale

Food fortification and vitamin supplementation are well-known to prevent vitamin deficiencies, but of more recent concern has been the occurrence of intakes above those recommended in well-nourished population. In developing countries, public health strategies have improved nutritional status in mothers and their progeny and prevented deleterious effects of vitamin deficiencies [38, 313, 424, 425]. However, in developed countries these strategies may lead to unintended high intakes close to or above the recommended upper limit of intake [353] and high concentrations in blood [354].

Both deficiency and excessive intakes of vitamins during pregnancy have deleterious consequences for fetal development [22, 281, 426], but little is known about the long term effects of high, non-toxic, intakes during pregnancy. Rodents have been useful models for exploring long-term outcomes related to deficient and high nutrient intakes because they have a short gestation period and life-span. Recent research in rats showed that multiple vitamin deficiency during pregnancy increases adiposity and serum lipids in adult offspring [29]. Similarly, in our laboratory, increased multivitamin content of the maternal diet during pregnancy was found to increase characteristics of metabolic syndrome in offspring of Wistar rats. Feeding 10-fold the recommended amount of multivitamins (i.e. HV-diet) resulted in higher food intake, weight gain and components of the metabolic syndrome in the adult offspring. However, the mechanisms underlying the observed effects are unknown. A possible explanation for the increased body fat and insulin resistance in the offspring may be the effects of high vitamin intakes during pregnancy on tissue fatty acid composition and the expression of PPARs, thus altering glucose regulation and fat mass. Therefore, this is the focus of Part 1 of the thesis, which took advantage of tissue available from a previous study of offspring fed an obesogenic diet and born to dams fed the HV-diet [33].
Although it is clear that the maternal diet affects development of offspring, there is a surprising lack of knowledge of the effect of interactions between diet and the physiological challenges of pregnancy on the post-pregnancy health of the mother. Therefore, the effects of high vitamin intakes during pregnancy on characteristics of metabolic syndrome in the dams post-weaning and their subsequent offspring are addressed in Part 2 of this thesis. Because folic acid is the most likely vitamin to be consumed above the upper limit by pregnant women [45], and because its function in methylation pathways [427], its role in determining the effects of the HV-diet on the dams was examined.

3.2. Hypotheses and Objectives

3.2.1. Overall hypothesis

High vitamin intakes during the first pregnancy increase body weight and food intake and promote characteristics of metabolic syndrome in Wistar rat dams as well as in both their first and second litter offspring.

3.2.2. Overall objective

To examine the effects of high vitamin intakes during the first pregnancy on body weight, food intake, and characteristics of metabolic syndrome in Wistar rat dams and their offspring from first and second pregnancies.
3.2.3. Specific hypotheses and objectives

Part 1. Effects of the high multivitamin diet (HV-diet) during pregnancy on tissue fatty acid concentration and PPAR gene expression in the offspring

**Hypothesis.** High multivitamin intakes during pregnancy alter tissue fatty acid concentrations, expression of PPAR genes in peripheral tissues, and their regulation of metabolism in the offspring.

**Objective 1.1. (Study 1).** To investigate the effect of high multivitamin intakes during pregnancy on tissue fatty acid concentration of the offspring at birth, weaning, 12 weeks and 48 weeks post-weaning when fed an obesogenic diet.

**Objective 1.2. (Study 2).** To investigate the effects of the HV-diet during pregnancy on gene expression of PPAR-γ, PPAR-β/δ and PPAR-α in adipose, liver and muscle and their relationship with insulin resistance and fat mass in the male offspring at birth, weaning and when fed either a RV or an obesogenic diet for 14 weeks post-weaning.

Part 2. Effects of the high vitamin intakes on the dams and their subsequent offspring

**Hypothesis.** High vitamin intakes during the first pregnancy increase weight gain, food intake, and insulin resistance in both the dams and their second as well as first litter male offspring.

**Objective 2.1. (Study 3).** To investigate the effect of the HV-diet only during the first pregnancy on body weight, food intake, fat mass and glucose metabolism in both the dams and their male offspring after their first and second pregnancies.

**Objective 2.2. (Study 4).** To compare the effect of the high multivitamin (HV) and high-folic-acid (HFol) diets during pregnancy on body weight gain, biomarkers of food intake regulation and insulin resistance in Wistar rat dams after their first pregnancy.
Chapter 4. High Vitamin Intake by Wistar Rats during Pregnancy Alters Tissue Fatty Acid Concentration in the Offspring Fed an Obesogenic Diet

This work was published in Metabolism Clinical and Experimental 2009; 58:772:730. *

4.0. Abstract

Diet during pregnancy affects the long-term health of the offspring. Vitamins are known to modulate lipid metabolism, which may be reflected in tissue fatty acid (FA) concentrations. The objective of this study was to investigate the effect of high-vitamin intake during pregnancy on tissue FA concentration of the offspring. Wistar rats were fed an AIN-93G diet with either the recommended vitamin or 10-fold higher amounts (HV) during pregnancy. Afterward, offspring were weaned onto an obesogenic diet. Liver, quadriceps, adipose and brain were collected over 48 weeks. Fatty acid concentration of tissue total lipids was analyzed by gas chromatography. At birth, the liver from HV offspring was higher in monounsaturated, stearic and arachidonic acids. At weaning, the liver from HV offspring was higher in stearic and oleic acids; and in adipose tissue, n-6 and n-3 FAs were lower only in the male HV offspring ($P<.05$). At 12 weeks, HV offspring had higher concentrations of total fat, saturates, monounsaturates, and n-6 FA in muscle ($P < .05$), but not in other tissues. At 48 weeks, gestational diet did not affect tissue total lipid FA concentrations; but differences remained in specific tissue phospholipids species. Liver phospholipids from HV offspring were lower in monounsaturates and n-6 FA. Brain

*This material has been reprinted from Metabolism Clinical and Experimental, Vol. 58, Sandra A. Reza-López, G. Harvey Anderson, Ignatius M.Y. Szeto, Ameer Y. Taha, David W.L. Ma. High vitamin intake by Wistar rats during pregnancy alters tissue fatty acid concentration in the offspring fed an obesogenic diet, Pages 722-730, Copyright (2009), with permission from Elsevier
phosphatidylethanolamine was higher in oleic, n-6 FA, and docosahexaenoic acid in the HV offspring. Phosphatidylinositol was lower in saturates, monounsaturates, arachidonic and docosahexaenoic acids only in HV female offspring. These observations demonstrate that high vitamin intake during pregnancy has short- and long-term effects on tissue FA concentration in the offspring.

4.1. Introduction

In humans and animals, the fat content of gestational diets is reflected in fetal tissues [428-430] and has been identified as a determinant of the risk for the offspring of developing obesity and chronic diseases [431-433]. Tissue fatty acid (FA) composition in the fetus is readily affected by the dietary fat in the maternal diet [434, 435] and mobilization of maternal FA stores during pregnancy [436, 437]. In addition to FA composition, the adequacy of energy and protein in the gestational diet affects fetal brain and liver FA content of n-3, n-6, and monounsaturated FA [125, 438], possibly with long-term effects [126]. However the effect of micronutrients in the maternal diet during pregnancy on tissue lipid metabolism and FA composition in the offspring has received little investigation.

A role for vitamins in FA metabolism and development is supported by 3 lines of evidence. First, vitamins influence gene expression and cell function, activating transcriptional factors or acting as methyl donors, coenzymes, or cofactors. For example, in the viable yellow Agouti mouse, feeding high intakes of folate, B12 and choline during pregnancy leads to changes in the expression of sensitive genes regulating coat color, adiposity and glucose metabolism in the offspring [205]. Second, many vitamins directly affect FA metabolism. Vitamin A interacts with nuclear receptors, thus activating transcriptional factors [439-441]. Two of the genes regulated by peroxisome proliferators-activated receptors (PPARs) are those that encode the Δ-5 and Δ-6 desaturases, involved in the synthesis of long-chain polyunsaturated fatty acids (PUFA) from their precursors; and vitamins, such as folic acid,
are involved in the regulation of the gene expression by PPARs through an epigenetic mechanism [442, 443]. The activity of Δ-5 and Δ-6 desaturases is also regulated by tocopherols [444] and probably B6 [445]. In addition, acyl-coenzyme A oxidase activity, a key enzyme involved in β-oxidation, is also involved in the conversion of eicosapentaenoic acid (EPA) to docosahexaenoic acid (DHA), which has been shown to be reduced by B6 deficiency [445]. Third, studies from our group have shown that high vitamin intake by Wistar rats during pregnancy leads to higher body weight and fat mass, and components of the metabolic syndrome in the adult offspring [32]. Because tissue FA content is used as a surrogate marker for diet-induced changes in lipid metabolism, the objective of this study was to investigate the effect of high multivitamin intake during gestation on tissue FA concentration of the offspring at birth, weaning, and 12 and 48 weeks postweaning (PW).

4.2. Materials and Methods

4.2.1. Experimental design. Pregnant (2nd to 3rd day of pregnancy) Wistar rats (Charles River, Montreal, Quebec, Canada) were randomly allocated to receive either the AIN-93G diet [446] containing either the regular amount of vitamins (RV) or a modified AIN-93G diet containing 10-fold higher vitamins (HV) during pregnancy. Except for the vitamin content, the rest of the components were the same in both diets (Table 4.1). Because the vitamin mix uses sucrose as a carrier (9.75g for 10 g) [446] we adjusted this component to provide similar amount in both diets; and it is presented separately.

After delivery, all dams received the RV diet; and the litters were culled to 10 pups per dam. At 3 weeks of age, the offspring from both groups were sexed and weaned to an obesogenic liquid diet, described in Table 4.1. [447]. Animals were housed individually in a temperature-controlled environment (22°C ±1°C) with a 12-hour dark-light cycle. The protocol was approved by the Animal Ethics Committee at University of Toronto. All diets were prepared in the laboratory and were provided ad libitum.
4.2.2. **Tissue collection.**

Animals were terminated by decapitation after overnight fasting. Tissues were collected at birth (liver and muscle), weaning (liver, muscle, abdominal adipose tissue), and 12 and 48 weeks PW (liver, brain, adipose, muscle). All tissues were immediately frozen in liquid nitrogen and stored at -80°C until their analysis. At birth the tissues from 2 pups per litter (when available) were analyzed, and their results were averaged. For each of the other time points, the tissues of 1 pup per dam were used for analyses, whereas part of the offspring was used for a separate experiment.

4.2.3. **Lipid analysis.**

4.2.3.1. **Chemicals.** All solvents and reagents, unless other specified were obtained from Sigma Chemical Co. (St. Louis, MO). Phospholipid standards were obtained from Matreya Biochemicals (Pleasant Gap, PA).

4.2.3.2. **Analysis.**

Liver, adipose tissue, and muscle (approximately 0.5 g of tissue) were homogenized (CH-6010 homogenizer, Kinematica, Kriens, Lucerne, Switzerland) in 2.5 mL of 0.88% of KCl. Lipids were extracted using the method of Folch et al [448]. In the case of brain, the whole organ was homogenized for lipid analysis; and the solvent volume was adjusted according to the organ weight. Heptadecanoic acid (17:0) either as a free FA or as cocktail of triglycerides, phospholipids, and cholesterol ester was added as an internal standard. Liver lipid classes (at 12 and 48 weeks PW) were separated by thin-layer chromatography (TLC) to obtain triglycerides, phospholipids, and cholesterol esters. Thin-layer chromatography plates were used to separate lipid classes using a solvent mixture of petroleum ether, ethyl ether, acetic acid (80:20:1vol/vol/vol). Bands corresponding to phospholipids, triglycerides and cholesterol esters were visualized under UV light after lightly spraying with 8-anilino-1-naphtalene-sulfonic acid (0.1% wt/vol).
Brain and muscle phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylinositol [PI]; phosphatidylethanolamine, [PE] and sphingomyelin from the offspring at 12 and 48 weeks PW were also separated by a similar procedure. The TLC plates were developed with a mixture of chloroform, methanol, 2-propanol, KCl (0.25% wt/wt), and triethylamine (30:9:25:6:18 vol/vol). Bands corresponding to the phospholipid fractions were visualized as previously described. Fatty acids from total lipid extracts, lipid classes, and phospholipid fractions were converted to methyl esters (FA methyl esters [FAME]) using 14% methanolic boron trifluoride. Fatty acid methyl esters were separated on an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector and separated on a fused-silica capillary column (SP2560, Sulpeco, Bellefonte, PA; 100 m, 0.25 µm film thickness, 0.25 mm internal diameter). Samples were injected in splitless mode. The injector and detector ports were set at 250°C. The FAMEs were eluted using a temperature program set initially at 60°C, increased at 10°C/min, and held at 170°C for 5 min; increased at 5°C/min, then at 2°C/min and at 1°C/min until reaching 175°/min, 185°C, and 190°C, respectively; and finally increased at 10°C/min until reaching 240°C and held for 18 minutes to complete the run. The carrier gas was helium, set to a 1.3 mL/min constant flow rate. Peaks were identified by comparison with authentic FA standards (GLC463; Nu-Chek Prep, Elysian, MN) and quantified using heptadecanoic acid as the internal standard. Peaks were quantified using ChemStation software (Version B.01.01, Agilent). Results from total lipid analyses are presented as milligrams per gram of wet tissue for the sum of saturated, monounsaturated, n-6 and n-3 FA, and the main individual FAs corresponding to these series.

4.2.4. Statistical analysis.

Values are presented as the mean and standard error of the mean. Means were compared by 2-way analysis of variance (ANOVA), including gestational diet and sex as main factors and their interaction term. Unpaired t test was used for comparison of means of the FA concentration at birth and to test means of FAs within each sex. Statistical significance was
declared at $P$ less than .05. The software SAS version 9.1 (SAS Institute, Cary, NC) was used for statistical analyses.

4.3. Results

4.3.1. FA concentration of the offspring at birth and weaning

Total FA, including many saturated FA, monounsaturated FA, and arachidonic acid (20:4n-6) concentrations (Table 4.2.) were higher in the liver of the unsexed offspring from HV dams relative to RV dams. Muscle FA concentration (not reported) was not different between groups. Abdominal adipose tissue was not detected at this age.

At weaning, the concentrations (milligrams per gram tissue) of stearic (males: RV 6.1±0.2, HV 6.6±0.2; females: RV 6.2±0.2, HV 6.6±2.2) and oleic acids (males: RV 1.3±0.1, HV 1.5±0.1; females: RV 1.4±0.1, HV 1.5±0.1) were higher in the liver of HV offspring ($P$<.05). No other significant changes were observed in other FAs. In muscle there was no effect of gestational diet on FA concentrations. In adipose tissue, an interaction was observed between gestational diet and sex for total n-6 FA and DHA (22:6n-3). Males, but not females from HV dams, were observed to have lower values of these FAs (Table 4.3.).

4.3.2. FA concentration of the offspring at 12 weeks PW

At 12 weeks PW, liver total FA concentration was different between sexes; but gestational diet had no effect. Therefore, data from RV and HV were pooled for further analysis to explore sex differences. Males had higher concentrations than females in the following FAs (milligrams per gram tissue): 16:0 (11.02±0.7 vs 7.6±1.0), 18:1 c11 (1.8±0.1 vs 0.8±0.1), 18:2 n-6 (7.3±0.7 vs 4.8±0.4), 20:3n-6 (0.3±0.01 vs 0.2±0.01), 22:4n-6 (0.1±0.01 vs 0.04±0.01), 18:3n-3 (0.5±0.1 vs 0.3±0.04), monounsaturates (12.9±0.9 vs 8.5±1.6), and n-6/n-3 ratio (5.4±0.2 vs. 3.8±0.1). Females were higher in 18:0 (7.5±0.2 vs 5.2±0.1) and the sum of n-3 (3.0±0.1 vs. 2.6±0.2) milligrams per gram tissue ($P$<.05). In addition to total
lipid analyses, liver lipid classes were separated; and as observed for total lipids, phospholipids and cholesterol esters FAs differed by sex, but were not influenced by the gestational diet (data not shown).

The gestational diet markedly affected muscle FA concentration (Table 4.4.). HV male offspring had higher concentration of total fat, saturates, monounsaturates, and n-6 FA (milligrams per gram tissue). Adipose tissue FA concentration showed a difference by sex, but no effect of gestational diet was detected. Males had higher concentration (milligrams per gram tissue) of 16:1 (41.7±1.9 vs 28.8±1.4), 18:1c11 (24.9±1.1 vs 17.0±0.5), and lower 22:4n-6 (0.1±0.05 vs 0.4±0.05) and DHA (0.2±0.2 vs 0.4±0.2) than females (p<.05).

Similarly, in the brain, differences by sex were observed in the concentration of 22:6 n-3 (females 4.7±0.2, males 4.2±0.11); but no differences by gestational diet were found in total lipids.

4.3.3. FA concentration of the offspring at 48 weeks PW

In the offspring at 48 weeks PW, there was no effect of gestational diet on liver total lipids. However, there were differences in FA concentration (milligrams per gram tissue) due to sex. Males were lower in 18:0 (6.0±0.2 vs 8.6±0.9) and 22:5n-6 (0.04±0.02 vs 0.13±0.04), and higher in 18:3n-3 (0.8±0.1 vs 0.4±0.1) than females. Further analysis of liver lipid classes showed that the gestational diet affected phospholipid FA concentrations in which monounsaturates, n-6 FAs, and n-6/n-3 ratio were lower in HV offspring (Table 4.5.). No effect of gestational diet was observed in liver triglycerides or cholesterol esters.

Brain total lipids at 48 weeks did not show differences by gestational diet. Small differences by sex were observed in several FAs. Males had higher concentration (milligrams per gram tissue) of 18:0 (7.1±0.1 vs 6.7±0.06), 18:1c11 (1.6±0.03 vs 1.6±0.05), 20:4n-6 (3.2±0.05 vs 3.0±0.4), 22:4n-6 (0.8±0.01 vs 0.9±0.02) and 18:3n-3 (0.3±0.01 vs 0.2±0.03) than females (P<.05). Additional analysis showed that gestational diet affected the FA concentration (micrograms per gram tissue) of phospholipid classes, specifically on brain PI and PE FA concentrations (Table 4.6.).
Muscle total lipid FA concentrations were influenced by sex, but not by gestational diet. Males had lower concentration (milligrams per gram tissue) than females in the following FAs: 14:0 (0.4±0.04 vs 0.7±0.07), 16:0 (5.9±0.4 vs 8.2±0.6), total saturates (8.1±0.04 vs 10.7±0.7), 16:1 (1.4±0.1 vs 2.1±0.2), 18:1c9 (5.7±0.4 vs 8.7 ±0.9), monounsaturates (8.2±0.6 vs 12.0±1.2), and total FAs (23.5±1.2 vs 30.6±2.1) in the total lipids \( (P<.05) \). Phospholipid FA concentration in this tissue also was influenced by sex, but not gestational diet (data not shown). Similarly, adipose tissue was not affected by gestational diet; and only sex was observed to affect the concentration of 18:1c11 (males 32.6±1.2, females 23.0±0.7 mg/g tissue) and 18:2n-6 (males 126.8±5.6, females 99.6±7.5 mg/g tissue) \( (P<.05) \).

### 4.4. Discussion

The results of this study support the hypotheses that high multivitamin intake during pregnancy affects tissue FA concentration and composition in the offspring from Wistar rats and that the effect is tissue, sex, and age dependent.

Effects of the gestational diet were evident in early life, as shown by the higher concentration of saturates, monounsaturates, and n-6 PUFA in the liver at birth of the offspring from dams fed the HV diet compared with those from dams fed the RV AIN-93G diet. In contrast, in the adipose tissue, the maternal high vitamin intake resulted in lower concentration of n-6 PUFA and DHA in male offspring at weaning. Long-lasting effects of the gestational diet were observed in specific tissues and classes of lipids in muscle (Table 4.4), liver (Table 4.5) and brain (Table 4.6) at 12 and 48 weeks PW.

Effects of the gestational diet at birth and through to 48 weeks of the life suggests that they were imprinted in utero. At birth, the FA concentration of the tissues would primarily reflect the effect of vitamin intake on the maternal, placental, and fetal lipid metabolism. The influence of the gestational period remained even though the offspring started to eat the
AIN-93G diet with the regular content of vitamins by their third week of lactation. Furthermore, both males and females received the same diet (same dam’s milk and diet); but only the male offspring of HV dams showed lower concentration of FAs in adipose tissue, particularly n-6, thus suggesting that gestational diet altered the offspring FA metabolism in a sex-dependant manner.

Moreover, after the pups were weaned to the obesogenic diet, which provided a higher content of saturates (35%) and lower n-6 (33.7%) and n-3 (6.7%) with traces of their elongated products than the AIN-93G diet, the gestational diet remained a factor in determining the FA concentration of their tissue. The FA of the diet is known to be reflected in tissues such as muscle [449], but this alone did not explain the differences in their elongated products of n-6 and n-3. Whereas, in RV males, the products from the precursor linoleic acid accounted for 37% of the total n-6, in the HV group, they accounted for only 23%. This suggests that the elongation and desaturation processes or the oxidation rate may have been altered by the gestational diet.

Functional changes in the peripheral tissues due to the HV diet may also be predicted from the phospholipid content of the tissues. We have reported that high vitamin intakes during pregnancy led to impairment of glucose metabolism [32] in male offspring consistent with the observed lipid composition of their peripheral tissues, as reported here. Phospholipids are mainly found in tissue cell membranes, and their FA composition modulates cell physiology [450-452]. For instance, studies in cell culture have shown that muscle cells treated with EPA (20:5 n-3) have higher uptake of glucose and FAs. Treatment with EPA has been shown to increase glucose transport more than 2-fold in the presence of insulin, demonstrating that long-chain FAs contribute to glucose uptake [451]. Furthermore, a lower concentration of PUFA is associated with lower insulin sensitivity in skeletal muscle [453]; and specifically, lower content of n-3 PUFA in membrane phospholipids is associated with reduced insulin sensitivity [35, 454, 455]. The male offspring (at 12 weeks) of HV dams had higher concentration of saturates and monounsaturates and higher n-6 FA in muscle. Although the n-6/n-3 ratio was not significantly different, the HV offspring tended to have
higher values \( (P=.078) \). The offspring of HV dams also had higher total fat (milligrams per gram tissue) in the muscle. Both a lower proportion of n-3 FA and higher intramuscular fat are related to insulin resistance [35] and support earlier observations that male HV offspring have impaired glucose metabolism [32].

