Examing a Developmental Approach to Childhood Obesity: The Fetal and Early Childhood Years
A Workshop

February 26-27, 2015
The National Academies Keck Center
500 Fifth Street, NW
Washington, DC 20001
Room 100

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Statement of Task

An ad hoc committee will plan a two-day public workshop exploring the body of evolving science that examines the nexus of biology, interaction between biology and environment, and developmental stage on risk for childhood obesity. The workshop will include attention to the prenatal period, infancy, and early childhood and will include evidence from animal and human studies. The committee will define the specific topics to be addressed, develop the agenda, and select and invite speakers and other participants. A summary of the workshop will subsequently be prepared by a rapporteur with the assistance of staff and then undergo the National Academies report review process prior to release.
Day 1
8:00–8:45 a.m. Registration

INTRODUCTION AND OPENING REMARKS

8:50 Welcome