The observed effects of the gestational diet are likely to result from its impact on in utero development of FA metabolism. However, the mechanism by which programming of lipid metabolism occurred in utero is unknown at present; but there are several possibilities. Both epigenetic regulation of transcriptional factors and its activation may provide a possible explanation [260]. Epigenetic regulation of gene expression is known to occur through changes in DNA methylation [439, 456], as observed in the offspring of animals supplemented with high amounts of folic acid and group B vitamins during pregnancy [204, 205, 456, 457]. Methylation of genes encoding transcriptional factors, such as PPARs is also possible [443], thus altering the expression of other genes encoding enzymes involved in lipid and glucose metabolism [458]. The PPARs are also regulated by vitamin A derivatives, which activate nuclear receptors such as retinoic acid receptor and retinoid X receptor [441]. Thus, the high dietary intake of vitamin A may also have contributed to the activation of these transcriptional factors and an interaction of retinoids with the 1-carbon metabolism [277]. However, we did not measure gene expression at any time point, therefore further research is needed to investigate this potential mechanism.

Another mechanism that has been proposed for in utero programming of metabolism is increased glucocorticoid concentrations [459-462]. Glucocorticoids have been shown to increase FA synthase expression [463], to mediate the programming effects of gestational diet on muscle and adipose tissue [464], and to mediate the effect of folic acid on the FA composition of brain in the context of a protein-deficient diet [30]. However, as previously reported [32], the corticosterone concentrations of the offspring from HV dams were not different from RV dams, suggesting that the observed changes in tissue FA concentration and composition in the offspring were not mediated by glucocorticoids.
Our results confirm previous studies reporting the influence of sex in tissue lipid profile [30, 124]. The earliest that we observed an effect of the sex of the offspring in the FA concentration of tissues in the offspring was at weaning because the pups were not sexed at birth. Mechanisms mediating sex differences in FA metabolism may involve sexual hormones [465], which also modulate the expression of hepatic transcriptional and fat oxidation rate [466]. Furthermore, hepatic clearance of FAs is faster in females, probably reflecting a higher concentration of FA transporters [467-469].

Fatty acid concentration of tissues also showed age-related changes. Adipose tissue shows a decrease of saturates and increase of monounsaturates with aging [470], and a similar age-related increase in monounsaturated FAs has been also reported in liver [471]. These reports are consistent with our observations that monounsaturates concentration was found to be higher in aged rats and that both groups followed similar pattern, thus confirming that this is probably an age-related effect. Others have found that gestational diet affects lipid metabolism in an age-dependant manner [472]. In our study, liver FA concentration was different by gestational diet at birth and at 48 weeks, whereas muscle only showed differences at 12 weeks PW. Fatty acid concentration of brain also showed differences at 48 weeks but not at 12 weeks PW, the 2 time points in which it was evaluated. However, the results from the offspring at 48 weeks PW must be interpreted with caution because the sample size was reduced at this time point.

The high-vitamin gestational diet had long-lasting effects on FAs concentrations in muscle, liver, and brain of the offspring, which may affect insulin sensitivity in peripheral tissues and possibly brain development and function [434, 453, 473-480]. Thus, changes in FA concentrations in these tissues may have long-term consequences for the health of the offspring. This is important in the context of high vitamin intakes observed in developed countries. The use of multivitamin supplements during pregnancy is a common practice; and many foods are fortified with vitamins, thus increasing the likelihood of intakes higher than current recommendations. A study in pregnant women in Boston reported vitamin intakes that exceed 2 to 5 times the recommendations in the upper quartile for several vitamins,
reaching levels that border or exceed the upper limit for several vitamins, including folic acid [353]. Recently, a study in humans showed that high-folate and low-B12 status in mothers was related to higher adiposity and risk for insulin resistance in their children [31]. Clearly, the effects of high intakes of vitamins during pregnancy by humans require further study.

We fed the offspring with an obesogenic diet. However, it may be that the effects on the offspring of the high vitamin intake during pregnancy would be ameliorated if they were fed a high-vitamin diet, as is proposed by the predictive adaptive hypothesis [481]. However, whether the effects of the gestational diet are modulated by exposure of the offspring to a high-vitamin diet deserves further investigation.

The vitamin content of the HV diet was high but less than toxic levels [482]. Although high amount of retinoic acid is teratogenic, the dose of vitamin A used in the HV diet was 8 to 10 times lower than the dose known to cause congenital malformations in mice [483, 484] Furthermore, the vitamin mix contains all-trans-retinyl palmitate, a form of vitamin A less toxic and less teratogenic than retinoic acid [482]. Vitamins involved in the 1-carbon metabolism (folic acid, B12) have been used in amounts 9 and 60 times the amount of control diets without an effect on litter size or weight [205]. Folic acid, however, when provided 20-fold the recommended amount during pregnancy, was found to decrease birth weight and length in Wistar rats [395]. We did not observe differences in birth weight or litter size with our 10-fold multivitamin diet.

In summary, high intake of multivitamins during pregnancy resulted in altered tissue FA concentration and composition in the offspring, suggesting that the diet during pregnancy has long-lasting effects in the lipid metabolism of the offspring. Furthermore, the effect is dependent on the sex and age of the offspring, and can vary between and within the tissues. However, the mechanisms underlying these effects of the gestational diet are still unclear. On the basis of these findings, further investigations are warranted.
Table 4.1. Composition of experimental diets

<table>
<thead>
<tr>
<th>Component (g/kg diet dry weight)</th>
<th>RV</th>
<th>HV</th>
<th>Ob-pup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch \textsuperscript{a}</td>
<td>529.5</td>
<td>529.5</td>
<td>278.4</td>
</tr>
<tr>
<td>Casein (&gt; 85% protein) \textsuperscript{a}</td>
<td>200.0</td>
<td>200.0</td>
<td>147.3</td>
</tr>
<tr>
<td>Sucrose (added) \textsuperscript{b}</td>
<td>100.0</td>
<td>10.0</td>
<td>397.2</td>
</tr>
<tr>
<td>Sucrose from Vitamin Mix</td>
<td>9.75</td>
<td>97.5</td>
<td>5.1</td>
</tr>
<tr>
<td>Sucrose from Mineral Mix</td>
<td>21.4</td>
<td>21.4</td>
<td>11.2</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.0</td>
<td>0.0</td>
<td>36.5</td>
</tr>
<tr>
<td>Fat \textsuperscript{c,e}</td>
<td>70.0</td>
<td>70.0</td>
<td>78.9</td>
</tr>
<tr>
<td>Fiber (Cellulose) \textsuperscript{a}</td>
<td>50.0</td>
<td>50.0</td>
<td>26.3</td>
</tr>
<tr>
<td>Mineral mix \textsuperscript{d,f}</td>
<td>13.6</td>
<td>13.6</td>
<td>7.2</td>
</tr>
<tr>
<td>Vitamin mix \textsuperscript{d,f}</td>
<td>0.25</td>
<td>2.5</td>
<td>0.2</td>
</tr>
<tr>
<td>L-Cystine \textsuperscript{d}</td>
<td>3.0</td>
<td>3.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Choline bitartrate \textsuperscript{d}</td>
<td>2.5</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>tert-Butylhydroquinone \textsuperscript{d} (mg/kg)</td>
<td>14.0</td>
<td>14.0</td>
<td>7.4</td>
</tr>
</tbody>
</table>

RV indicates regular vitamin diet; HV, high vitamin diet; Ob-pup, obesogenic liquid diet.
\textsuperscript{a} From Harlan Teklad (Madison, WI).
\textsuperscript{b} From Allied Food Service (Toronto, Ontario, Canada).
\textsuperscript{c} From Loblaws (Toronto, Ontario, Canada).
\textsuperscript{d} From Dyets (Bethlehem, PA).
\textsuperscript{e} Fat source in RV and HV was soybean oil; in obesogenic diet, fat was derived from condensed milk and soybean oil.
\textsuperscript{f} Absolute amount of mineral/vitamin, without sucrose used as a carrier. The content of vitamin mix is 10 and 100 g/kg in RV and H, respectively. The sucrose content of the diet was adjusted to provide similar amount of sucrose in the RV and HV diets and is presented separately.
Table 4.2. At birth, gestational diet influences liver FA concentrations (milligrams per gram tissue) of the unsexed offspring

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>From RV</th>
<th>From HV</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.0 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>.11</td>
</tr>
<tr>
<td>16:0</td>
<td>12.2 ± 1.3</td>
<td>18.2 ± 2.3</td>
<td>.05</td>
</tr>
<tr>
<td>18:0</td>
<td>4.3 ± 0.3</td>
<td>5.4 ± 0.3</td>
<td>.03</td>
</tr>
<tr>
<td>Saturates&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.6 ± 1.8</td>
<td>27.0 ± 3.2</td>
<td>.04</td>
</tr>
<tr>
<td>16:1 c9</td>
<td>0.7 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>.04</td>
</tr>
<tr>
<td>18:1 c9</td>
<td>7.2 ± 0.9</td>
<td>12.7 ± 2.0</td>
<td>.03</td>
</tr>
<tr>
<td>18:1 c11</td>
<td>1.0 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>.03</td>
</tr>
<tr>
<td>Monounsaturates&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.2 ± 1.1</td>
<td>16.0 ± 2.5</td>
<td>.03</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>10.8 ± 1.1</td>
<td>16.4 ± 2.5</td>
<td>.07</td>
</tr>
<tr>
<td>18:3 n-6</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>.05</td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>.06</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>10.2 ± 0.9</td>
<td>14.2 ± 1.6</td>
<td>.05</td>
</tr>
<tr>
<td>22:5 n-6</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>.10</td>
</tr>
<tr>
<td>Sum n-6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.9 ± 2.3</td>
<td>36.3 ± 4.8</td>
<td>.06</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>0.7 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>.11</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>1.3 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>.09</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>2.0 ± 0.2</td>
<td>2.6 ± 0.3</td>
<td>.10</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>5.6 ± 0.5</td>
<td>6.8 ± 0.4</td>
<td>.08</td>
</tr>
<tr>
<td>Sum n-3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.6 ± 0.9</td>
<td>12.3 ± 1.1</td>
<td>.07</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>2.6 ± 0.0</td>
<td>2.9 ± 0.2</td>
<td>.10</td>
</tr>
<tr>
<td>Total mg/g tissue&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.4 ± 6.0</td>
<td>91.8 ± 11.5</td>
<td>.04</td>
</tr>
</tbody>
</table>

n = 6 to 7 per group.
<sup>a</sup>By unpaired t test.
<sup>b</sup>Total includes other minor FAs not reported.
Table 4.3. At weaning, gestational diet and sex influence adipose tissue FA concentration (milligrams per gram tissue) of the offspring

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From RV</td>
<td>From HV</td>
</tr>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>14:0</td>
<td>44.6±2.0</td>
<td>35.6 ± 2.5</td>
</tr>
<tr>
<td>16:0</td>
<td>149.1±4.9</td>
<td>133.0 ± 8.5</td>
</tr>
<tr>
<td>18:0</td>
<td>18.7±0.6</td>
<td>17.1 ± 1.2</td>
</tr>
<tr>
<td>Saturates*</td>
<td>239.9±7.2</td>
<td>207.3 ± 13.6</td>
</tr>
<tr>
<td>16:1 c9</td>
<td>19.4±1.2</td>
<td>19.6 ± 1.9</td>
</tr>
<tr>
<td>18:1 c9</td>
<td>132.7±5.4</td>
<td>122.4 ± 8.1</td>
</tr>
<tr>
<td>18:1 c11</td>
<td>10.2±0.5</td>
<td>9.5 ± 0.7</td>
</tr>
<tr>
<td>Monounsaturates*</td>
<td>165.0±6.9</td>
<td>154.0 ± 10.3</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>157.2±6.1</td>
<td>138.4 ± 11.3</td>
</tr>
<tr>
<td>18:3 n-6</td>
<td>1.0±0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>1.7±0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>4.9±0.2</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>Sum n-6</td>
<td>168.4±6.5</td>
<td>147.7 ± 12</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>19.9±0.9</td>
<td>17.9 ± 1.5</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>0.7±0.1</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>1.5±0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Sum n-3</td>
<td>23.9±1.2</td>
<td>20.8 ± 1.7</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>7.1±0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>Total mg/g</td>
<td>597.7±19.3</td>
<td>530.3 ± 36.3</td>
</tr>
</tbody>
</table>

*P less than .05 for *gestational diet effect, †sex of the pup effect, and ‡gestational diet × sex interaction, by 2-way ANOVA; n = 7 to 9 per group. * Includes other minor FAs not reported.
Table 4.4. At 12 weeks PW, gestational diet and sex influence muscle FA concentration (milligrams per gram tissue) of the offspring

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From RV Mean ± SEM</td>
<td>From HV Mean ± SEM</td>
</tr>
<tr>
<td>14:0</td>
<td>0.2 ± 0.02</td>
<td>0.5 ± 0.10</td>
</tr>
<tr>
<td>16:0</td>
<td>2.5 ± 0.20</td>
<td>4.8 ± 0.90</td>
</tr>
<tr>
<td>18:0</td>
<td>1.0 ± 0.00</td>
<td>1.3 ± 0.10</td>
</tr>
<tr>
<td>Saturates</td>
<td>3.7 ± 0.30</td>
<td>6.6 ± 1.20</td>
</tr>
<tr>
<td>16:1</td>
<td>0.6 ± 0.10</td>
<td>1.2 ± 0.30</td>
</tr>
<tr>
<td>18:1 c9</td>
<td>1.7 ± 0.20</td>
<td>4.5 ± 1.20</td>
</tr>
<tr>
<td>18:1 c11</td>
<td>0.4 ± 0.03</td>
<td>0.7 ± 0.10</td>
</tr>
<tr>
<td>Monounsaturates</td>
<td>2.7 ± 0.30</td>
<td>6.6 ± 1.70</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>1.7 ± 0.20</td>
<td>3.3 ± 0.70</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>0.9 ± 0.10</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>Sum n-6</td>
<td>2.7 ± 0.20</td>
<td>4.3 ± 0.70</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>0.7 ± 0.10</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td>Sum n-3</td>
<td>0.9 ± 0.10</td>
<td>1.0 ± 0.04</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>3.0 ± 0.10</td>
<td>4.5 ± 0.80</td>
</tr>
<tr>
<td>Total mg/g</td>
<td>10.1 ± 0.90</td>
<td>18.6 ± 3.60</td>
</tr>
</tbody>
</table>

* P less than .05 for gestational diet effect, † sex of the pup effect, and ‡ gestational diet × sex interaction, by 2-way ANOVA; n = 7 to 9 per group.

a Includes other minor FAs not reported
Table 4.5. At 48 weeks PW, gestational diet and sex influence FA concentrations (milligrams per gram tissue) of liver phospholipids in the offspring

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Males From RV Mean±SEM</th>
<th>Males From HV Mean±SEM</th>
<th>Females From RV Mean±SEM</th>
<th>Females From HV Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.8±0.21</td>
<td>0.4±0.12</td>
<td>2.9±2.10</td>
<td>0.8±0.21</td>
</tr>
<tr>
<td>16:0</td>
<td>3.8±0.12</td>
<td>3.5±0.15</td>
<td>2.6±0.12</td>
<td>2.8±0.10†</td>
</tr>
<tr>
<td>18:0</td>
<td>4.5±0.52</td>
<td>4.5±0.62</td>
<td>6.9±0.37</td>
<td>6.4±0.22†</td>
</tr>
<tr>
<td>Saturatesa</td>
<td>9.2±0.42</td>
<td>8.6±0.80</td>
<td>12.6±2.08</td>
<td>10.2±0.34†</td>
</tr>
<tr>
<td>141 c9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>161 c9</td>
<td>0.3±0.03</td>
<td>0.2±0.04</td>
<td>0.2±0.03</td>
<td>0.2±0.03</td>
</tr>
<tr>
<td>181 c9</td>
<td>0.8±0.04</td>
<td>0.7±0.03</td>
<td>0.7±0.07</td>
<td>0.6±0.03†</td>
</tr>
<tr>
<td>181 c11</td>
<td>0.7±0.06</td>
<td>0.5±0.04</td>
<td>0.4±0.04</td>
<td>0.4±0.03†</td>
</tr>
<tr>
<td>Monounsatuaes</td>
<td>1.8±0.11</td>
<td>1.4±0.06</td>
<td>1.3±0.11</td>
<td>1.3±0.09*†</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>1.9±0.12</td>
<td>1.4±0.09</td>
<td>1.4±0.27</td>
<td>1.1±0.10†</td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>0.3±0.04</td>
<td>0.2±0.02</td>
<td>0.2±0.03</td>
<td>0.2±0.03*</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>5.7±0.18</td>
<td>5.4±0.29</td>
<td>5.9±0.28</td>
<td>5.7±0.19</td>
</tr>
<tr>
<td>22:5 n-6</td>
<td>0.04±0.01</td>
<td>0.1±0.02</td>
<td>0.1±0.05</td>
<td>0.2±0.03†</td>
</tr>
<tr>
<td>Sum n-6a</td>
<td>8.0±0.15</td>
<td>7.2±0.28</td>
<td>7.7±0.34</td>
<td>7.2±0.24*</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>0.01±0.01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>0.05±0.02</td>
<td>0.01±0.01</td>
<td>0.1±0.04</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>1.8±0.10</td>
<td>2.03±0.07</td>
<td>2.0±0.27</td>
<td>2.2±0.16</td>
</tr>
<tr>
<td>Sum n-3a</td>
<td>2.0±0.09</td>
<td>2.2±0.08</td>
<td>2.2±0.22</td>
<td>2.4±0.14</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>4.1±0.18</td>
<td>3.3±0.17</td>
<td>3.5±0.39</td>
<td>3.1±0.19*</td>
</tr>
<tr>
<td>Total (mg/g)a</td>
<td>21.1±0.56</td>
<td>19.4±1.07</td>
<td>23.9±2.26</td>
<td>21.1±0.66*†</td>
</tr>
</tbody>
</table>

Males: RV n = 9, HV n = 7; females: RV n = 4, HV n = 7. ND indicates not detected. 
*P less than .05 for *gestational diet effect and †sex of the pup effect, by 2-way ANOVA.  
aIncludes other minor FAs not reported.
Table 4.6. At 48 weeks PW, gestational diet and sex influence FA concentration (micrograms per gram tissue) of brain phospholipids in the offspring

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Males</th>
<th></th>
<th></th>
<th>Females</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From RV</td>
<td>From HV</td>
<td>From RV</td>
<td>From HV</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean ±SEM</td>
<td>Mean ±SEM</td>
<td>Mean ±SEM</td>
<td>Mean±SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>92.7 ±5.9</td>
<td>120.7 ±10.5</td>
<td>130.7 ±30.9</td>
<td>87.5 ±4.9‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>407.4 ±38.4</td>
<td>384.2 ±27.3</td>
<td>700.4 ±86.4</td>
<td>420.7 ±55.1*†‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturates&lt;sup&gt;a&lt;/sup&gt;</td>
<td>502.9 ±41.1</td>
<td>509.5 ±32.0</td>
<td>855.3 ±130.0</td>
<td>508.2 ±57.8*†‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 c9</td>
<td>133.3 ±10.5</td>
<td>147.2 ±8.8</td>
<td>228.2 ±60.4</td>
<td>137.8 ±15.7‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 c11</td>
<td>25.3 ±2.4</td>
<td>33.8 ±2.8</td>
<td>42.6 ±12.0</td>
<td>18.3 ±6.2‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monounsaturates&lt;sup&gt;a&lt;/sup&gt;</td>
<td>180.2 ±19.7</td>
<td>206.6 ±10.7</td>
<td>316.7 ±94.5</td>
<td>175.4 ±32.4‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>2.6 ±1.8</td>
<td>2.9 ±1.9</td>
<td>7.2 ±4.0</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>323.7 ±21.5</td>
<td>347.2 ±33.2</td>
<td>489.4 ±48.9</td>
<td>321.7 ±39.4‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:4 n-6</td>
<td>20.7 ±6.3</td>
<td>15.3 ±2.9</td>
<td>54.6 ±7.7</td>
<td>24.9 ±8.3*†‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:5 n-6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum n-6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>347.0 ±25.5</td>
<td>365.5 ±33.3</td>
<td>551.3 ±59.2</td>
<td>346.5 ±45.7*†‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>ND</td>
<td>ND</td>
<td>4.5 ±4.5</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>98.8 ±23.1</td>
<td>67.0 ±6.4</td>
<td>247.0 ±31.6</td>
<td>119.5 ±30.7*†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum n-3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.8 ±23.1</td>
<td>73.9 ±11.0</td>
<td>251.6 ±35.6</td>
<td>119.5 ±30.7*†‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>5.12 ±1.25</td>
<td>5.42 ±0.69</td>
<td>2.23 ±0.19</td>
<td>3.92 ±1.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>Males RV</td>
<td>Females RV</td>
<td>Males HV</td>
<td>Females HV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>----------</td>
<td>------------</td>
<td>----------</td>
<td>------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>254.4 ±31.5</td>
<td>291.7 ±44.5</td>
<td>197.4 ±3.4</td>
<td>273.5 ±30.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>642.9 ±76.3</td>
<td>864.7 ±183.1</td>
<td>508.5 ±6.0</td>
<td>724.2 ±79.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturates</td>
<td>1117.5 ±133.5</td>
<td>1437.5 ±294.1</td>
<td>888.7 ±15.5</td>
<td>1234.2 ±137.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>12.9 ±2.5</td>
<td>17.2 ±1.9</td>
<td>10.7 ±0.7</td>
<td>16.5 ±1.9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 c9</td>
<td>725.8 ±86.0</td>
<td>1137.8 ±285.7</td>
<td>536.0 ±12.2</td>
<td>771.0 ±85.4*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 c11</td>
<td>137.5 ±15.9</td>
<td>217.0 ±54.8</td>
<td>99.8 ±4.1</td>
<td>137.8 ±16.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monounsaturates</td>
<td>1192.0 ±131.5</td>
<td>1846.9 ±412.8</td>
<td>893.0 ±20.4</td>
<td>1248.9 ±129.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>25.5 ±3.4</td>
<td>28.0 ±3.4</td>
<td>16.9 ±1.7</td>
<td>23.1 ±2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>16.5 ±1.9</td>
<td>25.7 ±6.1</td>
<td>13.5 ±1.4</td>
<td>19.4 ±2.0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>451.1 ±49.8</td>
<td>616.9 ±131.5</td>
<td>354.3 ±8.2</td>
<td>508.7 ±56.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:4 n-6</td>
<td>190.9 ±21.2</td>
<td>307.5 ±81.5</td>
<td>151.1 ±2.7</td>
<td>216.7 ±24.4*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:5 n-6</td>
<td>19.3 ±2.4</td>
<td>22.7 ±3.8</td>
<td>16.2 ±0.9</td>
<td>25.1 ±3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum n-6a</td>
<td>716.4 ±79.0</td>
<td>1016.3 ±227.5</td>
<td>561.1 ±13.2</td>
<td>803.0 ±88.3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>37.2 ±3.6</td>
<td>68.6 ±21.7</td>
<td>25.6 ±0.7</td>
<td>32.9 ±4.3†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>580.0 ±65.9</td>
<td>851.0 ±202.1</td>
<td>497.1 ±11.8</td>
<td>697.9 ±76.0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum n-3a</td>
<td>640.4 ±72.6</td>
<td>945.5 ±223.1</td>
<td>542.7 ±9.0</td>
<td>756.3 ±81.0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>1.12 ±0.01</td>
<td>1.09 ±0.02</td>
<td>1.03 ±0.02</td>
<td>1.06 ±0.01†‡</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Males: RV n = 9, HV n = 7; females: RV n = 4, HV n = 7.
P less than .05 for *gestational diet effect, †sex of the pup effect, ‡gestational diet × sex interaction, by 2-way ANOVA.
a Includes other minor FAs not reported.
Chapter 5. High Multivitamin Intakes during Pregnancy and Post-Weaning Obesogenic Diets Interact to Affect the Relationship between Expression of PPAR Genes and Glucose Regulation in the Offspring

5.0. Abstract

High multivitamin intake (HV) during pregnancy increases body fat and weight and alters glucose and fatty acid metabolism in Wistar rat offspring. This study investigated the expression of peroxisome-proliferator activated receptors (PPARs) genes involved in regulation of glucose and fatty acid metabolism in their tissues. Dams received the AIN-93G diet with either the regular (RV) or 10-fold multivitamins (HV) during pregnancy. Male offspring were weaned to either the RV-diet (RV-RV and HV-RV) or to an obesogenic diet (RV-Ob and HV-Ob). Gene expression in tissues from offspring at birth, weaning, and 14 wk post-weaning were analyzed by real time RT-PCR. Gestational diet (GD) did not affect PPARs gene expression in offspring at either birth or weaning. In liver, at 14 wk post-weaning, PPAR-γ was 30% lower in the HV-RV and 30% higher in HV-Ob, than in the RV-RV group (GD p=0.76, PD p=0.19, interaction p=0.02, by 2-way ANOVA). In muscle, PPAR-α expression was affected by both gestational and post-weaning diets (GD=0.05, PD=0.01, interaction p=0.32). In adipose tissue, PPAR-α gene expression was higher in all groups compared to RV-RV (GD=0.25, PD=0.85, interaction=0.03). PPAR-γ mRNA levels were directly correlated with abdominal fat (r=0.45, p<0.05) and insulin resistance index (r=0.39, p<0.05). In the liver, PPAR-γ mRNA were inversely correlated with insulin resistance index in offspring from RV (r=0.61, p<0.05), but not in those from HV-dams (r=0.13, p=N.S). In conclusion, the HV-diet during pregnancy interacts with post-weaning diets in determining the expression of PPARs genes in a tissue and age dependent manner and uncouples the relationship between these genes and glucose regulation and abdominal fat mass in the offspring of Wistar rats.
5.1. Introduction

Maternal nutrition during pregnancy plays a fundamental role in fetal development and growing evidence suggests a linkage to later outcomes in life associated with the risk of metabolic disturbances in the offspring [3]. Whereas altered metabolic responses due to gestational nutrient-deficient diets are commonly recognized [7], nutrient excess during pregnancy also has adverse consequences in offspring and increases their risk for chronic diseases [485]. As shown by animal studies, maternal overnutrition due to high-fat diet consumption, affected offspring responses to postnatal diets, resulting in higher body weight and alterations in glucose response in the offspring, effects that were amplified by feeding offspring a high-fat diet after weaning [11, 485, 486]. Other nutrients, such as vitamins, when consumed above recommendations during pregnancy also result in higher body weight and components of the metabolic syndrome in offspring of Wistar rats [32]. These effects were further amplified by feeding the offspring a liquid obesogenic diet after weaning and resulted in higher body weight, abdominal fat mass, as well as altered glucose homeostasis and tissue fatty acid composition [487, 488].

Glucose and fatty acid metabolism are partially regulated by nuclear transcription factors known as peroxisome-proliferator activated receptors (PPARs) [259]. Each PPAR isotype, namely PPAR-α, PPAR-β/δ, and PPAR-γ, has specific actions according to the tissue in which it is expressed, but in general PPAR-α and PPAR-β/δ regulate the expression of enzymes and transporters that promote fatty acid uptake and oxidation, whereas PPAR-γ is related to increased adipogenesis and insulin sensitivity [36]. Thus, metabolic disturbances may be related to changes in the gene expression of these transcriptional factors.

Gene expression of PPARs has been shown to be affected by obesity and dietary factors in different tissues of animal models. For example, gene expression of PPAR-γ is altered in cardiac tissue of obese lambs [489], and a high-fat post-weaning diet increases PPAR-γ expression in mouse liver and adipose tissue [490]. PPARs gene expression is also sensitive to the nutrient content of maternal diets, as exemplified by studies in sheep showing that a
global nutrient restriction results in higher expression of PPAR-α in adipose tissue of the lambs [261]. In rats, a protein restricted diet during pregnancy also increases the expression of PPAR-α in the offspring liver, but the expression of this gene was comparable to that from offspring of control mothers when the protein-restricted diet given to the dams was supplemented with folic acid [442]. However, whether the vitamin content of the maternal diet, without protein or energy restriction, alters the expression of PPARs in the offspring tissues is unknown.

Because we previously observed that a maternal HV diet followed by a post-weaning obesogenic diet results in alterations in glucose and fatty acid metabolism, the objective of this study was to investigate the effects of the HV-diet during pregnancy on gene expression of PPAR-γ, PPAR-β/δ and PPAR-α in adipose, liver and muscle and their relationship with fat mass and insulin resistance and fat mass in the male offspring at birth, weaning and when fed either a RV or obesogenic diet for 14 weeks post-weaning (PW).

5.2. Material and Methods

Animals and diets. All procedures were approved by the Animal Ethics Committee at University of Toronto. Twenty Wistar rats in the 2-3rd day of their first pregnancy were purchased from Charles Rivers (Quebec, Canada). Animals were housed individually in transparent cages with free access to water in facilities with controlled temperature (22±1°C) and illumination (12-hour dark-light cycle, lights on at 7:00 A.M.). The regular AIN-93G diet [446] with either the regular amount (RV), or 10 times the amount of vitamin mix (HV) and a liquid obesogenic diet (Ob) were used. The composition of these diets has been previously reported [488].

Experimental design. Dams were randomly allocated to receive either the RV or the HV-diet during pregnancy (gestational diet). During lactation, all dams received the RV-diet. The litters were culled to 10 animals per dam on post-natal day 1 and were weaned at post-
natal day 21. From weaning to 14 wk post-weaning the offspring (1 pup/dam) were fed either the RV-diet or the Ob-diet (post-weaning). Thus, after weaning, four groups of animals were formed: 1) From RV-dams, weaned to RV-diet (RV-RV); 2) from RV-dams weaned to Ob-diet (RV-Ob); 3) from HV-dams weaned to RV-diet (HV-RV); and 4) from HV-dams weaned to Ob-diet (HV-Ob).

**Tissue collection and analysis:** Tissues from the unsexed offspring at birth (liver), from males at weaning and 14 wk PW (liver, quadriceps muscle and abdominal fat) were collected, immediately frozen in liquid nitrogen, and kept at -80 ºC until analysis. Samples of tissues were homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) and mRNA isolated. After quantification using a UV-Agilent 8453, a High Capacity cDNA archive kit (Applied Biosystems Inc, Foster City, CA, USA) was used for cDNA synthesis, by incubating the mix for 10 min at 25ºC, followed by 120 at 37ºC in an ABI Gene Amp PCR System 2700. Real-time RT-PCR was performed using Taqman essays for the following genes: PPAR-α (Cat # Rn00566193_m1), PPAR- β/δ (Cat # Rn00565707_m1), PPAR- γ (Cat # Rn00440945_m1), glucokinase (GK, Cat# Rn00688285_m1), Carnitine-palmitoyl transferase 1 (CPT1, Cat #Rn00580702_m1) and glucose transporter 4 (Glut4, Cat# Rn00562597_m1) from Applied Biosystems, in the 7900HT Fast Real-Time RT-PCR System also from Applied Biosystems (Foster City, CA, USA). The cycle conditions were: 50ºC for 2 minutes, 95ºC for 10 minutes, 40 cycles for 95ºC for 15 seconds, and 60ºC for 1 minute. The relative quantification method was performed, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cat # Rn99999916-s1) as an endogenous control for liver samples and β-2-microglobulin for muscle and adipose tissue samples (Cat #4331182-Rn00560865-m1). Results are expressed as fold-change, obtained by the $2^{-\Delta\Delta CT}$ method [491] and using the mean of RV-RV group as the reference.

**Statistical analyses.** The effects of gestational diet and/or post-weaning diet were analyzed by unpaired t-test and general linear models. Two-way analysis of variance was used to analyze the effects of the gestational and post-weaning diets and their interaction on mRNA levels of the selected genes. Pearson correlation coefficients were calculated to
analyze the relationship between gene expression and the insulin resistance index (IRI, previously calculated as glucose * insulin plasma concentrations [32]), and abdominal fat mass. Correlation coefficients were calculated for the entire group of animals (pooled), as well as within each gestational and post-weaning diet groups. SAS v. 9.2. (SAS Institute Inc., Cary, NC, USA) was used for the statistical analysis of the data.

5.3. Results

Results describing the phenotypic characteristics of the offspring (body weight, glucose metabolism and fatty acid concentration of tissues) have been previously reported [32, 487, 488]. In brief, the gestational HV-diet did not affect litter size and birth weight, but resulted in greater weight gain after weaning and differences in fatty acid composition in different tissues than in offspring of dams fed the AIN-93G diet with the regular amount of vitamins [32, 487, 488]. At 14 weeks, male offspring of HV-dams also showed higher abdominal fat mass and higher IRI [32, 487]. In this study, we further analyzed the tissues for the expression for selected genes related to glucose and lipid metabolism.

Gene expression of PPARs in tissues from the offspring at birth and weaning did not differ by gestational diet (Table 5.1). However, mRNA levels of PPAR-β/δ in the liver of offspring at weaning were correlated with the IRI (r=0.73, p<0.01) in the whole group of animals (pooled data) and the direction of the association did not differ by gestational diet (RV r=0.6, p=0.08; HV r=0.78, p=0.01). The correlation between the expression of PPAR-α or PPAR-γ in this tissue and IRI was not significant (r=0.35, p=0.15; and r=0.04, p=0.88, respectively). Abdominal fat mass (g) was not correlated with the expression of any of the PPARs in the liver (PPAR-α r=0.15, p=0.60; PPAR-β/δ r=0.34, p=0.26; PPAR-γ r=0.39, p=0.16). Glucose concentrations, but not insulin or IRI, were negatively correlated with the expression PPAR-α (r=−0.52), PPAR-β (r=−0.55), and PPAR-γ (r=−0.57) in the muscle of the
entire group of animals at weaning but did not differ by gestational diet. mRNA levels of PPARs in adipose tissue were not correlated with any metabolic measures.

At 14 wk PW, the gestational diet did not have independent effects on the expression of any of the genes that were measured, except for PPAR-\(\alpha\) in muscle, in which HV-offspring showed lower levels than RV-offspring (\(p=0.05\)). The obesogenic post-weaning diet resulted in lower expression of CPT1 (<0.01) in the liver, higher PPAR-\(\alpha\) (\(p<0.01\)) in muscle and a trend towards higher PPAR-\(\gamma\) in adipose tissue (\(p=0.07\)). Gestational and post-weaning diet interactions were significant for the gene expression of PPAR-\(\gamma\) and PPAR-\(\alpha\) in the liver and adipose tissue, respectively (Table 5.2).

Table 5.3 shows the correlation between gene expression and abdominal fat and IRI for all the animals (pooled). mRNA levels of PPAR-\(\beta/\delta\) in the liver were inversely correlated with IRI. In contrast, expression of PPAR-\(\gamma\) in adipose tissue was directly correlated with the amount of abdominal fat and the IRI. An inverse correlation was also observed between the expression of CPT1 in the liver and the amount of abdominal fat and IRI (\(p<0.05\)).

Because differences by gestational and/or post-weaning diet were observed in the expression of PPAR-\(\gamma\) in the liver and in PPAR-\(\alpha\) in muscle and adipose tissue, we analyzed the relationship between either fat mass or IRI, within dietary groups (e.g. by gestational diet or by post-weaning diet). The correlation between IRI or abdominal fat mass and the expression of PPAR-\(\gamma\) in the liver was not statistically significant in the entire group of animals. However, in the subgroup of animals from RV-dams, the expression of PPAR-\(\gamma\) in the liver was inversely correlated with IRI (\(r=-0.62, p=0.04\)), but not in those from HV-dams (\(r=0.13, p=0.67\)). In contrast, in the offspring from HV-dams the expression of PPAR-\(\gamma\) in the liver was correlated with fat mass (\(r=0.51, p=0.04\)), but this correlation was not observed in animals from RV-dams (\(r=-0.34, p=0.21\)). Gene expression of PPAR-\(\alpha\) in adipose tissue was correlated with the IRI only in offspring from RV-dams (\(r=0.86, p<0.01\)) but not in those from HV-dams (\(r=-0.01, p=0.96\)) (Table 5.3).
mRNA levels of PPAR-γ in adipose tissue were correlated with IRI in the entire group (r=0.39, p=0.05). However, when analyzed by post-weaning diet, this correlation was significant only in those fed the RV-diet post-weaning (weaned to RV r=0.7, p<0.01; weaned to Ob-diet r=0.07, p=0.81). Gene expression of CPT1 in the liver was negatively correlated with abdominal fat mass in pups fed the obesogenic diet post-weaning (r=-0.5, p=0.04) but not in those fed the RV-diet (r=0.23, p=0.42), irrespective of the gestational diet. The correlation between CPT1 and IRI was stronger in offspring fed Ob-diet (r=-0.5, p=0.06) than in those from RV-diet post-weaning (r=0.26, p=0.48). PPAR-α gene expression in muscle was not correlated with IRI in the entire group of animals or within subgroups by gestational or post-weaning diet.

5.4. Discussion

The results suggest that the HV-diet during pregnancy and the post-weaning obesogenic diets affect gene expression of PPARs in a tissue and age dependent manner and modify their relationship with metabolic measures in the offspring.

Whereas the expression of these genes was found not significantly different in tissues from offspring at birth or at weaning, the response to a post-weaning dietary challenge (i.e. obesogenic diet) was dependent upon the vitamin content of the maternal diet during pregnancy, as suggested by the interactions between gestational and post-weaning diets on PPAR-γ in the liver and PPAR-α in adipose tissue, respectively.

Feeding pups an obesogenic diet increased PPAR-α gene expression in skeletal muscle, compared with offspring fed RV-diet post-weaning. This outcome may be due to two reasons. First, the expression of PPAR-α is sensitive to the macronutrient component of the diet (e.g. sucrose and saturated fat) in humans and rats [492] [493-495]; and second, it is also likely that the expression of this transcriptional factor is related to body weight and fat
mass in the offspring fed the obesogenic diet [494]. This is supported by the results showing higher expression of PPAR-α in animals fed the Ob- diet, which is higher in saturated fat and sucrose content than the RV-diet. In addition, these offspring were also heavier and had higher fat mass than their siblings weaned to the RV-diet [32, 487].

The post-weaning obesogenic diet also resulted in a trend towards higher expression of PPAR-γ in adipose tissue. This is probably related to the increased abdominal fat mass found in these animals [487] as it is known that PPAR-γ is involved in adipogenesis and lipid storage [496]. This is further supported by the significant correlation found between the amount of abdominal fat mass and the expression of PPAR-γ in this tissue (Table 5.3). Our results are consistent with those from other studies feeding a cafeteria diet that have resulted in a ~5-fold increase the gene expression of PPAR-γ in adipose tissue [497]. Our results show the same direction but the difference in mRNA levels was about ~20-30% by the post-weaning diet. This can be explained by the different composition of the diets (65 vs. 17% energy from fat) as it is also known that PPAR-γ is sensitive to the fat content of the diet [495].

The effects of gestational diet on gene expression were not independent, except for the lower expression of PPAR-α in muscle in offspring of HV-dams. The hepatic expression of PPAR-α in the offspring at day 6 PW has been found to be modified by the protein and folate levels of gestational diets. Whereas a protein-restricted diet led to a 10-fold increase in PPAR-α mRNA levels, the addition of folic acid (5-fold the amount than in the control group) prevented this increase [442]. In our experiment, the gestational diet containing 10-fold of multivitamins, including folic acid, did not alter the gene expression of PPAR-α in the liver at any age, but resulted in decreased PPAR-α mRNA levels in muscle from offspring of HV-dams at 14 wk PW, primarily in those weaned to the obesogenic diet.

The gestational diet modulated the effects of the obesogenic post-weaning diet as shown by the significant interactions between gestational and post-weaning diet on gene expression (Table 5.3). Interactions between gestational and post-weaning diets have been previously
reported. For instance, the effects of maternal overnutrition and post-weaning diets have been shown to be additive when both dams and offspring are fed a high-fat diet [498]. Similarly, we have previously reported that the post-weaning obesogenic diet exacerbates the effects of feeding high-multivitamin diet during pregnancy on body weight, food intake and metabolic measures [487]. Thus, our results are consistent in showing an interaction between the gestational and post-weaning diet that affects the phenotype of the offspring.

Because of their widespread effects on different tissues, changes in PPAR expression may be related to the metabolic regulation in the offspring. We explored the relationship between the mRNA levels of PPARs and the abdominal fat mass and insulin resistance index as illustrated in figure 5.1. The gestational diet modified the relationship between gene expression of PPAR-γ and the insulin resistance index and fat mass of the offspring over a wide range of mRNA levels. Although the expression of PPAR-γ in the liver has been reported to be about 50-fold lower than that in adipose tissue, it plays an important role in improving insulin sensitivity [499]; thus a lower expression is expected to result in decreased insulin sensitivity, which is one of the characteristics observed in the offspring of dams fed the HV-diet during pregnancy [32]. Our findings are in line with these outcomes, since we observed that in offspring from RV-dams the mRNA levels of PPAR-γ in the liver were inversely correlated with the IRI. Conversely, in offspring from HV-dams, this correlation was not statistically significant. This may suggest a disruption in the downstream response to PPAR-γ regulating glucose metabolism in the offspring of HV-dams when challenged with Ob-diet post-weaning. Furthermore, the strong correlation between IRI and PPAR-α mRNA in adipose tissue only in the offspring of dams fed the RV but in those from the HV-diet also suggests an effect of the gestational diet on the relationship between the PPARs gene expression and glucose metabolism, in a tissue-dependent manner. However, the specific pathways that could be affected remain to be determined.

We did not establish a correlation between lipid metabolism and gene expression of PPARs because fatty acid concentrations were measured in offspring fed obesogenic diet only [488]. However, the decreased mRNA levels of PPAR-α in the offspring of HV-dams are in line
with previous findings that male offspring from HV-dams weaned to an obesogenic diet have increased concentrations of fatty acids in skeletal muscle at 12 wk PW when compared with offspring from RV-dams [488]. Whether these results are similar in offspring weaned to the RV-diet or in female offspring is currently unknown.

In conclusion, the HV-diet during pregnancy interacts with post-weaning diets in determining the expression of PPARs genes in a tissue and age dependent manner and uncouples the relationship between these genes and glucose regulation and abdominal fat mass in the offspring of Wistar rats.
Table 5.1. Gene expression of PPARs on tissues from offspring at birth (unsexed) and males at weaning

<table>
<thead>
<tr>
<th>Gene</th>
<th>From RV-dams</th>
<th>From HV-dams</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIRTH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>1.0±0.1</td>
<td>0.8±0.2</td>
<td>0.36</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>1.0±0.1</td>
<td>0.9±0.1</td>
<td>0.27</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>1.0±0.1</td>
<td>1.2±0.2</td>
<td>0.34</td>
</tr>
<tr>
<td>WEANING</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>1.1±0.2</td>
<td>1.5±0.2</td>
<td>0.24</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>1.0±0.1</td>
<td>1.2±0.2</td>
<td>0.51</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>1.2±0.2</td>
<td>1.5±0.2</td>
<td>0.39</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>1.3±0.3</td>
<td>1.6±0.5</td>
<td>0.63</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>1.1±0.2</td>
<td>1.3±0.2</td>
<td>0.66</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>1.1±0.2</td>
<td>1.7±0.4</td>
<td>0.26</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>1.1±0.3</td>
<td>0.9±0.1</td>
<td>0.48</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>1.1±0.2</td>
<td>1.2±0.1</td>
<td>0.57</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>1.2±0.3</td>
<td>1.7±0.3</td>
<td>0.26</td>
</tr>
</tbody>
</table>

RV=From dams fed AIN-93G regular diet HV=From dams fed HV-diet (AIN-93G + 10-fold vitamin mix), n=7-10 per group, except adipose tissue n=5 per group. Results are presented as fold change with RV group as reference. *unpaired t-test
Table 5.2. Gene expression of male offspring 14 wk PW in selected tissues

<table>
<thead>
<tr>
<th>Gene</th>
<th>From RV-dams Mean ± SEM</th>
<th>From HV-dams Mean ± SEM</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From RV-dams Ob</td>
<td>From HV-dams Ob</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RV</td>
<td>Ob</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>1.1 ± 0.05</td>
<td>1.1 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 ± 0.02</td>
<td>1.2 ± 0.08</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>1.0 ± 0.03</td>
<td>0.9 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.9 ± 0.02</td>
<td>0.9 ± 0.03</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>1.0 ± 0.03</td>
<td>0.9 ± 0.06</td>
<td>0.7 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>1.3 ± 0.04</td>
<td>0.7 ± 0.02</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.19</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td>CPT1</td>
<td>1.0 ± 0.01</td>
<td>0.6 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.9 ± 0.03</td>
<td>0.7 ± 0.02</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td>GK</td>
<td>1.1 ± 0.07</td>
<td>0.7 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 ± 0.06</td>
<td>1.0 ± 0.07</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>1.4 ± 0.22</td>
<td>4.5 ± 0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.3 ± 0.15</td>
<td>2.1 ± 0.24</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>1.2 ± 0.10</td>
<td>1.1 ± 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 ± 0.03</td>
<td>1.2 ± 0.06</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.48</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>1.0 ± 0.05</td>
<td>1.3 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 ± 0.03</td>
<td>1.2 ± 0.06</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.56</td>
</tr>
<tr>
<td>GLUT4</td>
<td>1.1 ± 0.07</td>
<td>1.3 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 ± 0.04</td>
<td>1.3 ± 0.07</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>Adipose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>1.0 ± 0.06</td>
<td>1.7 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0 ± 0.12</td>
<td>1.4 ± 0.05</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>1.1 ± 0.04</td>
<td>0.8 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8 ± 0.02</td>
<td>0.8 ± 0.03</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>1.0 ± 0.04</td>
<td>1.3 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 ± 0.05</td>
<td>1.3 ± 0.02</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td>GLUT4</td>
<td>1.2 ± 0.13</td>
<td>1.5 ± 0.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.8 ± 0.15</td>
<td>1.3 ± 0.09</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.30</td>
</tr>
</tbody>
</table>

RV=From dams fed AIN-93G regular diet HV=From dams fed HV diet
Pups were fed either RV or Ob-diet (liquid obesogenic)
Results are presented as fold-change with RV-RV group as reference

*2-way ANOVA, with gestational diet (GD), post-weaning diet (PD) and their interaction as factors, n=7-9 per group
Table 5.3. Correlation between PPARs gene expression and fat mass and insulin resistance index at 14 wk PW, in all animals and by gestational diet group

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abdominal fat (g)</th>
<th>Insulin resistance index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALL</td>
<td>From RV</td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>-0.10</td>
<td>-0.09</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>-0.3†</td>
<td>-0.34</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>0.15</td>
<td>-0.34</td>
</tr>
<tr>
<td><strong>Muscle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>0.28</td>
<td>0.31</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>-0.03</td>
<td>-0.43</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>0.16</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Adipose tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAR-α</td>
<td>0.10</td>
<td>0.39</td>
</tr>
<tr>
<td>PPAR-β/δ</td>
<td>-0.30</td>
<td>-0.20</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>0.45*</td>
<td>0.34</td>
</tr>
</tbody>
</table>

r=Pearson correlation coefficient including all four groups. n=7-9 per group

*p<0.05   †p<0.10
**Figure 5.1.** PPARs mRNA levels in the liver and adipose tissue and insulin resistance index and fat mass in male offspring at 14 weeks post-weaning, by gestational diet

A. PPAR-γ gene expression in the liver and insulin resistance index (PPAR-γ p=0.14, gestational diet p=0.28, interaction=0.057, by PROC GLM), n=7-9 per group.

RV r=-0.62, p=0.04
HV r=0.13, p=0.67

B. PPAR-γ gene expression in the liver and abdominal fat mass (PPAR-γ p=0.72, gestational diet p=0.05, interaction p=0.03, by PROC GLM), n=7-9 per group

RV r=-0.34, p=0.21
HV r=0.51, p=0.04

C. PPAR-α gene expression in adipose tissue and insulin resistance index (PPAR-α p=0.03, gestational diet p=0.01, interaction p=0.02, by PROC GLM), n=7-9 per group

RV r=0.86, p<0.01
HV r=-0.01, p=0.96
Chapter 6. High Vitamin Intakes During the First Pregnancy Affect the Dams and their Offspring through Two Pregnancies

6.0. Abstract

A diet with a 10-fold increase in multivitamins consumed by Wistar rats during pregnancy results in obesity and characteristics of metabolic syndrome in the offspring. **Objective:** To investigate the effect of feeding a high multivitamin diet (HV) only during the first pregnancy on body weight, food intake, fat mass and glucose metabolism in both the dams and their male offspring after their first (L1) and second (L2) pregnancies. **Design:** Wistar rats were fed either the HV-diet or the regular (RV) AIN-93G diet during their first pregnancy only. They were maintained on the RV-diet and mated again at 12 wk post-weaning. **Results:** HV-dams gained more weight after both their first (30% more) and second (3-fold) pregnancies than the RV-dams (p<0.05). The HV-diet during the first pregnancy resulted in 3% and 11% higher weight gain (weaning to 15 wk post-weaning) in males from L1 and L2, respectively (MD p=0.04, parity p=0.01, interaction p=0.10). Those from L2 had 30% higher plasma concentrations of leptin at weaning and 16 wk post-weaning. Hypothalamic gene expression of pro-opiomelanocortin (POMC) was 30% higher in L1 at weaning in offspring from HV-dams. POMC and neuropeptide Y were 50 and 30% higher in L2 at 16 wk PW in offspring of HV-dams. Blood glucose and insulin concentrations at weaning were higher in both L1 and L2 offspring of dams fed the HV-diet, but they were lower (p<0.02) in L2 than in L1. In conclusion, consumption of the HV-diet only during the first pregnancy increases body weight and food intake in the dams and promotes characteristics of the metabolic syndrome in both their first and second litter male offspring.
6.1. Introduction

Diet and parity during pregnancy play fundamental roles in fetal development in humans and animals [1, 54]. Diets containing either inadequate or excessive amounts of energy and nutrients alter the development of fetal tissues and energy and nutrient metabolism [8, 122, 243, 433, 500], leading to higher body weight and increased risk for chronic diseases later in life [60, 501]. However, these effects vary according to the nutrients involved [128, 168]. For instance, animal studies have shown that feeding dams high-protein diets during pregnancy increased body weight, adiposity [500] and blood pressure in a sex-dependent manner [10], whereas high-fat diets resulted in glucose intolerance [147], higher insulin [433] and leptin concentrations [486], and increased hypothalamic expression of genes involved in energy homeostasis in the offspring [104]. Similarly, increased multivitamin content of diets consumed during pregnancy by Wistar rats leads to increased food intake and characteristics of the metabolic syndrome in the adult offspring. These effects were only observed in male offspring when they were weaned to a regular AIN-93G diet [32]. However, the maternal high multivitamin intake during pregnancy increased body weight and impaired in glucose metabolism in both male and female offspring when they received an obesogenic diet post-weaning [33].

Parity has also been associated with the risk of obesity in the offspring in human and animal studies [162, 502]. However, the direction of this association has not been consistent. For instance, increased risk of late-onset obesity (i.e. after 8 year old) was observed in second or third-born U.S. children [502]. In contrast, a higher risk of becoming overweight was reported in first-born girls than their middle siblings in Japan [503]. Similarly, in animal studies, lambs (within 1 month of age) from multiparous ewes were found to have less adipose tissue mass, lower leptin concentrations, and lower gene expression of the glucocorticoid receptor in adipose tissue [504] and of growth factor receptors in the liver than lambs from first-time pregnant ewes, independent of gestational diet [505].
Parity influences maternal physiological adaptations to pregnancy. Pregnancy induces changes in plasma concentration of hormones such as prolactin, insulin and leptin and their response in target organs [169, 506-509]. These changes result in transient leptin and insulin resistance, allowing a positive energy balance and redirection of nutrients for maternal weight gain and fetal growth [510, 511]. However, these physiological responses are different depending on maternal parity. For example, increases in plasma concentrations of leptin during pregnancy are less pronounced in multiparous than in nulliparous mothers [512, 513] but insulin concentrations are higher [513] which may account in part for differences in fetal growth and development [184].

Despite their known independent effects, the interaction of maternal diet and parity on offspring outcomes is unclear. Epidemiological data indicate that the effects of maternal undernutrition differ by parity in humans. First borns were heavier and second born infants were approximately 100g lighter than controls when the mother was exposed to famine during the first trimester of pregnancy [514]. In contrast, in sheep, the effects of parity and maternal undernutrition during late pregnancy on offspring outcomes were found to be independent [505]. Weight gain during previous pregnancies may also play a role in modifying the phenotype of subsequent offspring, but there are very limited data [515]. However, whether dietary factors during pregnancy have long-term effects for the mother and the subsequent offspring is unknown. Therefore, the objective of this study was to investigate the effect of feeding a high multivitamin diet only during the first pregnancy on body weight, food intake, fat mass, glucose metabolism and expression of genes regulating food intake in the hypothalamus in both the dams and their male offspring after their first and second pregnancies.
6.2. Material and Methods

Animals and diets. All procedures were approved by the Animal Ethics Committee at University of Toronto. Nineteen Wistar rats on day 2-3 of their first pregnancy were purchased from Charles Rivers (Quebec, Canada). Animals were housed individually in transparent cages with free access to water in facilities with controlled temperature (22±1°C) and illumination (12-hour dark-light cycle, lights on at 7:00 A.M.). Nineteen Wistar males (12 wk-old) were also purchased from the same provider. The regular AIN-93G diet [446] with either the regular amount (RV), or 10 times the amount of vitamin mix (HV) was used, as reported previously [32].

Experimental design. Dams were randomly allocated to receive either the RV or the HV-diet (RV n=10, HV n=9) during only the first pregnancy. After delivery, dams were maintained on the RV-diet and the pups from both litters were fed only the RV-diet. After 12 wk post weaning (PW), the dams were mated with the 12 week-old males purchased from the same provider (one male per dam). Sixteen dams (RV n=9, HV=7) were pregnant for second time. They all received the RV-diet during the second pregnancy. Two dams from the RV group were terminated shortly after weaning, leaving 7 dams per dietary group. Body weight was recorded within 24 hours after delivery, at weaning (21st day after delivery) and then weekly up to 20 wk PW of their second litters. Following the second litters, their total weekly food intake was measured from the week PW2-14. One-hour food intake was measured after overnight fasting at 18 wk PW. A glucose tolerance test (GTT) was conducted at 6 wk PW. The dams were terminated by decapitation at 20 wk PW for blood and tissue collection.

Within the first day after delivery, offspring were counted, weighed and litters were culled to 12 pups/dam. Eight males per group were terminated at weaning, 1-2 males/dam were kept for follow up, and the rest were used for separate experiments. Males from L1 (n=11/dietary group) and L2 (n=14/dietary group) were weaned to the RV-diet and body weight and food intake were recorded weekly for 15 weeks (L2). The first litters were terminated at 35
weeks, as previously described (Szeto et. al. JDOHaD 2011, *In press*) and the second litters at 16 weeks post-weaning for blood and organ collection.

Glucose tolerance tests (GTT) were performed at 5, 9, and 13 weeks in the pups. A single dose (0.5 g/kg body weight) of glucose (Sigma-Aldrich Cat # G-5767, St. Louis, MO) solution was gavaged after overnight fasting. A blood sample was withdrawn from the capillary bed of the tail tip at fasting and again at 15, 30, and 60 minutes after the gavage.

Blood glucose, insulin and leptin, were measured in a subset of males terminated at weaning and in males from L2 at 16 wk PW 30 min after water or glucose gavage, and in the dams (fasting) at 20 wk PW L2. Blood glucose was measured using a commercial glucose oxidase kit (MediSense® Precision Xtra TM, Alameda, CA). The glucose incremental area under the curve was calculated as in previous studies [32]. Insulin and leptin were measured using a Milliplex map Kit (Rat Gut Hormone panel, cat#RGT-88K, Millipore Corporation, Billerica, MA). Corticosterone concentrations in plasma of the L2 and of the dams were analyzed by radioimmunoassay (MP Biomedicals, Cat#07-120103, Orangeburg, NY).

*Tissue collection and hypothalamic gene expression: Fat pads from the abdominal cavity were collected from the dams and L1 and L2 males at weaning and from L2 at 16 wk PW. Brains from the dams and the male offspring at weaning (L1 and L2) and 15 PW (L2) were removed and immediately frozen. The hypothalami were excised, homogenized in Trizol reagent (Invitrogen, Cat#15596-026, Carlsbad, CA) and mRNA isolated following the protocol from the manufacturer. After quantification using a UV-Agilent 8453, a High Capacity cDNA Archive Kit (Applied Biosystems Inc, Foster City, CA) was used for cDNA synthesis (High Capacity cDNA Archive Kit Protocol from Applied Biosystems), by incubating the mix for 10 min at 25°C, followed by 120 min at 37°C in an ABI Gene Amp PCR System 2700. Real-time RT-PCR was performed using Taqman assays for the following genes: Leptin receptor, LepR (Cat # Rn01433205-m1); pro-opiomelanocortin, POMC (Cat # Rn00595020-m1); Agouti-related peptide, AgRP (Cat# Rn01431703-g1); and neuropeptide Y, NPY (Cat # Rn01410146-m1), obtained from Applied Biosystems, on the
7900HT Fast Real-Time RT-PCR System also from Applied Biosystems (Foster City, CA). The cycle conditions were: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles for 95°C for 15 seconds, and 60°C for 1 minute (35). The relative quantification method was performed, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cat # Rn99999916-s1) as an endogenous control. Results are expressed as fold-change, obtained by the $2^{-\Delta\Delta CT}$ method (36) and using the mean of RV-diet from L1 as the reference group.

Statistical analyses. To analyze results from the dams, unpaired t-test was used to compare group means of food intake, 1-hour food intake, glucose tolerance test, hormone plasma concentration, abdominal fat mass and hypothalamic gene expression. The PROC MIXED procedure (repeated measures) was used to analyze the weight gain from delivery to 12 weeks post-weaning, with parity and diet as the main factors. Body weight and food intake were also analyzed by PROC MIXED for repeated measures for the entire follow up period (up to 12 wk PW the first litters, and 20 wk PW the second litters). Results from the offspring at birth, weaning were analyzed by PROC GLM, with maternal diet and parity as the main factors. For the analyses of glucose and hormone plasma concentrations, the gavage condition (water/glucose) was also included as a factor. When two animals per dam were kept for follow up, their average on the post-weaning measures was obtained. Then, only one value for each litter was used for further analyses. The effects of parity, maternal diet (and gavage when appropriate) on food intake and weight gain were analyzed by PROC GLM. Variables with non-normal distribution were transformed to their natural logarithm and geometric means are presented as indicated in their respective table. The PROC MIXED procedure was used for analyses of repeated measures of body weight, glucose response and food intake data, as well as for the results of the glucose tolerance test to include the age of the animal in the model. Measures of gene expression were compared by t-test or PROC GLM. However, when the gene expression measures or their transformed values did not show normal distribution non-parametric tests were used (Mann-Whitney or Kruskal-Wallis test). Pearson correlation was used to establish the relationship between selected variables.
Statistical significance was declared at p<0.05. SAS v. 9.2. (SAS Institute Inc., Cary, NC) was used for the statistical analysis of the data.

6.3. Results

Dams

Body weight and food intake. As shown in Figure 6.1, weight gain of dams fed the HV-diet during the first pregnancy was approximately 30% higher after the first and 3-fold higher after the second pregnancy than in the RV group, from delivery to 12 wk PW (MD p=0.036; parity p<0.01; interaction p=0.48). Despite not showing significant differences by dietary group in their body weights at delivery (L1: RV 288±8 g, HV 290±9, p=0.87; delivery of L2: RV 411±21 g, HV 410±20 g, MD p=0.95, parity p<0.01; interaction p=0.94) or at weaning (L1: RV 279±7, HV 277±9 g; at weaning L2: RV= 353±13 g, HV 349±13, MD p=0.85, parity p<0.01, interaction p=0.91), the post-weaning body weight of dams increased more rapidly in those initially fed HV-diet from weaning to 12 wk PW L1 and up to 20 wk PW L2 (PW L1: Diet p=0.37, time p<0.01, interaction p=0.01; PW L2: diet p=0.48, time p<0.01, interaction p=0.004). Consistent with the weight gain, food intake of dams fed the HV-diet during the first pregnancy was, on average, 5% higher after weaning the second litters (Table 6.1.).

Metabolic measures. Exposure to HV-diet during the first pregnancy did not affect glucose response of the dams after the second pregnancy (6 weeks after weaning L2). Similarly, it did not affect plasma concentrations of corticosterone (p=0.98), insulin (p=0.44) or leptin (p=0.34). Insulin and leptin concentrations were correlated with body weight (r=0.6 and r=0.9, p<0.05, respectively) and with abdominal fat pad mass (r=0.7 and r=0.9, p<0.05, respectively). The maternal diet did not affect gene expression of leptin receptor, POMC, AgRP or NPY in the hypothalamus of the dams 20 weeks after weaning L2 (Table 6.1.).
Litters

Litter size was not affected by maternal diet during the first pregnancy or parity (MD p=0.19, parity p=0.10, interaction p=0.40) (Table 6.2.). Birth weight was lower in L2 pups from dams previously fed the HV-diet (MD p=0.01, parity p=0.06, interaction p=0.35), but weaning weight was not affected (MD p=0.84, parity p=0.22, interaction p=0.47). However, in the males terminated at weaning, abdominal fat content was 20% higher in L1 than in L2, irrespective of the maternal diet (MD p=0.4, parity p=0.03, interaction p=0.96). At 16 wk PW L2 offspring had higher abdominal fat if born to dams from the HV-diets (RV 5.8±0.3, HV 7.3±0.3, p<0.01). The HV-diet during the first pregnancy resulted in 3% and 11% higher weight gain in L1 and L2, respectively from weaning to 15 wk PW (MD p=0.04, parity p=0.01, interaction p=0.10) (Table 6.2.).

Average of daily food intake was higher from 11 to 15 wk PW (MD p=0.01) in offspring from dams fed the HV-diet. In L1 those from HV-dams consumed 6% more food than from RV-dams. This difference was 13% in L2. In the overall period from 2-15 wk PW, offspring from HV-dams tended to have higher food intake and L1 tended to consume more food (MD p=0.06, parity p=0.07, interaction p=0.37) (Table 6.2.).

Metabolic measures. Fasting blood glucose at 5, 9 and 13 wk was affected by time (p<0.01), maternal diet (p=0.05) and parity (p=0.06) with a significant time by parity interaction (p<0.05). Fasting blood glucose was 7-8% higher in offspring from dams fed the HV-diet in L1. In L2, only at 5 wk PW HV-males had higher values of fasting glucose. The glucose iAUC was higher in L2 than in L1 (parity p=0.05) independently of animal age or maternal diet (Table 6.3.).

Blood glucose and insulin concentrations following gavage with glucose solution were higher at weaning in all the animals than in those gavaged with water (p<0.05). However, glucose concentrations were higher and insulin values were lower in L1 than in L2 (p<0.01), thus the glucose/insulin ratio was more than 2-fold higher in the first litters than in L2 (parity p<0.01). Maternal diet was not a significant factor. Maternal diet affected leptin
concentrations. Offspring from the dams fed the HV diet had a higher concentration of leptin (p=0.02), which was more apparent in L2 (p<0.01) which had much higher leptin concentrations than L1 (Table 6.4.). Plasma concentrations of corticosterone in L2 offspring at weaning were not affected by maternal diet during the first pregnancy (RV=205±38, HV 226 ±46 ng/ml, p= 0.73).

At 16 wk PW, L2 males from HV-dams had higher plasma insulin (p=0.04) and leptin (p=0.056) concentrations than those from RV-dams. As expected, glucose gavage increased blood glucose (p<0.05), insulin (p<0.01) and decreased the glucose/insulin ratio (p<0.01). An interaction (p=0.03) between maternal diet and composition of the gavage was explained by a much larger increase in leptin concentrations following glucose gavage in the offspring from RV-dams, and by the much higher concentration in those from HV-dams in both water and glucose-gavaged animals (Table 6.5.).

Glucose and insulin were not measured in L1 at 16 wk PW. However, at 30 wk PW L1 blood glucose concentrations were higher in the offspring from HV-dams before (RV=4.9±0.1, HV=5.4±0.3 mmol/L, p=0.01) and 30 min (RV=7.7±0.4, HV=10.8±0.7, p<0.01) after glucose gavage. No differences were observed in plasma insulin concentrations (fasting RV=1.8±0.3, HV=2.4±0.3, p=0.17; 30 min RV=7.0±0.9, HV=8.3±0.6 ng/ml, p=0.25). The glucose/insulin ratio was not different by maternal diet (fasting RV=2.8 ±0.4, HV=2.5±0.5, p=0.65; 30 min RV=1.2±0.2, HV=1.4±0.3, p=0.62).

Gene expression. In the offspring at weaning, the maternal diet did not have independent effects on hypothalamic gene expression. However, the offspring from L2 had higher expression of leptin receptor, NPY and POMC compared to L1 (parity p<0.05). A trend towards an interaction between MD and parity was observed in POMC. Therefore, we compared the effects of the MD on the expression of this gene in each litter. The offspring of HV-dams showed significantly higher expression of POMC (p<0.05) than those from RV-dams in L1 only, but this effect of the MD was not observed in L2 (Table 6.6.).
The expression of POMC and NPY was higher in the offspring of dams fed the HV-diet during the first pregnancy (p<0.05) and in the offspring of L2 at 16 wks post-weaning. (Figure 6.2.). Hypothalamic gene expression (in fold-change from L1-RV group) of L1 males at 35 wk PW was not altered by the maternal diet (Leptin receptor RV=1±0.05, HV=0.9±0.04, p=0.27; POMC RV=1±0.08, HV=1.1±0.13, p=0.79; AgRP RV=1.1±0.17, HV=1.4±0.15, p=0.26; NPY RV=1±0.05, HV=0.9±0.05, p=0.14).

6.4. Discussion

The results of this study show that the composition of the diet consumed only during the first pregnancy has sustained effects after the first and second pregnancies in the offspring and the dams. The HV-diet during the first pregnancy, independently of parity, affected weight gain, and food intake in the offspring and these effects were enhanced in the second litters. Parity affected glucose metabolism and modified the effects of the HV-diet on leptin concentrations and hypothalamic gene expression. In the dams, while the HV-diet increased body weight after weaning the first and second litters, no effects on metabolic measures or gene expression in the hypothalamus were found.

Consistent with the present results, previous research from our group has shown that rats born to dams fed a high multivitamin diet during pregnancy exhibited higher weight gain, food intake, and altered glucose metabolism when fed either the regular AIN93-G or obesogenic diets [32, 487]. A novel observation of the present study was that the effects of the maternal HV-diet during the first pregnancy were observed in the second litters even though the dams were then maintained on RV-diet. This suggests that the HV-diet fed to the dams only during the first pregnancy may have carry-over effects that affect subsequent litters.
In contrast to the first litters in previous studies [32, 487], the second offspring from the HV-dams were lighter at birth and this may be a factor in their more rapid weight gain than in the first litters. Lower birth weight followed by a rapid catch up growth has been related to obesity and chronic diseases later in adulthood [516, 517] which may partially explain their faster weight gain after weaning. The further development of overweight in L2 from HV-dams may also be as a result of the increased maternal body weight at conception, in line with rodent studies showing that preconceptional maternal obesity results in increased food intake, body weight and altered glucose metabolism in adult offspring [90, 154].

Because leptin is produced by adipose tissue, its concentrations are correlated with fat mass in adult animals. However, this correlation is not observed in animals at early life [518, 519]. Similarly, our results showed that leptin concentrations were not correlated with abdominal fat mass in offspring at weaning. This observation is consistent with results from other studies that had shown that leptin plasma concentrations during early postnatal life are not correlated with body weight or food intake, but the mechanisms regulating this postnatal leptin surge are still poorly understood [519, 520]. However, in L2 at 16 wk PW, the increased leptin concentrations found in offspring from HV-dams was consistent with the increased fat mass also found in these animals.

The effects of parity have been noted in other animal models. Although there are obvious differences between animal models, our results are consistent with those from other studies showing that parity affects the development of the offspring. Similar to our results, studies in sheep have reported the effect of parity status of ewes on fat mass of lambs in early life, being lower in offspring within 1 month of age from multiparous ewes [504, 505]. Also in sheep, parity has been reported to affect gene expression of growth factors in the liver, in which lambs from primiparous ewes exhibit higher gene expression [505].

The HV-diet resulted in higher weight gain of the dams after delivering both the first and second litters, probably as a result of their increased food intake. Increased weight gain in the dams fed the HV-diet has been shown after weaning the first litters (Reza-Lopez et al.
2011, unpublished). Similar to the weight gain and increased food intake in the L2 offspring, the effects of the HV-diet fed only during the first pregnancy on body weight gain persisted after the second pregnancy also in the dams, despite having similar weight at delivery and at weaning. After the second pregnancy, HV-dams were consuming more food, even after adjusting for body weight. However, their higher food intake could not be attributed to plasma concentrations of insulin and leptin, or expression of the hypothalamic genes related to food intake regulation measured 20 wk post-weaning, because they were not different between the two groups. In this experiment, hormone concentrations were not measured at earlier stages in order to minimize stress on the dams and thus impact on outcomes in L1 and L2 offspring. However, the HV-diet also resulted in increased ghrelin concentrations in the dams at weaning (Reza-Lopez et al. 2011, unpublished).

Both groups of dams were heavier after the second pregnancy, consistent with the known effect of parity on maternal weight in animals and humans [521-523]. In this study, the higher maternal weight gain in the HV-groups was independent of the parity effect. This observation is in line with the results of cohort studies in humans. Parity is an important predictor of overweight later in life in women from developed countries [524] but the role of the maternal diet during pregnancy in their long-term weight gain has not been reported.

Maternal diet during the first pregnancy also interacted with the effects of parity in the offspring. Evidence for an interaction between these two factors was observed in the plasma concentrations of leptin and the hypothalamic expression of POMC in the offspring. Plasma concentrations of leptin did not differ by maternal diet in the first litters, they were higher in the second litters, and were the highest in L2 born to HV-dams, suggesting that the HV-diet received by the dams during the first pregnancy modulated the effects of parity on the offspring. In contrast, the gene expression of POMC at weaning was increased by the HV-diet only in the first litters and although the second litters also had increased POMC values, they were not affected by maternal diet when measured at weaning. However, POMC mRNA levels were higher in L2 from HV-dams at 16 wk PW, but not in L1 when measured at 35 wk PW.
The carryover effect of the diet during the first pregnancy on the phenotype of the second litters is intriguing. Previous pregnancy outcomes have been associated with outcomes from subsequent pregnancies. However, only one study has illustrated this relationship by analyzing data from two subsequent pregnancies in women. The results of this epidemiological study showed that the excess weight gain during the first pregnancy significantly affected the birth weight of the subsequent one, an association that remained significant even after adjusting for maternal age and body mass index by the time of the second pregnancy [515]. In our rat study it was clear that characteristics of both dams and offspring differed in the long-term by the high vitamin content of the diet given to the dams only during the first pregnancy.

Several mechanisms have been proposed to explain the influence of the gestational diets on their long-term health status of offspring, including the influence of glucocorticoids and modifications in DNA methylation patterns that alter gene expression [224, 525, 526]. However, corticosterone concentrations in the offspring were not affected by the HV-diet in the offspring, as previously reported [32] nor in the second litters or the dams in the present experiment. Because of the known role of several vitamins in DNA methylation pathways and because a higher hypothalamic gene expression was found in L1 at weaning, changes in DNA methylation patterns could have occurred as a consequence of the HV-diet.

Our study had several limitations to take into consideration. First, we did not obtain metabolic measures of the dams during pregnancy and lactation that could have shed light on the effects of the high multivitamins on the hormonal status of the offspring. However, this prevented increased stress of the dams due to handling procedures. Second, because not all dams became pregnant a second time, the number of pups from the second litters was reduced. However, despite the small sample size (e.g. 6-9 per group) for several measures, and in particular those involving previous gavage, we were able to detect significant differences due to both maternal diet and parity. Third, because the primary objective of the previously reported study (Szeto et. al. J DOHaD 2011, In press) was to investigate the effect of the mismatch between gestational and pup diets, the experiment was not terminated
until L1 reached 35 wk post-weaning. However, the measures at week 15 show that more rapid weight gain occurred in L2 compared to L1.

The relevance of these observations to humans is uncertain at present. However, in association with the high prevalence of obesity, high vitamin intakes are observed in developed countries as a result of consumption of supplements and fortified foods [44, 527]. In North America, the median intake of several vitamins (A and B vitamins) by supplement users was reported to be above that recommended, and the established upper limit of intake of vitamin A, niacin and folate was exceeded by about 10 percent of the adult population [351]. Among pregnant women in Boston, MA, USA, the reported intake of certain vitamins ranged from 2 to 7 times the recommended values for pregnant women in the third quartile of intake [353]. In non-pregnant women, the use of supplements has been associated with a decrease in appetite [528]. However, in overweight or undernourished pregnant women, micronutrient supplementation during pregnancy resulted in higher post-partum weight retention [23, 529], but it is unknown whether similar results could be observed in a well-nourished population. Thus, the short- and long-term effects of high intakes of vitamins by pregnant women and their progeny warrant further investigation. In addition, the maternal reproductive history is a factor that should be taken into consideration when analyzing the effects of nutritional interventions during pregnancy on the mother and progeny.

In conclusion, consumption of the HV-diet only during the first pregnancy increases body weight and food intake in the dams and promotes characteristics of the metabolic syndrome in both their first and second litter male offspring. Further research on the mechanisms underlying these effects is warranted.
Table 6.1. Food intake, glucose response and hypothalamic gene expression of the dams after weaning their second litters

<table>
<thead>
<tr>
<th>Measure</th>
<th>RV</th>
<th>HV</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Food intake</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food intake&lt;sup&gt;1&lt;/sup&gt;, g/kg body weight (average/wk)</td>
<td>271±3.9</td>
<td>284±2.7</td>
<td>0.02</td>
</tr>
<tr>
<td>1 hour food intake, g</td>
<td>3.4±0.6</td>
<td>3.7±0.6</td>
<td>0.74</td>
</tr>
<tr>
<td><strong>Glucose tolerance test</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting blood glucose, mmol/L</td>
<td>4.1±0.2</td>
<td>4.0±0.1</td>
<td>0.91</td>
</tr>
<tr>
<td>Glucose iAUC&lt;sub&gt;0-60 min&lt;/sub&gt;, mmol·min/L</td>
<td>137.9±20.8</td>
<td>141.4±31.9</td>
<td>0.93</td>
</tr>
<tr>
<td><strong>Glucose and hormones&lt;sup&gt;2&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>5.3±0.3</td>
<td>5.1±0.3</td>
<td>0.53</td>
</tr>
<tr>
<td>Fasting insulin, ng/ml</td>
<td>0.6±0.1</td>
<td>0.8±0.1</td>
<td>0.44</td>
</tr>
<tr>
<td>Fasting glucose/insulin ratio</td>
<td>9.7±1.7</td>
<td>7.6±1.0</td>
<td>0.31</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>23.3±4.5</td>
<td>31.8±7.2</td>
<td>0.34</td>
</tr>
<tr>
<td>Corticosterone ng/ml</td>
<td>466±90.5</td>
<td>469±52</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>Abdominal Fat&lt;sup&gt;2&lt;/sup&gt;, g</strong></td>
<td>33.4±3.8</td>
<td>40.7±5.2</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>Hypothalamic gene expression&lt;sup&gt;3&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin Receptor</td>
<td>1.0±0.1</td>
<td>0.9±0.1</td>
<td>0.37</td>
</tr>
<tr>
<td>POMC</td>
<td>1.0±0.1</td>
<td>1.1±0.1</td>
<td>0.28</td>
</tr>
<tr>
<td>AgRP</td>
<td>1.0±0.1</td>
<td>1.1±0.1</td>
<td>0.52</td>
</tr>
<tr>
<td>NPY</td>
<td>1.0±0.1</td>
<td>1.2±0.1</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Mean ± SEM, n=7/group

<sup>1</sup> Average of food intake from weeks 2-14 after weaning the second litters

<sup>2</sup> At sacrifice (20 wk after weaning their second litters)

<sup>3</sup> Fold change, using RV-diet group as reference
Table 6.2. Effect of vitamin intake during the first pregnancy on litter size, body weight, food intake and abdominal fat of the male offspring from first and second litters

<table>
<thead>
<tr>
<th>Measure</th>
<th>MD(^1)</th>
<th>First Litters</th>
<th>Second Litters</th>
<th>P value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MD</td>
<td>Parity</td>
<td>Interaction</td>
<td></td>
</tr>
<tr>
<td>Litter size</td>
<td>RV</td>
<td>13.4±0.9</td>
<td>14.1±0.8</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>13.9±0.6</td>
<td>16.0±0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Birth weight, g (^3)</td>
<td>RV</td>
<td>6.8±0.13</td>
<td>6.7±0.12</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>6.6±0.15</td>
<td>6.1±0.16</td>
<td>0.35</td>
</tr>
<tr>
<td>Body weight at weaning(^4), g</td>
<td>RV</td>
<td>66.2±1.2</td>
<td>62.6±2.9</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>64.5±1.2</td>
<td>63.6±1.7</td>
<td>0.47</td>
</tr>
<tr>
<td>Abdominal fat (^5), g</td>
<td>RV</td>
<td>0.91±0.08</td>
<td>0.75±0.07</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>0.85±0.05</td>
<td>0.70±0.07</td>
<td>0.96</td>
</tr>
<tr>
<td>Food intake, average g/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 2-6</td>
<td>RV</td>
<td>24.3±0.5</td>
<td>22.0±0.9</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>24.2±1.0</td>
<td>23.5±1.0</td>
<td>0.09</td>
</tr>
<tr>
<td>Week 7-10</td>
<td>RV</td>
<td>27.2±0.6</td>
<td>24.9±0.7</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>27.6±1.1</td>
<td>27.2±1.1</td>
<td>0.26</td>
</tr>
<tr>
<td>Week 11-15</td>
<td>RV</td>
<td>25.5±0.3</td>
<td>23.5±0.9</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>27.1±1.2</td>
<td>26.6±0.8</td>
<td>0.16</td>
</tr>
<tr>
<td>Average 2-15 wk</td>
<td>RV</td>
<td>25.6±0.4</td>
<td>23.5±0.7</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>26.3±1.0</td>
<td>25.6±0.8</td>
<td>0.37</td>
</tr>
<tr>
<td>Weight gain(^6), g (from weaning to 15wk PW)</td>
<td>RV</td>
<td>517.4±9.1</td>
<td>539.4±18.4</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>533.5±26.1</td>
<td>600.1±13.4</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Mean ± SEM, n=6-9 per group and litter

\(^{1}\) RV: Regular, HV: High vitamin, \(^{2}\) Two-way ANOVA, \(^{3}\) Unsexed offspring; \(^{4}\) male offspring; \(^{5}\) male offspring terminated at weaning, n=6-8 per maternal diet and litter
Table 6.3. Blood glucose concentration following a glucose load in first and second litters at 5, 9 and 13 wk post-weaning

<table>
<thead>
<tr>
<th>Age (wk PW)</th>
<th>Maternal diet$^1$</th>
<th>Fasting glucose, mmol/L</th>
<th>Glucose iAUC$^3$, mmol·min/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>RV</td>
</tr>
<tr>
<td>First litter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second litter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5wk</td>
<td>6.2±0.2</td>
<td>6.7±0.2</td>
<td>5.9±0.2</td>
</tr>
<tr>
<td>9wk</td>
<td>5.8±0.1</td>
<td>6.2±0.2</td>
<td>5.4±0.5</td>
</tr>
<tr>
<td>13wk</td>
<td>5.4±0.2</td>
<td>5.8±0.3</td>
<td>5.3±0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SEM, n=6-9 per group and litter

$^1$Maternal diet during the first pregnancy. RV= Regular diet; HV= High vitamin

$^2$From PROC MIXED procedure, with time, maternal diet during the first pregnancy (MD) and parity as main factors. Interactions were not significant (p>0.05) unless otherwise indicated

$^3$iAUC= incremental area under the curve from 0-60 min after glucose load (5g of glucose/kg body weight).
Table 6.4. Glucose and hormone concentrations of the male offspring at weaning, 30 min following water or glucose gavage

<table>
<thead>
<tr>
<th>Maternal diet</th>
<th>First litter</th>
<th>Second litter</th>
<th>p-value&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Glucose</td>
<td>Water</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>7.7±0.2</td>
<td>10.5±0.5</td>
<td>6.8±0.2</td>
</tr>
<tr>
<td>HV</td>
<td>8.3±0.5</td>
<td>10.9±0.7</td>
<td>7.2±0.2</td>
</tr>
</tbody>
</table>

*Glucose (mmol/L)*

| RV | 0.3±0.1 | 0.7±0.2 | 0.8±0.1 | 1.4±0.1 | 0.75 | <0.01 | <0.01 |
| HV | 0.4±0.1 | 0.9±0.2 | 0.8±0.2 | 1.4±0.5 |

*Insulin (ng/ml)*

| RV | 30.9±1.3 | 17.2±1.3 | 9.3±1.2 | 6.0±1.1 | 0.51 | 0.07 | <0.01 |
| HV | 25.0±1.3 | 16.0±1.4 | 11.5±1.4 | 11.0±1.4 |

*Glucose/Insulin ratio<sup>4</sup>*

| RV | 2.1±0.5 | 0.9±0.1 | 3.5±0.5 | 3.8±0.9 | 0.02 | 0.20 | <0.01<sup>5</sup> |
| HV | 1.6±0.5 | 1.1±0.5 | 6.6±0.9 | 5.2±0.5 |

Mean ± SEM, n=6-9 per group and litter.

1 Maternal diet during the first pregnancy. RV= Regular diet; HV= High vitamin
2 3-way ANOVA. Interactions were p>0.05, unless otherwise indicated
3 Animals were gavaged with a glucose solution (5g of glucose/kg body weight) or similar volume of water 30 min before the sacrifice
4 Glucose/insulin ratio was log-transformed for analysis. Then values correspond to geometric means
5 Interaction MD X Parity p=0.01
Table 6.5. Blood glucose and plasma hormone concentrations of the second litters at 16 weeks post-weaning following glucose or water gavage

<table>
<thead>
<tr>
<th>Maternal diet¹</th>
<th>Gavage²</th>
<th>p-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>MD¹</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 7.2±0.4 8.0±0.4</td>
<td>0.15</td>
<td>0.06</td>
</tr>
<tr>
<td>HV 8.3±0.5 9.4±0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 1.2±0.2 4.3±1.2</td>
<td>0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HV 1.9±0.6 6.2±0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose/insulin ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 5.0±0.4 2.2±0.8</td>
<td>0.61</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HV 4.9±0.9 1.6±0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 11.1±1.7 18.8±2.3</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td>HV 20.6±2.4 18.0±1.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SEM, n=6-8 per dietary group.
¹Maternal diet during the first pregnancy. RV=Regular diet; HV=High vitamin diet
²Two-way ANOVA
³Gavaged with water or glucose solution 30 min before sacrifice
Table 6.6. Hypothalamic gene expression from the L1 and L2 offspring at weaning

<table>
<thead>
<tr>
<th>Gene</th>
<th>LepR</th>
<th>POMC</th>
<th>AgRP</th>
<th>NPY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>RV</td>
<td>HV</td>
</tr>
<tr>
<td></td>
<td>1.0±0.04</td>
<td>1.1±0.03</td>
<td>1.1±0.07</td>
<td>1.2±0.04</td>
</tr>
<tr>
<td></td>
<td>1.0±0.10</td>
<td>1.3±0.06*</td>
<td>1.5±0.14</td>
<td>1.3±0.06</td>
</tr>
<tr>
<td></td>
<td>1.0±0.12</td>
<td>1.1±0.03</td>
<td>1.2±0.07</td>
<td>1.2±0.08</td>
</tr>
<tr>
<td></td>
<td>1.0±0.08</td>
<td>0.9±0.05</td>
<td>1.1±0.05</td>
<td>1.2±0.04</td>
</tr>
</tbody>
</table>

Mean ± SEM, n=6-8 per group and litter
Data are expressed as fold-change using as a reference RV group from L1
1Maternal diet during the first pregnancy. RV= Regular diet; HV= High vitamin diet
2 2-way ANOVA
*p<0.05 by t-test within the litter
Figure 6.1. Effect of HV diet during the first pregnancy on body weight gain of the dams from delivery to 12 weeks after weaning the first and the second litters.

After first litters (RV n=10, HV n=9) and after second litters (RV n =7, HV n=7)
RV= Regular diet; HV= High vitamin diet during the first pregnancy.
Maternal diet during the first pregnancy (MD) p=0.03; parity p<0.01; interaction p=0.81
Data is presented as mean ± SEM
Figure 6.2. Hypothalamic gene expression from the second litters at 16 wk post-weaning

Mean±SEM of fold change, n=6-8 per group, with the RV group as a reference. RV: From dams fed regular diet; HV: From dams fed high vitamin diet during the first pregnancy
*p<0.05 by t-test †p<0.05 by Mann-Whitney test
Chapter 7. Effects of High Multivitamin and Folic Acid Intakes during Pregnancy on Body Weight and Food Intake Regulation in Wistar Rat Dams

7.0. Abstract

High vitamin intake during pregnancy by Wistar rats results in higher body weight and development of components of the metabolic syndrome in their offspring; however the impact on the dams is poorly understood. The objective of the study was to compare the effect of high multivitamin and high folic acid diets during pregnancy on maternal body weight, biomarkers of food intake regulation and insulin resistance in the dams. Pregnant Wistar rats (2-3rd day of pregnancy, n=13-14 per group) received the AIN-93G diet with either the regular (RV) or 10-fold vitamin mix (HV) or RV+10-fold folic acid (HFol) during pregnancy. After delivery they received only the RV-diet. Food intake of rats fed the HFol-diet was lower during pregnancy (p<0.05) and higher during lactation than in rats fed either RV or HV-diets (p<0.05). Rats fed the HV or HFol-diets gained more weight from weaning to 20 wk post-weaning than those fed RV-diet (diet p=0.13, time p<0.01, diet*time interaction p=0.03), but food intake was not significantly different. Plasma concentrations of ghrelin and leptin were higher at weaning and at 20 wk post-weaning, respectively, in HV-dams than in those from RV and HFol-diets. Gene expression of NPY in the hypothalamus was 20% higher in HFol-dams than in RV and HV-dams. No effects on blood glucose regulation were found. In conclusion, high multivitamin and high folic acid diets during pregnancy increase maternal post-weaning body weight gain, without the appearance of insulin resistance, but differ in their effects on biomarkers of food intake regulation.
7.1. Introduction

Obesity and overweight have been associated with genetic and lifestyle factors. In women, the risk of overweight and obesity is also associated with factors such as parity, gestational weight gain, breastfeeding duration, and post-partum weight retention/gain [530-534]. Weight changes during the first year after delivery are a strong predictor of women’s body weight 15 years later [535].

Whereas changes in postpartum body weight have been related to lifestyle [536] and pregnancy-related factors such as gestational weight gain [537], little is known about the role of interactions between dietary components and physiological adaptations during pregnancy on body weight and metabolic regulation. Evidence from animal and human suggests that adaptive changes during pregnancy and lactation are aimed to meet the increased metabolic demands of the mother by increasing food intake and promoting energy storage [171, 173] and are influenced by the fat and protein content of the diet [122, 538]; but the influence of other components of nutritionally adequate diets on post-partum body weight is unclear.

We have shown that a diet containing 10-fold the recommended multivitamins during pregnancy by Wistar rats results in higher body weight and food intake and development of characteristics of the metabolic syndrome in the offspring [32]. However, in previous studies we examined only the effects on the offspring and there are no reports of the effects of high vitamin intakes during pregnancy on the dams. The HV-diet contained a complete multivitamin mix, leaving uncertain the effects of individual vitamins. Folic acid is of particular interest for two reasons. First, intakes of this vitamin are more likely to exceed the upper limit of recommendation [353]; and second, folic acid is known to play a role in epigenetic modulation of gene expression, not only during early development [442], but also in later stages of life [539, 540]. Therefore, the objective of this study was to compare the effects of a high multivitamin and a high folic acid diet fed during pregnancy on body weight, biomarkers of food intake regulation and insulin resistance in Wistar rat dams after their first pregnancy.
7.2. Material and Methods

Design and procedures. Forty-four Wistar rats on day 2-3 of pregnancy were purchased from Charles Rivers (Quebec, Canada) and housed individually in transparent cages with free access to water in facilities with controlled temperature (22±1°C) and illumination (12-hour dark-light cycle, lights on at 7:00 A.M.). Dams were fed the regular AIN-93G diet [446] with either the regular amount (RV), 10 times the amount of vitamin mix (HV) or RV+10-fold folic acid (20 mg/kg diet) (H Fol) during pregnancy.

Within 24 hours of delivery, the litters were culled to 10 pups per dam. All dams received the RV-diet during lactation and for 20 wk after weaning the litters at post-natal day 21. Food intake was measured weekly throughout 20 wk and was also measured for the 1 hour of feeding after an overnight fast at 18 wk PW. Body weights of the dams were recorded 2-3 days before and within 24 hours after delivery and then weekly up to 20 wk PW.

A glucose tolerance test (GTT) was conducted at 6 wk PW and an insulin tolerance test (ITT) at 12 wk PW as in previous studies [32]. Plasma concentrations of insulin, leptin, and active ghrelin were measured at weaning and at 20 wk PW using a Milliplex map Kit (Rat Gut Hormone panel, cat#RGT-88K, Millipore Corporation, Billerica, MA). All procedures were approved by the Animal Ethics Committee at University of Toronto.

Hypothalamic gene expression. At 20 weeks PW, dams were euthanized and their brains removed and immediately frozen. The hypothalami were excised, homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) and mRNA isolated following the protocol from the manufacturer. After quantification using a UV-Agilent 8453, a High Capacity cDNA Archive Kit (Applied Biosystems Inc, Foster City, CA, USA) was used for cDNA synthesis (High Capacity cDNA Archive Kit Protocol from Applied Biosystems), by incubating the mix for 10 min at 25°C, followed by 120 min at 37°C in an ABI Gene Amp PCR System 2700. Real-time RT-PCR was performed using Taqman essays for the following genes: Leptin receptor, LepR (Cat # Rn01433205-m1); pro-opiomelanocortin, POMC (Cat # Rn00595020-m1); Agouti-related peptide, AgRP (Cat # Rn01431703-g1); and neuropeptide Y, NPY (Cat # Rn01410146-m1),
obtained from Applied Biosystems, on the 7900HT Fast Real-Time RT-PCR System also from Applied Biosystems (Foster City, CA, USA). The cycle conditions were: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles for 95°C for 15 seconds, and 60°C for 1 minute. The relative quantification method was performed, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cat # Rn99999916-s1) as an endogenous control. Results are expressed as fold-change, obtained by the $2^{-\Delta\Delta CT}$ method [491] and using the mean of RV-diet as reference group.

Statistical analyses. Group means were compared by analysis of variance. The PROC MIXED procedure was used for analyses of repeated measures. Pearson correlation coefficients were used to establish the relationship between selected variables. Statistical significance was declared at $p<0.05$. SAS v. 9.2. (SAS Institute Inc., Cary, NC, USA) was used for the statistical analysis of the data.

7.3. Results

Body weight of dams fed the RV and HV-diet did not differ at the end of pregnancy, at delivery, during lactation or at weaning, but dams fed the HFol-diet during pregnancy were lighter at delivery ($p<0.05$) and tended to be lighter also at weaning ($p=0.06$) than RV-dams. Food intake (g/day) was also lower during weeks 1 and 2 of pregnancy in the HFol group ($p<0.05$). However, HFol-dams consumed more food (g/kg body weight) than RV-dams during week 1 ($p<0.05$) of lactation, and tended to have higher food intake also during week 2 ($p=0.08$). These differences in food intake were not observed in the HV-group (Table 7.1.).

HV and HFol diets during pregnancy resulted in higher body weight gain over time than the RV-diet (diet $p=0.13$, time $p<0.01$, diet*time interaction $p=0.03$, Figure 7.1.), but the average of food intake after weaning was not different between groups (RV=19.1±0.4, HV=18.4±0.2, HFol=18.7±0.5 g/day, $p=0.51$) even after being adjusted by body weight (RV: 50.4±1.1; HV 49.1±0.9; HFol 50.9±0.9 g/kgBW/day, $p=0.46$). However, as shown in Figure 7.2., the relationship between the total amount of food consumed and body weight gain from weaning to
20 wks post-weaning was modified by the diet consumed during pregnancy; whereas total food intake was highly correlated with body weight gain post-weaning in RV and HV groups (r=0.66 and r=0.85, p<0.01, respectively), the correlation was not statistically significant in HFol-dams (r=0.2, p=0.46). Food intake (1 hour) after an overnight fast was not different among the groups (RV 4.9±0.4g, HV 5.2±0.6g, HFol, 4.8±0.5g, p=0.80).

Glucose response (iAUC) following a glucose load was not statistically different between groups (RV 147±9, HV 123±11, HFol 142±15 mmol/min/L, p=0.30). There was also no difference in individual time points (e.g. baseline, 15, 30, 45 min), except at 60 min after glucose gavage, when HV-dams had lower blood glucose concentrations than RV and HFol-dams (RV 8.4±0.3 mmol/L, HV 7.3±0.3 mmol/L, HFol 8.3±0.2 mmol/L, p=0.02). Glucose response (iAUC) after intraperitoneal insulin injection (ITT) was not different between groups (RV -112±10.0, HV -83±11, HFol -93±12 mmol·min/L, p=0.24). Dams fed the HV-diet, but not the HFol-diet had higher plasma concentrations of ghrelin at weaning (p<0.05) and higher plasma concentrations of leptin (p<0.05) at 20 wk PW, compared to the RV-dams (Table 7.2.).

Dams fed the HFol-diet during pregnancy had 20% higher expression of NPY in the hypothalamus (RV 1.0±0.06, HV 1.1±0.03, HFol 1.2±0.05, p=0.05) at 20 weeks PW, but the relative expression of LepR (RV 1.0±0.04, HV 1.0±0.03, HFol 1.0±0.04, p=0.48), POMC (RV 1.0±0.07, HV 1.0±0.09, HFol 1.0±0.05, p=0.96) and AgRP (RV 1.0±0.04, HV 1.0±0.04, HFol 1.1±0.11, p=0.81) was not different between groups.

### 7.4. Discussion

Both the high multivitamins and high folic acid diets fed during pregnancy affected the phenotype of the dams beyond the gestational period, but their effects on biomarkers of food intake regulation were different. While both high multivitamins and high folic acid intake affected body weight gain over time, only the HV-diet affected hormone concentrations whereas
the HFol-diet affected food intake and hypothalamic gene expression. Thus, folic acid alone may account for some but not all the effects of the HV-diet.

Our findings are consistent with previous studies in our laboratory showing that the HV-diet did not result in higher body weight at the end of pregnancy or at delivery [32]. In the present study we included a high folic acid diet, because intakes of this vitamin are likely to exceed recommended amounts during pregnancy [353] due to the consumption of supplements and fortified foods. The HFol group of dams tended to have lower body weight at the end of pregnancy and lactation. Whether this difference in body weight is a direct effect of the high folic acid content of the diet is unclear and might be due to the lower food intake, a lower gestational weight gain, or a lower pregestational weight. The lower body weight of the HFol group at delivery was not due to difference in litter size (RV 13±0.5, HV 13±0.5, HFol 13±0.5, p =0.87) or in birth weight between the groups (RV 6.5±0.1g, HV 6.3±0.1g, HFol 6.4±0.1g, p=0.55) (Cho CE, et al, unpublished).

The higher food intake of the HFol-dams during lactation (per kg body weight), may have been due to compensation for their lower weight at delivery, because the litters were culled to provide equal number of pups per dam and the weight of pups at weaning was not different among groups. Furthermore, because this HFol group did not gain more weight than HV and RV-dams during lactation (calculated as the absolute difference between delivery and weaning body weight, g) we could speculate that the increased energy consumed was related to lactation nutrient demands [541]. However the litters gained similar weight from birth to weaning as those in the HV and RV groups (weaning weight of unsexed offspring RV=60±2g, HV=56±2g, HFol 59±2g, p=0.49) (Cho CE, et al, unpublished). After weaning their litters, dams fed the HFol-diet during pregnancy gained more weight than RV-dams, following a similar pattern to the HV group.

Body weight gain in the high vitamin groups may have resulted from either higher caloric intakes, lower energy expenditure, or both. Although no difference was observed in the overall food intake between groups after correcting for body weight, we observed that greater proportion of the variability of the post-weaning total weight gain could be explained by the total food
intake in RV- and HV-dams, but not in the HFol group. In this latter group, either lower or higher food intake led to similar weight gain, whereas in the RV and HV groups higher weight gain was observed with increasing food intake. This suggests that although both HV- and HFol-dams gained more weight over time than the RV-dams, the mechanisms are different and might involve differences in energy expenditure, which is in line with the higher expression of NPY in the hypothalamus of the HFol group but not in HV group hypothalamus, since in addition to its known role in food intake regulation, NPY also decreases energy expenditure primarily by affecting thermogenesis [542].

The multivitamin, but not the folic acid content of the diet during pregnancy also affected hormone concentrations at weaning (ghrelin) and 20 wk PW (leptin). Thus our results suggest that the excess of other vitamins in the multivitamin mix during pregnancy, but not high intakes of folic acid alone, affect the plasma concentrations of active ghrelin and leptin. Both ghrelin and leptin are involved in food intake regulation [543] and changes in their blood concentrations are observed during pregnancy and lactation [544, 545]. However, plasma/serum concentrations may be affected by synthesis, secretion, or clearance (and acylation in the case of active ghrelin) of these hormones and both ghrelin and leptin seem to be responsive not only to energy but to nutrient composition of the diet in non-pregnant humans and animals [546, 547]. The effect of vitamin intake during pregnancy on post-pregnancy blood concentration of these hormones has not been previously reported.

The effects of high multivitamin or folic acid alone intake during pregnancy on hormone and vitamin status of the dams during pregnancy and lactation were not evaluated in this experiment. These measures were not taken to minimize the stress due to handling and/or fasting on the dams that could modify the outcomes [548-550]. However, it is plausible that vitamins affect hormone status. Thus it is possible that the effects of the multivitamin diet on leptin and insulin are affected by vitamin A and D. Vitamin A derivatives have been found to reduce leptin secretion by adipocytes and placental cell lines [411], and to stimulate insulin secretion by pancreatic cells in culture [551]. Vitamin D concentrations have been found to correlate with insulin sensitivity during pregnancy in humans [24].
The relevance of the results for humans is uncertain. However, the results highlight the importance of evaluating the effects of high intakes of vitamins due to the ready availability of vitamin supplements and fortified food [351, 527]. Studies in human populations have shown that vitamin A supplementation increases weight retention after pregnancy in women with HIV [23] which can be beneficial in malnourished population. Similarly, a multivitamin mix provided during pregnancy increased post-partum weight retention in overweight vitamin deficient women [529], but whether this was directly due to increased food intake or indirectly due to increased nutrient utilization through better nutritional status is unclear. However, the effects of vitamin intakes above the recommendations during pregnancy in well-nourished women have not been investigated beyond the perinatal period.

In summary, high multivitamin and high folic acid diets during pregnancy increase maternal post-weaning body weight gain, without the appearance of insulin resistance, but differ in their effects on biomarkers of food intake regulation. Further investigation on the long-lasting effects and the mechanisms of action of intakes of single vitamins or their combination above recommended levels during pregnancy on both mother and offspring is warranted.
Table 7.1. Body weight and food intake of the dams during pregnancy and lactation

<table>
<thead>
<tr>
<th>Period</th>
<th>Diet during pregnancy</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>HFol</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy</td>
<td>408±9</td>
<td>391±9</td>
<td>380±10</td>
<td></td>
</tr>
<tr>
<td>Delivery</td>
<td>334±9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>320±7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>306±8&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Lactation wk1</td>
<td>359±10</td>
<td>345±8</td>
<td>333±9</td>
<td></td>
</tr>
<tr>
<td>Lactation wk2</td>
<td>372±10</td>
<td>363±8</td>
<td>345±10</td>
<td></td>
</tr>
<tr>
<td>Weaning</td>
<td>355±11</td>
<td>346±8</td>
<td>323±11</td>
<td></td>
</tr>
<tr>
<td>Food intake during pregnancy,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>20±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19±0.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>18±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Week 2</td>
<td>27±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26±0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>25±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Week 3</td>
<td>27±0.7</td>
<td>26±0.5</td>
<td>26±0.7</td>
<td></td>
</tr>
<tr>
<td>Food intake during lactation, g/kgBW/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>117±2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>125±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>139±4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Week 2</td>
<td>177±4.8</td>
<td>179±3.6</td>
<td>199±10.7</td>
<td></td>
</tr>
<tr>
<td>Week 3</td>
<td>220±7.6</td>
<td>228±6.8</td>
<td>242±8.7</td>
<td></td>
</tr>
</tbody>
</table>

Body weight during pregnancy was obtained 2-3 days before delivery. Different letters denote p<0.05 by Tukey’s post-hoc after ANOVA, n=14-16 per group. RV= Regular AIN-93G diet, HV=High vitamin diet, HFol=High folic acid diet.
Table 7.2. Glucose and hormone concentrations in blood from dams at weaning and 20 wk PW

<table>
<thead>
<tr>
<th>Measure</th>
<th>Diet during pregnancy</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>H Fol</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>4.6 ± 0.3</td>
<td>4.6±0.4</td>
<td>4.9±0.2</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.7 ± 0.4</td>
<td>2.5±0.6</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>2.3 ± 0.4</td>
<td>2.2±0.3</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>Ghrelin, pg/ml</td>
<td>134±24a</td>
<td>243±21b</td>
<td>186±26ab</td>
</tr>
</tbody>
</table>

Weaning

20 wk PW

<table>
<thead>
<tr>
<th>Measure</th>
<th>Diet during pregnancy</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>H Fol</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.1 ± 0.2</td>
<td>4.8±0.2</td>
<td>4.9 ±0.2</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.5±0.2</td>
<td>2.1±0.2</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>7.7±0.6a</td>
<td>10.5±0.8b</td>
<td>7.8±0.3ab</td>
</tr>
<tr>
<td>Ghrelin, pg/ml</td>
<td>139±17</td>
<td>158±31</td>
<td>120±18</td>
</tr>
</tbody>
</table>

Different letters denote p<0.05, by Tukey post-hoc after one-way ANOVA, n=10-14 per group
RV= Regular AIN-93G diet, HV=High vitamin diet, H Fol=High folic acid diet
Figure 7.1. Weight gain of dams after weaning their litters

RV= Regular AIN-93G diet, HV=High vitamin diet, HFol=High folic acid diet

Diet p=0.13, time p<0.01, diet*time interaction p=0.03, by PROC MIXED procedure, n=13/14 per group
**Figure 7.2.** Total food intake and weight gain from weaning to 20 wks PW.

RV = Regular diet  HV = High-vitamin diet  HFol = High folic acid diet during pregnancy

Total food intake $p<0.01$, diet $p<0.001$, interaction $p<0.01$, by GLM procedure

(n=11-13 per group).
Chapter 8. General Discussion

The studies support the overall hypothesis that high-vitamin intakes during the first pregnancy increase body weight and promote characteristics of metabolic syndrome in Wistar rat dams as well as in their first and second litter offspring. Specifically, the Part 1 hypothesis that high multivitamin intakes during pregnancy alter tissue fatty acid concentrations, expression of PPAR genes in peripheral tissues and their regulation of metabolism in the offspring fed an obesogenic diet post-weaning was supported. Similarly, the Part 2 hypothesis that high-vitamin diets during the first pregnancy increase weight gain, food intake, and insulin resistance in the dams and their subsequent male offspring was partially supported. Insulin resistance was not observed in the dams but was indicated in the second as well as the first litter male offspring.

Novel findings derived from this thesis work are four. First, high multivitamin intakes during pregnancy altered the fatty acid composition of the liver, adipose and muscle from the offspring at several ages (Study 1, Chapter 4). Second, the maternal HV-diet interacted with the post-weaning obesogenic diet of the offspring in determining the expression of PPARs and uncoupled the relationship between PPARs and measures of fat mass and insulin resistance in male offspring (Study 2, Chapter 5). Third, the results are the first to show that high vitamin diets affect body weight, food intake, and hormone concentrations in the dams, and had carryover effects to their second litter offspring (Study 3, Chapter 6). Fourth, the effects of the HV-diet on the dams were not fully explained by its folic acid content (Study 4, Chapter 7). Each of these observations is discussed in the following.

8.1. Part 1: The Effect of the HV-Diet on Tissue Fatty Acids and PPAR Gene Expression

Metabolic syndrome is characterized by an impairment of lipid and glucose metabolism, obesity and insulin resistance being the most important underlying risk factors [34]. Previous studies in our laboratory showed that feeding the HV-diet of the dams during pregnancy increased body
weight, food intake, fat mass and insulin resistance in the offspring [32, 33]. Fatty acid composition and PPAR gene expression in tissues was the focus of Part 1 several reasons. First, because the development of metabolic syndrome is accompanied by alterations in fatty acid metabolism; second, a dysregulation of fatty acid metabolism has been found to precede insulin resistance in adults [552, 553]; and third, PPARs are major regulators of fatty acid and glucose metabolism [259]. Studies of Part 1 took advantage of tissues available from previous experiments. Taken together, the two studies of Part 1 support the hypothesis that the HV-diet during pregnancy alters tissue fatty acid concentrations, expression of PPAR genes in peripheral tissues and their regulation of metabolism in offspring fed an obesogenic diet, favouring insulin resistance.

8.1.1. The effect of the HV diet on tissue fatty acid concentrations

In study 1, high multivitamin intakes during pregnancy altered fatty acid concentrations in the liver, adipose and muscle from the offspring, but these effects interacted with age, sex and the post-weaning diet. This is an important observation because fatty acid concentrations in tissues can be considered a surrogate marker of fatty acid metabolism, in which vitamins are involved. In these experiments, the fat amount, source and composition were the same for both RV and HV-diets. Yet, the concentrations of essential fatty acids were higher in the liver and lower in the adipose tissue from the offspring of HV-dams at birth and males at weaning, respectively, supporting the hypothesis that these changes occurred as a result of the high vitamin content of the maternal diet during pregnancy.

The differences in the concentration of these fatty acids in the offspring tissues due to the maternal diet at birth and weaning may be explained by the effects of HV-diet intake on any one or, a combination of, actions on maternal fatty acid metabolism, placental transport, milk fatty acid content or altered fatty acid metabolism in the offspring. The maternal circulating fatty acids are the only source of the essential n-6 and n-3 fatty acids for the fetus and are often correlated with the fatty acid composition of the maternal diet [554]. However, there is little
information to which to link the maternal effects of vitamins during pregnancy to fatty acid composition of tissues in organs of the offspring even though vitamins are involved in regulation of genes and enzymes related to fatty acid uptake [555], desaturation [445], synthesis and oxidation [556]. Only folic acid has been reported to play a role in changing fatty acid composition [30]. In rats, increasing folic acid (4-fold the control) in maternal diets lowered ALA by ~5-fold and saturated fatty acids by 10% in the placenta [557], increased n-6 and n-3 in maternal milk [558], and decreased the proportion of DHA in fetal brains [336]. Similarly, DHA concentration in the fetal brain (20 d gestation) was found to be lower in offspring born to a rat dam fed normal B12 with high folic acid (4-fold) diet [336]. The extent to which folic acid in the HV-diet contributed to the results observed in the present study, or whether other vitamins contributed is unknown, but deficiency of other methyl donors in addition to folic acid in maternal diets up-regulate the expression of proteins related to fatty acid β-oxidation, and down-regulate those related with fatty acid uptake and synthesis in rat fetal livers [298]. This may suggest a role for other vitamins involved in one-carbon group metabolism in modulating fatty acid metabolism in the offspring, perhaps through epigenetic mechanisms.

The effects of the maternal HV-diet on fatty acid concentration of tissues of the offspring were also evident at later stages in life. At 12 wk PW, the male offspring from HV-dams had higher concentration of fatty acids (study 1) in muscle. This may be due to two reasons. First, lipid accumulation in muscle is associated with alterations in glucose metabolism [559], one of the characteristics observed in the adult offspring of HV-dams; and second, it can be due to increased fatty acid uptake or a decreased rate of fatty acid oxidation. The lower mRNA levels of PPAR-α in muscle (study 2) in males from HV-dams support this latter idea, as this nuclear factor plays a fundamental role in the catabolism of fatty acids [560].
8.1.2. Effects of HV-diet on PPAR gene expression

Altered glucose metabolism in the adult offspring of dams fed the HV-diet is consistent with previous reports [32, 487], but the mechanisms are unclear, which is why Study 2 examined PPAR gene expression in their tissues. PPARs regulate glucose metabolism in several ways. First, by activating the expression of genes such as insulin receptor substrate 1 [458]; second, by promoting GLUT 4 translocation [458, 561] thus increasing glucose uptake by muscle and adipose tissue; and third, by reducing gluconeogenesis in the liver [562]. In study 2, mRNA levels of PPAR-γ in the liver correlated with lower insulin resistance only in the offspring from RV-dams, which suggests that hepatic pathways of glucose control regulated by PPAR-γ genes were impaired in offspring from HV-dams.

8.1.3. Effects of the post-weaning obesogenic diet

A novel observation is that the obesogenic diet had independent effects and interacted with the prenatal (i.e. maternal) diet leading to differences in gene expression in peripheral tissues. Previous studies from our laboratory showed that the post-weaning obesogenic diet exacerbated the effects of the maternal HV-diet [487]. In study 2, the post-weaning diet showed an independent effect increasing the mRNA levels of PPAR-α in the muscle. In addition, the post-weaning obesogenic diet modified the effects of the maternal diet, as suggested by the significant interactions between these two factors in the gene expression of PPAR-α in the offspring at 14 weeks PW. Whereas the obesogenic diet markedly increased mRNA levels of PPAR-α in muscle in offspring from RV-dams, the same increase was not observed in those from HV-dams.

The effects of the obesogenic post-weaning diet on gene expression may be explained in part by its saturated fat and milk (condensed) content, since the gene expression of PPARs responds to dietary components. In humans, PPAR-α mRNA levels in muscle responded to a 5-day high-fat diet, but the direction of response was different in obese and normal weight subjects. In obese subjects the high-fat diet decreased the gene expression of PPAR-α whereas in non-obese
subjects its expression was increased [563]. However, in our rat study, the mRNA levels of PPAR-α were not significantly correlated with the abdominal fat mass. In mice, dairy-based diets fed for 6 weeks increased the expression of PPAR-α in muscle, a result that was not observed when they were supplemented with calcium alone, thus milk components may play a role in modifying the expression of this gene [564]. PPARs are responsive to fasting and re-feeding states [440]. But whether the maternal and post-weaning diets affected the expression of PPARs in the fed state was not evaluated in this thesis.

The post-weaning obesogenic diet increased the expression of PPAR-α. This increase was higher in offspring weaned to obesogenic diet and born from RV-dams than in those from HV-dams, but the mismatch in the vitamin content of the maternal and the post-weaning diet is not likely to be responsible for the interaction between the maternal and the post-weaning diet. According to the predictive adaptive response hypothesis, the mismatch between the nutrient content of the maternal and the offspring diets is responsible for many of the programming effects [481]. Although the obesogenic diet contained ~30% less multivitamins and had lower choline content than the RV-diet, thus creating a potential vitamin mismatch, we have shown that post-weaning diets containing even lower amounts of multivitamins (i.e. 1/3 the recommendations) had no effect on growth and food intake of the offspring from HV-dams (Szeto et. al. JDOHaD 2011, In press). An alternative explanation is that the increased fat mass and insulin resistance in offspring fed the obesogenic diet could have increased mRNA levels of PPAR-α, which regulates genes that encode for enzymes involved in fatty acid β-oxidation in skeletal muscle [458] but these responses were attenuated as a result of the gestational HV-diet.

8.1.4. Effect of sex of the offspring

Sex of the offspring was as a strong modifier of the effects of the maternal diets in fatty acid concentration of tissues analyzed in study 1 in which males and females exhibited a different fatty acid profile in the liver and brain phospholipids. In addition, weight gain, food intake and glucose response were analyzed for males (study 3) and females (appendix #2). Whereas males
from HV-dams had higher weight gain over time, females showed no effect. Similarly, higher food intake and glucose response was found only in males, but not in females.

Sex as a factor modulating the response to the maternal diet is consistent with results from our previous studies [32]. Males are more sensitive to the effects of the maternal HV-diet as shown by their higher food intake, body weight, and glucose response than females when they were fed the RV-diet after weaning [32]. However, when the offspring was weaned to the obesogenic diet, both males and females from HV-dams had increased body weight, food intake and glucose response [487]. Other models of fetal programming in mice and rats have found sex of the offspring to be a factor affecting the outcomes of maternal diets [88, 565-567]. Males consistently show more sensitivity to maternal diets restricted in energy, protein or methyl-donors and to maternal high-fat diets. Male, but not female offspring of energy restricted dams have higher body weight, HOMA values, leptin concentrations [568] and altered lipid metabolism in the liver [121]. A low-protein diet alters several biomarkers of pancreatic structure and function [569] and lipid metabolism [139] in a sex-dependent manner, with the male offspring more affected than females. Similarly, an increased HOMA index and insulin peak in an oral glucose tolerance test have been found only in male, but not female offspring of dams fed a methyl-deficient diet (folic acid, choline and methionine) periconceptionally [301] and increased glucose plasma concentrations after a glucose tolerance test have been found in males of dams fed a high fat diet [570].

In contrast, females are more sensitive to the effects of diets high in fructose or salt. Females but not males, from dams fed a high fructose diet during pregnancy had higher blood concentrations of leptin and glucose [571]. Females were also more sensitive to the effects of high salt content of maternal diets on their response to stress, a response that was not observed in males [572]. Furthermore, the results of one study suggest that the effect of the maternal diet on the offspring also depends on the gestational period of the intervention, and show that females are more sensitive to nutritional insults during middle pregnancy, whereas males are more sensitive during late pregnancy [206]. Thus, the effects of sex of the offspring is clearly a variable in determining
phenotypes in response to maternal diets, but the mechanisms by which this occurs has been less examined.

8.1.5. Conclusion and significance

The results of Part 1 lead to the conclusion that the HV-diet during pregnancy increases fatty acid concentrations in the liver and muscle and interacts with the post-weaning obesogenic diet in determining the expression of PPARs genes in a tissue and age dependent manner in the offspring. The HV-diet during pregnancy also uncouples the relationships between PPAR genes and insulin resistance and fat mass in male offspring of Wistar rats. This suggests that some of the characteristics of metabolic syndrome in offspring of the HV-diet fed dams are the result of alterations of tissue function and not only due to increased food intake by the offspring. These findings encourage further examination of the effect of the HV gestational diet on cellular and tissue regulation of energy metabolism, as discussed later in the section on future directions.


Taken together, the results of studies 3 and 4 support the hypothesis that high vitamin intakes during the first pregnancy increase weight gain and food intake in the dams and their second litter male offspring. Insulin resistance due to the HV-diet was observed only in the litters, but not in the dams.
8.2.1. Effects on the second litters.

The results of study 3 provide evidence that experiences from previous pregnancies promote characteristics of metabolic syndrome in the subsequent offspring. The maternal HV-diet had carryover effects to their second litters, independent of the effect of parity which also accentuated the characteristics of metabolic syndrome. Within the second litters, males from dams fed the HV-diet during their first pregnancy had higher food intake, weight gain, abdominal fat mass, plasma insulin and leptin concentrations, and mRNA levels of POMC and NPY, than those from dams that were fed only the RV-diet. Irrespective of the maternal diet, the second litters also gained more weight post-weaning despite their lower food intake, than their siblings from the first litters, had lower glucose and higher insulin plasma concentrations and mRNA levels of hypothalamic genes at weaning than males from the first litters.

The observed differences in the offspring of dams fed RV in comparison with those from dams fed the HV-diet during the first pregnancy may be due to their early catch up growth or to the effects of maternal obesity. The offspring of HV-dams were lighter at birth, caught up during lactation, gained more weight post-weaning and had higher abdominal fat mass by 16 wk post-weaning. These results are consistent with other studies in humans and rats showing that early catch up increases body weight and fat during adulthood [85, 573]. This increased body weight and fat of the second litters from HV-dams probably results from their increased food intake. The litters from HV-dams had higher food intake that those from RV-dams, despite having higher plasma concentrations of leptin and increased hypothalamic mRNA levels of POMC, known to decrease food intake [98]. They also had increased mRNA levels of the orexigenic NPY that may explain why they consumed more food. Taken together these results suggest a disruption in the mechanisms regulating food intake in the second litters. An alternative explanation is that the carryover effects of the HV-diet on the second litters were mediated by the higher body weight of the dams at conception [90, 154], consistent with results from other studies showing that maternal obesity leads to hyperphagia [154] and increased NPY mRNA levels in the hypothalamus in 3 month and 16 week-old rat offspring, respectively [89].
8.2.2. Effects of the maternal HV-diet on the dams

The main effect of the increased amount of vitamins during pregnancy on the dams was on their weight gain. However, despite increasing their body weight, the dams did not show insulin resistance as measured by glucose and insulin tolerance tests performed after their first and second pregnancies, in their fasting glucose and insulin concentrations at weaning or at 20 weeks PW of their first or second litters. The contribution of this weight gain to other metabolic effects was not evaluated.

It is surprising that a single exposure period to the HV-diet affected mechanisms regulating energy homeostasis, but this is suggested by several lines of evidence. First, body weight gain was higher in dams fed the HV-diet during their first pregnancy. Despite not being different at the end of pregnancy, at delivery or at weaning, HV-dams increased their body weight after both weanings. Second, food intake was higher in HV-dams, during the period of follow up after weaning their second litters (study 3). Third, HV-dams had higher concentrations of ghrelin at weaning. And finally, HV-dams gained more weight with the same amount of food consumed as the RV-dams after their first pregnancy (study 4).

In study 4, the HFol-diet fed during pregnancy had similar effects to the HV-diet on body weight gain after weaning. However HFol-dams were lighter at delivery and weaning, ate less during pregnancy and more during lactation compared with the RV-dams. The HFol diet, but not the HV, resulted in higher mRNA levels of NPY in the hypothalamus but did not affect hormone concentrations. Furthermore, food intake did not explain the post-weaning weight gain in dams fed HFol-diet. These differences in the outcomes suggest that other vitamins, in addition to or interacting with folic acid, may explain the effects of the HV-diet. Studies in humans have reported that the nutritional status of folic acid and vitamin B12 interact in maternal outcomes 5 years post-partum [25] in a B12 deficient population but it is unknown if similar interactions occur between these vitamins when consumed in excess.

There are no studies of the effects of high vitamin intakes during pregnancy on human mothers. However, providing multiple micronutrient supplements (including multivitamins, 1-1.5-fold
RDA) to pregnant women in a vitamin deficient population increased their caloric intake (~100 kcal) [41]. This supplement also increased one-month post-partum weight-retention, but only in overweight women [529]. Similarly, supplementation with vitamin-A only increased 3-6 months post-partum weight retention in HIV-infected women [23]. Retaining post-partum weight in malnourished population may be beneficial for the mother and future offspring. However, there are no studies evaluating the effects (beneficial or adverse) of intakes above the recommendations in pregnant women from a well-nourished population. Given the potential for vitamin intakes of 2-7 –fold the recommendations [353] and high concentrations of vitamin biomarkers [354] found in pregnant women from U.S.A. and Canada, respectively, the long-term effects of vitamins during pregnancy on the mothers warrant further investigation.

8.2.3. Effects of parity

The parity status of the mother is commonly used as a potential confounder of many pregnancy outcomes in epidemiological studies, but whether first and later borns differ in metabolic regulation and subsequent risk for disease is poorly understood. Differences in metabolic regulation are suggested by studies in humans that have shown that the risk for being overweight and obese may differ according to the order at birth [166, 502]. Similarly, studies in sheep have found differences in gene expression, adiposity and hormone concentrations in < 1 month-old lambs by parity status of the ewes [504, 505], but not at later ages.

The effects of parity can be confounded with those deriving from maternal age and pregestational weight. In study 3, the dams were heavier and ~16 weeks older by the time when they became pregnant for second time. However, studies in sheep comparing measures in lambs from nulliparous and multiparous ewes of similar age showed that parity is a major factor influencing the metabolic phenotype of the offspring [504, 505]. Because in study 3 first and second litters were from the same mothers, the study allowed comparison of the effect of parity and the effects of a single exposure to the high-vitamin diet on the first and second litters.
Only one study has reported the effects of previous pregnancies on outcomes in human siblings, which was only limited to measures in the newborn [515]. In this epidemiological study, excess of gestational weight gain during the first pregnancy was associated with an increase of the Z-score of birth weight in the subsequent offspring [515], but measures from the infants in later stages were not reported.

Thus the importance of evaluating the impact of dietary interventions during previous pregnancies and the parity status of the mothers when evaluating outcomes of the offspring are supported by the literature and the present studies.

8.2.4. Conclusion and significance

The results of Part 2 lead to the conclusion that high-vitamin diets during the first pregnancy increase weight gain and food intake in the dams and their second as well as first litter male offspring. The results from study 3 suggest that both maternal diet during pregnancy and parity interact to determine the metabolic phenotype of the dams and their offspring. However, insulin resistance was not observed in the dams but was indicated in their litters. A single exposure to HV-diet during the first pregnancy affects the dams post-weaning and increases expression of characteristics of metabolic syndrome in their second litters. The comparison of the HV-diet and the HFol-diet (study 4) showed that folic acid is not the only vitamin to affect the dams. Thus as for Part 1, these results encourage further examination of the effect of the maternal HV-diet and specific vitamins involved on cellular and tissue regulation of energy metabolism, as well as the regulation of energy homeostasis in the dams, as discussed in the section on future research.

8.3. Strengths and Limitations of the Studies

The studies included in this thesis aimed to further characterize the phenotype of the offspring of dams fed high multivitamin intake during pregnancy and to examine the effects of high vitamin
intakes during pregnancy on the dams and subsequent offspring. Therefore, similar designs to previous studies from our laboratory were used, including the composition of maternal diet (i.e. 10-fold the content of the regular vitamin mix) and the post-weaning diets. For the first objective, the offspring were fed the obesogenic diet post-weaning, because this diet is known to exacerbate the effects of the maternal diet. For the second objective the design was aimed to isolate the effects of one single exposure (i.e. the maternal diet), without manipulating any other variable in the dams or in the offspring. The experimental designs used have several strengths and weaknesses as discussed in the following.

The strengths include the clearly defined period of exposure to the maternal diet, the same number of pups per dam during lactation, the administration of vitamins as part of the diet and limited handling of the dams during pregnancy and lactation. Starting the diets on 2-3rd day of pregnancy provided exposure to vitamins during the pre-implantation period, important for methylation changes in the forming embryo [574], without interfering in the fertilization process. The exposure to the experimental diet was targeted to a specific period (from day 2-3rd of pregnancy to delivery) to investigate the effects of the high vitamin intake during pregnancy only. Studies in the field of developmental origins of disease have used a variety of dietary approaches. Some have started by feeding the diet of interest to females before pregnancy (some studies starting at weaning of the future mothers), during the entire gestational period and during lactation, making difficult to distinguish between the effects of maternal chronic under or overnutrition, from the effect of the specific diet during pregnancy. Feeding the pregnant dams the experimental diet only during pregnancy allowed attributing the outcomes to the exposure to the high vitamin diets in this particular period. Therefore, the observed effects in the offspring are not due to dietary influences in oocyte maturation or fertilization rate, in which vitamins are also involved (Figure 2.3).

By maintaining a constant number of pups per dam during lactation, we controlled for the effect of litter size on nutritional status and on the energy cost of lactation of the offspring and dams, respectively. Standardizing the litter size is a common practice in studies of the effects of maternal nutrition on rat offspring [59, 575]. In addition, because the experimental diet was
given to the pregnant dam, the litter was considered the experimental unit and then only one offspring value was used from each litter.

Another advantage of the experimental design was that we minimized handling of the dams. Restraining results in elevated glucocorticoids [576] that in turn, are associated with programming effects in the offspring. Thus, providing the vitamins in the diet minimized the handling of the dams during pregnancy and the influence that glucocorticoids could have on the offspring outcomes due to maternal stress. The concentrations of corticosterone were measured in the dams and the offspring and were not statistically different between dietary groups (study 3), permitting the conclusion that corticosterone levels and maternal stress are unlikely mediators of the effects of the HV-diet. Furthermore, because the vitamins were provided in the diet, keeping the rest of the nutrients in almost identical amounts, we prevented the influence of a vehicle (often oils or other solutions), that could have contributed to the outcomes.

Some limitations also have to be recognized, including the lack of measures before, and during pregnancy and lactation, the reduced sample size for some measures, and having tested only one dose of multivitamins (10-fold the recommended amounts of vitamin mix). The pregnant dams were directly purchased from a provider and their age and pregestational weight were not obtained. These two maternal factors have been related to birth weight in humans and have been related to postnatal growth and adiposity in the offspring later in life in both humans and animal models. Because the dams were randomized to receive either the HV or the RV-diet, it is expected that the variation in age and weight would be similarly distributed in both groups, as supported by the fact that the weight of the dams one day before and after delivery was not different between groups.

Because the experimental diets were provided only during pregnancy, their effect during lactation is unknown. It is possible that higher vitamin exposure occurred in the offspring of HV-dams during lactation via maternal milk. In humans, maternal status of vitamins A and from B group affect their concentrations in milk, but this has been related to maternal deficiencies, but not with intakes above the recommended [577]. However, in our model of high vitamin intake, the extent of exposure to increased vitamin levels during lactation has not been determined.
The small sample size in several of the outcomes measures may have been a limiting factor in assessing some effects of HV-diets. Although overall the sample size provided enough statistical power to detect significant differences in the primary dependent measures, in some cases the numbers in a group were small. This was particularly observed in the female offspring at 48 wk PW in study 1, and in the dams and offspring from study 3. In this latter study, the sample size was 9-10 per dietary group in the dams. However, the sample size was reduced to 7 dams per group for the second pregnancy, because 3 dams did not become pregnant for a second time, and 2 more required to be terminated after weaning their litters. Thus reducing the number of dams and pups evaluated. However, due to the magnitude of the differences in weight gain in the second litters they reached statistical significance.

Another limitation is perhaps the high dose and composition of vitamins in the experimental diet. The HV-diet provides 10-fold the amount of vitamin mix than regular formulation of the AIN-93G diet. Although this dose is high, it is below the levels considered toxic or teratogenic, according with the Subcommittee on Laboratory Animal Nutrition from the National Research Council (1995, Nutrient Requirements of Laboratory Animals). However, it is unclear at the moment if the observed effects of high multivitamins occur at lower doses. Recent studies in our laboratory have shown that hypothalamic genes are altered in offspring from mice fed 2.5 and 5-fold the recommended amount of vitamins during pregnancy and fed a high-fat diet post-weaning, but the effects on body weight and metabolic measures were not confirmed [578]. In contrast, in rats, increasing folic acid during pregnancy and lactation by 2.5-fold increased body weight, but decreased the expression of hypothalamic genes in the adult offspring (Pedro Huot, unpublished PhD thesis).

The effects of the HV-diet during pregnancy could be due to any of the vitamins or their combination in the mix. Therefore, the contribution of single vitamins or their combinations to these outcomes is unknown. However, this thesis began to investigate the role of high intake of single vitamins (folic acid) in the dams. The multivitamin mix was used since it is the most common form of supplementation during pregnancy in women from developed countries. However, women may also have high intakes of folic acid alone, from fortified food and single
vitamin supplements and high plasma concentrations of folate have been found in Canadian women [354]. Although the relevance for humans is unknown, in study 4 the effect of 10-fold folic acid on maternal weight and metabolism was investigated. The effects of this high folic acid diet on the offspring are currently under investigation as part of a Ph.D. and a M.Sc. thesis projects.

Finally, the exploration of mechanisms by which the HV-diet affects the offspring at the level of peripheral tissue is reported only in Part 1. However some additional work is reported in Appendix 3. It was hypothesized that the observed changes in glucose metabolism in the offspring were mediated by a decreased caveolin expression in muscle, subcellular fractions (i.e. plasma membrane and caveolae). Skeletal muscle plays a fundamental role in energy homeostasis and glucose metabolism [579]. Insulin binds to its receptor and promotes the traslocation of GLUT 4 to the plasma membrane to promote glucose uptake [580, 581]. In these pathways, plasma membrane specialized regions, namely caveolae, and their structural proteins (caveolin-1 and caveolin-3) are reported to be involved [582, 583]. However, protein expression of caveolins was not found to be significantly affected by the HV-diet in any of the subcellular fractions (Appendix 3). Similarly, the mRNA levels of GLUT 4 were not different by maternal diet (Study 2). Perhaps further examination of the traslocation of GLUT 4 or the expression and activity of enzymes involved in glucose uptake will provide more insight.

Despite the limitations discussed above, the combined results of these studies show that high vitamin diets fed during pregnancy altered mechanisms regulating energy homeostasis, and suggest that it impacts both food intake regulation by hypothalamic mechanisms in the offspring and fatty acid and glucose metabolism in peripheral tissues. This is the first time that high multivitamin intakes during the first pregnancy have been shown to have carryover effects for the dam during the post-weaning period and for their second litters. In addition this is the first time that impaired metabolism in peripheral tissues of offspring of dams fed HV-diet has been suggested based on tissue fatty acid and transcriptional factor (PPARs) expression, thus encouraging a fruitful area of investigation in both the dams and their offspring.
8.6. Conclusions

8.6.1. Specific conclusions

Study 1. High multivitamin intake during pregnancy has short and long-term effects on tissue fatty acid concentrations in the offspring from Wistar rats. These effects are tissue, sex, and age dependent.

Study 2. The HV-diet during pregnancy interacts with post-weaning diets in determining the expression of PPARs genes in a tissue and age dependent manner and uncouples the relationship between these genes and glucose regulation and abdominal fat mass in the offspring of Wistar rats.

Study 3. Consumption of the HV-diet only during the first pregnancy increases body weight and food intake in the dams and promotes characteristics of the metabolic syndrome in both their first and second litter male offspring.

Study 4. High multivitamin and high folic acid diets during pregnancy increase maternal post-weaning body weight gain, without the appearance of insulin resistance, but differ in their effects on biomarkers of food intake regulation.

8.6.2. Overall conclusion

The HV-diet, when consumed during the first pregnancy, increases post-weaning body weight and food intake in Wistar rat dams, uncouples tissue regulation of glucose metabolism in their offspring and promotes characteristics of metabolic syndrome in their second as well as their first litters. Folic acid is not the only vitamin involved.
8.7. Future Directions

This work has been mostly descriptive in nature and several limitations have been recognized. Future research focusing on mechanisms is needed to explain the effects of the high vitamin intake in the dams and the offspring, and to identify the vitamin or group of vitamins and their dose responsible for these effects.

The HV-diet is directly consumed by the pregnant rat. Whereas wide ranges of vitamins have been studied and high doses are known to affect non-pregnant humans and animals, the effects of increased (non-toxic, non-teratogenic) intakes during pregnancy are unknown. Pregnancy involves a series of changes in the brain and in peripheral tissues. Importantly, the maternal physiology adapts its energy homeostasis regulation and the distribution of nutrients during this stage. The role that vitamin content of the diet plays in this adaptation and how that relates to long-term outcomes in the offspring and to post-partum metabolism in the mother is currently unknown. Moreover, it is still unknown whether vitamins directly affect fetal development or whether their effects are mediated by maternal and/or placental metabolism.

Previous research showed that the HV-diet results in increased body weight, food intake and altered glucose metabolism in the offspring, and suggested that mechanisms regulating food intake could be altered in utero. This research further investigated the expression of four main hypothalamic genes known to regulate food intake in the offspring. However, investigation on downstream genes and the effects of derived neuropeptides on other brain regions related to nutrient sensing and the regulation of energy expenditure is lacking. Other signaling pathways in the brain remain to be investigated. For instance, the brain-derived-neurotrophic factor (BDNF), mainly involved in cognitive process, is also known to interact with dietary factors, and dopaminergic and gabaminergic systems also play a role in the regulation of food intake. Since several vitamins are involved in cell proliferation, differentiation and migration in the nervous system, it is likely that brain structures could be affected by the high vitamin intake, but these effects have not been evaluated. Previous experiments and those presented in this thesis examined the effects of the HV-diets on food intake, but not in energy expenditure.
The increased body weight and fat consistently observed in the dams and male offspring may be partially explained by the increased food intake, but it is also likely that pathways regulating energy expenditure are also involved (i.e. decreasing energy expenditure). Furthermore, the results from this thesis suggest that peripheral tissues may be involved in the regulation of energy substrates and further research is warranted to investigate the cellular and molecular pathways underlying the effects of the HV-diets on both the dams and their offspring.

Further work should also address the effect of high intakes of single vitamins or combination of them during pregnancy. This research started exploring the effects of high folic acid alone on the dams, and its effects on the offspring are currently under investigation. Of particular interest are those vitamins involved in one-carbon metabolism necessary for DNA and histone methylation, and therefore epigenetic regulation of gene expression. The choline content of the maternal diets was kept constant; therefore the effects of exceeding the amounts of this vitamin alone or in combination require further research.

The effects of maternal diets during pregnancy have been defined as “programming” effects. However, studies have shown that some effects are reversible by supplementation of leptin, folic acid or by feeding the offspring the same maternal diet [61, 584, 585]. Whether the effects of the maternal HV-diet can be reversed or attenuated by the addition of vitamins, either the complete vitamin mix, or single vitamins and the necessary dose, also require investigation. This question was taken by other members of the laboratory and it is currently under investigation.

The effects of the HV diets are differentially expressed in males and females. The mechanisms underlying these differences by sex are currently unknown. Whether these differences are programmed in utero or are related with postnatal hormone status is unknown. Finally, the research has focused on the effects of modifying the maternal diet. However, it is unknown whether the vitamin content of the paternal diet has any effect on programming the metabolism of the offspring.
## References


13. Muhlhausler BS, Duffield JA, McMullen IC. Increased Maternal Nutrition Stimulates Peroxisome Proliferator Activated Receptor-Gamma, Adiponectin, and Leptin Messenger


49. About Materna (R). *Available at: [http://www.materna.ca/About/Default.asp]*


138. Shahkhalili Y, Moulin J, Zhinden I, Aprikian O, Mace K. Comparison of Two Models of Intrauterine Growth Restriction for Early Catch-up Growth and Later Development of


419. Ghoshal K, Li X, Datta J, Bai S, Pogribny I, Pogribny M, Huang Y, Young D, Jacob ST. A Folate- and Methyl-Deficient Diet Alters the Expression of DNA Methyltransferases...


442. Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary Protein Restriction of Pregnant Rats Induces and Folic Acid Supplementation Prevents


549. Sugden MC, Langdown ML, Munns MJ, Holness MJ. Maternal Glucocorticoid Treatment Modulates Placental Leptin and Leptin Receptor Expression and Materno-Fetal Leptin Physiology During Late Pregnancy, and Elicits Hypertension Associated


APPENDIX 1. Protein Abundance of PPARs in Muscle from Offspring at Weaning and 14 Week Post-Weaning

In study 2, in addition to the mRNA levels of PPARs, the protein abundance in muscle of the offspring at weaning and at 14 weeks PW of those weaned to the RV-diet post-weaning were analyzed by Western blot as follows.

After determining the total amount of protein by a commercial kit (Thermo Scientific Pierce Coomassie Plus Protein Assay, Thermo Scientific Cat#23236), an equal amount of protein was mixed with Laemmli buffer (Biorad Cat#161-0737) with 5% beta-mercaptoethanol (Sigma-Aldrich Cat#M3148-25ml) and separated by electrophoresis in a 10% acrylamide (Sigma-Aldrich Cat #A7168) gel for 1 hour at 150V. Gels were then transferred in a Transblot SD (Biorad Cat#170-3940) for 1 hour to a PVDF membrane (Biorad, Cat #162-0184) and blocked in a 5% milk (Bioshop, Cat # SKI400.500), TBST (Trizma base 0.5mM, Sigma-Aldrich, Cat # T-1503; NaCl, 7.5mM, Bioshop Cat#SOD002.205, pH 7.4, Tween 20 0.10%, Sigma-Aldrich Cat#P-9416) solution for 1 hour, washed for 10 min with TBST and incubated with primary antibody (anti PPAR-ɑ, β/δ or γ, from Santa Cruz Biotechnologies) for 1 hour RT (PPAR-γ) or 16-18 hours (PPAR-ɑ, and β/δ.) After washing the membranes several times with TBST, they were incubated with the corresponding secondary antibody (anti-rabbit or anti-goat, Santa Cruz Biotechnology) for 50 min, then washed again and incubated with Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Cat # sc-2048) and developed on film. Band density was quantified by densitometry. Values are given in fold-change from the mean of the RV-diet group. Equal loading was confirmed by Ponceou staining after transferring to PVDF and before the blocking step.

Values are expressed in fold-change relative to RV-group. Unpaired t-test was used to compare the means in density values.
**Results**

Consistent with the results of RT-PCR, the maternal HV-diet did not affect the protein quantities of PPARs in muscle from the offspring at weaning (Figure A.1.1). Similarly, at 14 week post-weaning, the maternal diet did not affect the protein abundance of PPARs in the offspring weaned to the RV-diet (Figure A.1.2). However, whether the protein abundance reflects the mRNA values found in the offspring weaned to the obesogenic diet, or that in other tissues require further confirmation.
Figure A.1.1. Protein abundance of PPARs in muscle from male offspring at weaning (p>0.05, by t test)

A. PPAR-α
B. PPAR-β/δ
C. PPAR-γ

Values are mean ±SEM of the relative (%) integrated density value (IDV), with mean of IDV in the group from dams fed RV-diet as reference.
RV= From dams fed Regular AIN-93G diet during pregnancy
HV= From dams fed High vitamin diet during pregnancy
Figure A.1.2. Protein abundance of PPARs in muscle from male offspring weaned to the RV-diet, at 14 wk post-weaning weaning (p>0.05, by ttest)

A. PPAR-α
B. PPAR-β/δ
C. PPAR-γ

Values are mean ±SEM of the relative (%) integrated density value (IDV), with mean of IDV in the group from dams fed RV-diet as reference.
RV= From dams fed Regular AIN-93G diet during pregnancy weaned to RV-diet
HV=From dams fed High vitamin diet during pregnancy weaned to RV-diet
APPENDIX 2. Effect of HV-diet on Females from the First and Second Litters

The effect of maternal HV-diet during the first pregnancy on the phenotype of the male offspring of the first and second litters was presented on Chapter 6. On female offspring, body weights, food intake and glucose tolerance test measurements were also collected as described in the section of methods of Chapter 6.

Results

The maternal HV-diet had no effect on the body weight gain of females. However, those from the second litters, gained more weight after 15 weeks post-weaning than their siblings from L1 (p<0.05). No significant differences were observed by maternal diet or parity in their food intake (Table 1).

Results of the glucose tolerance tests are shown in Table 2. L2 offspring had higher AUC$_{0-60}$ values at 5 and 9 weeks post-weaning, irrespective of the maternal diet.

Glucose and hormone concentrations were not evaluated at weaning. However, in the L2 offspring at 15 wk post-weaning, fasting blood glucose concentrations were not different by maternal diet (RV =5.5±0.4, HV=5.1±0.4, p>0.05). Plasma insulin (RV=0.9±0.2, HV=1.3±0.2) or leptin (RV=14.4±3.2, HV=15.6±5.2) were not statistically different (p>0.05) by maternal diet. Samples of female offspring of L1 were not analyzed, because these animals were kept until 35 weeks post-weaning for a separate experiment.
Table A.2. 1. Weight gain and food intake of female offspring from first and second litters

<table>
<thead>
<tr>
<th>Period</th>
<th>Maternal diet</th>
<th>First pregnancy</th>
<th>Second pregnancy</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MD</td>
<td>Parity</td>
<td>Interaction</td>
</tr>
<tr>
<td>Weight gain from weaning to 15 wk PW</td>
<td>RV</td>
<td>275.8±11.9</td>
<td>301.4±17.8</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>245.4±6.6</td>
<td>314.1±22.4</td>
<td></td>
</tr>
<tr>
<td>Food intake, g/week</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 2-6</td>
<td>RV</td>
<td>136.7±4.2</td>
<td>131.1±10.6</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>130.0±5.6</td>
<td>132.5±5.6</td>
<td></td>
</tr>
<tr>
<td>Week 7-10</td>
<td>RV</td>
<td>139.0±8.8</td>
<td>154.1±12.3</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>135.4±9.3</td>
<td>143.8±7.7</td>
<td></td>
</tr>
<tr>
<td>Week 11-15</td>
<td>RV</td>
<td>136.2±8.2</td>
<td>135.4±6.6</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>133.7±9.2</td>
<td>147.9±5.8</td>
<td></td>
</tr>
<tr>
<td>Average 2-15 wk</td>
<td>RV</td>
<td>137.2±6.6</td>
<td>139.7±7.2</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>132.9±7.0</td>
<td>141.2±5.4</td>
<td></td>
</tr>
</tbody>
</table>

*From 2-way ANOVA, n=6-10 animals per dietary group and litter.
Table A.2.2. Results from glucose tolerance tests of the female offspring from first and second litters.

<table>
<thead>
<tr>
<th>Age</th>
<th>First litters</th>
<th></th>
<th>Second litters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>RV</td>
<td>HV</td>
</tr>
<tr>
<td>5wk</td>
<td>5.0±0.1</td>
<td>4.9±0.2</td>
<td>5.3±0.2</td>
<td>5.3±0.5</td>
</tr>
<tr>
<td>9wk</td>
<td>5.2±0.2</td>
<td>5.3±0.2</td>
<td>5.2±0.3</td>
<td>4.7±0.5</td>
</tr>
<tr>
<td>13wk</td>
<td>5.2±0.2</td>
<td>5.6±0.2</td>
<td>5.2±0.2</td>
<td>5.4±0.5</td>
</tr>
</tbody>
</table>

*Fasting glucose, mmol/L*

*Glucose iAUC0-60 min, mmol·min/L*

<table>
<thead>
<tr>
<th>Age</th>
<th>First litters</th>
<th></th>
<th>Second litters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>RV</td>
<td>HV</td>
</tr>
<tr>
<td>5wk</td>
<td>165±14</td>
<td>164±16</td>
<td>224±12</td>
<td>255±28†</td>
</tr>
<tr>
<td>9wk</td>
<td>148±13</td>
<td>186±16</td>
<td>204±13</td>
<td>204±24†</td>
</tr>
<tr>
<td>13wk</td>
<td>151±9</td>
<td>130±9</td>
<td>173±23</td>
<td>161±60</td>
</tr>
</tbody>
</table>

† parity effect p<0.05. MD and interaction effects were not significant (p>0.05) by 2-way ANOVA.

N=6-10 per dietary group and litter.
APPENDIX 3. Effects of maternal HV-diet during pregnancy on caveolin protein abundance in cell fractions of muscle of the male offspring

Introduction

Maternal diet during pregnancy influences fetal development with consequences observed later in life [501]. Energy and macronutrient deficiency or excess during pregnancy has demonstrated to alter tissue structure and function of the offspring, thus influencing their risk for chronic diseases later in life [7, 59]. Vitamins have also shown similar effects. Restriction of vitamins (50%) has resulted in higher body fat and plasma triglycerides in the adult offspring [29] and a maternal diet high in multivitamins resulted in higher body weight, abdominal fat, and altered glucose metabolism [32].

Muscle plays a fundamental role in insulin-mediated glucose disposal. Glucose uptake is mediated by a signalling cascade that facilitates glucose uptake that starts with the insulin binding to the insulin receptor located in the sarcolemma [580]. The insulin receptor in adipocyte and muscle is found in caveola [586, 587], specialized regions of the plasma membrane. Caveolins, structural proteins of caveolae enhance GLUT 4 translocation to the plasma membrane, allowing the internalization of glucose into the cell [582, 583]. The down-regulation or the lack of caveolins results in impaired glucose metabolism, hyperinsulinemia, increased hepatic lipid content, fat mass and body weight [582]

Modifications in diet or the nutritional environment seem to affect the expression of caveolins. For example, a high-fat diet leads to up-regulation of caveolin-2 and down-regulation of caveolin-1 expression, in adipose tissue of male rats (Lopez et al., 2005). High glucose concentrations decreased caveolin-1 expression by about 50% and reduced the number of caveolae in in vitro studies (Hayashi, Juliet, Miyazaki, Ignarro, & Iguchi, 2007). However, it is unknown whether maternal diet affects the expression of caveolins in the offspring tissues.
Because the offspring of dams fed a high multivitamin diet during pregnancy shows a phenotype characterized by increased body weight, fat mass, glucose and insulin plasma concentrations [32] and fatty acid content in the liver [488], we hypothesized that this maternal diet also affects the expression of caveolins. Therefore, the objective of this study was to investigate the effects of a high multivitamin diet during pregnancy on the protein abundance of caveolin 1 and 3 in muscle from the male offspring.

Material and methods

Wistar rats on their 2-3rd day of pregnancy (Charles Rivers, Quebec, Canada) were fed either the AIN-93G diet with the regular (RV-diet) or 10-fold the amount of multivitamins (HV-diet) during pregnancy. At delivery they were all fed the RV-diet and the litters were culled to 12 pups/dam. At weaning, eight males from each dietary group were gavaged with either water or glucose solution (5 g/kg body weight) and sacrificed by decapitation 30 minutes after.

Tissue collection and caveolae isolation

The quadriceps (both sides) were excised and ~1 g or tissue was homogenized (Homogenizer CH6010, Kriens-Lu, Switzerland) in 2 ml of buffer A, containing 0.25 mM of sucrose (EMD Chemicals, Cat #SX10753), 1 mM ethylenediamene-tetraacetic tetrasodium tetrahydrate (Sigma-Aldrich Cat #E5391), and 20 mM tricin(Sigma-Aldrich, Cat# T5816), pH 7.8, with 100 μl of protease inhibitor cocktail (Sigma-Aldrich. Cat # P8340). Plasma membrane and caveolae fractions were isolated according with a non-detergent modified method [588, 589]. Briefly, the tissue homogenate was spun for 20 min at 1200 g, the supernatant was collected and the debris was rehomogenized in 1.5 ml of buffer A with 50 μl of protease inhibitor cocktail and spun for 20 min. The supernatant were combined on top of 9 ml of 34.86% Percoll (Amersham-Biosciences, Cat # 17089102) in Beckman Ultracentrifuge tubes and centrifuged for 30 min at 4⁰ in a Beckman SW41 Ti rotor at 84,000g. The fraction containing the plasma membrane was collected and placed in a new tube, then sonicated twice for 2 seconds. An OptiPrep (Sigma-
Aldrich, Cat #D1556) gradient 20 to 10% (3.75ml) was then added on top of the sample and centrifuged at 52,000g for 90 min. The top 5.95 ml were collected and mixed with 4.76 ml of 50% OptiPrep and 1.19 ml of 15% OptiPrep was added, followed by 0.6 ml of 5% OptiPrep and then centrifuged at 52,000g for 90 min. The top band, identified as caveolae, was collected. Samples were kept at -80°C until used.

Western blot

After determining the total amount of protein by a commercial kit (Thermo Scientific Pierce Coomassie Plus Protein Assay, Thermo Scientific Cat#23236), an equal amount of protein was mixed with Laemmli buffer (Biorad Cat#161-0737) with 5% beta-mercaptoethanol (Sigma-Aldrich Cat#M3148-25ml) and separated by electrophoresis in a 12% acrylamide (Sigma-Aldrich Cat #A7168) gel for 1 hour at 150V. Gels were then transferred in a Transblot SD (Biorad Cat#170-3940) for 1 hour to a PVDF membrane (Biorad, Cat #162-0184), and blocked in a 5% milk (Bioshop, Cat # SKI400.500), TBST (Trizma base 0.5mM, Sigma-Aldrich, Cat # T-1503; NaCl, 7.5mM, Bioshop Cat#SOD002.205, pH 7.4, Tween 20 0.10%, Sigma-Aldrich Cat#P-9416) solution for 1 hour, washed for 10 min with TBST and incubated with primary antibody (caveolin-3 (BD-laboratories Cat #610420), caveolin-1 (Santa Cruz Biotechnology Cat#sc-894) for 16-18 hours. After washing the membranes several times with TBST, they were incubated with the secondary antibody (Santa Cruz Biotechnology) for 50 min, then washed again and incubated with Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Cat # sc-2048) and developed in film. Band density was quantified by densitometry. Values are given in fold-change from the mean of the RV-diet group. Equal loading was confirmed by staining the membrane with Coomassie blue [590].

Statistical analysis. Results are presented as mean±SEM. Unpaired t-test was used to compare the group means.
Results

As shown in figures A.3.1. and A.3.2, the abundance of caveolin-1 and caveolin-3 proteins was not affected by the vitamin content of the maternal diet or glucose gavage in the male offspring at weaning.
Figure A.3.1. Caveolin 1 protein abundance in male offspring at weaning. A. Whole tissue homogenate; B. Plasma membrane fraction; C. Caveolae fraction

A. Whole tissue homogenate; B. Plasma membrane fraction; C. Caveolae fraction

Animals were gavaged with either water or glucose solution (5 g/kg body weight) 30 min before sacrifice. Values are mean ±SEM of the relative (%) integrated density value (IDV) with mean of IDV in the group from dams fed RV-diet and gavaged with water as reference. RV= From dams fed Regular AIN-93G diet during pregnancy. HV=From dams fed High vitamin diet during pregnancy.
Figure A.3.2. Caveolin 3 protein abundance in male offspring at weaning.

B. Whole tissue homogenate; B. Plasma membrane fraction; C. Caveolae fraction

Animals were gavaged with either water or glucose solution (5g/kg body weight) 30 min before sacrifice.

Values are mean ±SEM of the relative (%) integrated density value (IDV) with mean of IDV in the group from dams fed RV-diet and gavaged with water as reference.

RV= From dams fed Regular AIN-93G diet during pregnancy. HV=From dams fed High vitamin diet during pregnancy
Appendix 4. Glossary

Developmental Origins of Health and Disease (DOHaD). A multidisciplinary field aimed to understand “how events in early life shape later morbidity risk, especially of non-communicable chronic diseases”[591].

Developmental Plasticity. The property of a given genotype to produce different phenotypes in response to distinct environmental conditions[73]. This property allows the organism to adapt with later environment, based on environmental cues.

Epigenetics. Refers “the study of mitotically (and potentially meiotically) heritable alterations in gene expression that are not caused by changes in DNA sequence”[245]

Fetal and Early Programming – The term ‘programming’ refers to the “process whereby a stimulus or insult at a critical period of development has lasting or lifelong significance” [4]. It refers as “fetal programming”, when this stimulus or insults occur in utero, and further extends to “early programming” to include other sensitive periods during development in early life (i.e. lactation).

Insulin Resistance. Refers to the decreased response of cells tissues to insulin[592]. There are numerous indexes proposed to measure insulin resistance in humans [593]. For the studies in this thesis, insulin resistance was evaluated in relation to the control group and assessed by glucose tolerance tests, by the ratio of blood glucose and plasma insulin concentrations, and by their product (glucose X insulin (fasting))[594]

Metabolic Syndrome. Cluster of conditions including obesity, hypertension and altered glucose and lipid metabolism [34]. Characteristics of metabolic syndrome evaluated in this thesis are increased abdominal fat mass and insulin resistance. Other characteristics associated with metabolic syndrome [35] are also included in this thesis: plasma concentrations of leptin, tissue fatty acid concentrations and peroxisome-proliferator activated receptors (PPARs) expression in peripheral tissues. All measures are compared to those in animals fed a control diet.
Predictive Adaptive Response Hypothesis. This hypothesis proposes that the risk of disease depends on the degree of match/mismatch between developmental and later life environment. It emphasize the effect of nutritional factors during critical periods of development

Thrifty Phenotype Hypothesis. This hypothesis proposes that “poor fetal and early post-natal nutrition impose mechanisms of nutritional thrift upon the growing individual” and that inadequate nutrition in early life increases the susceptibility to type 2 diabetes, by affecting the development of the pancreas [60].
Copyright Acknowledgements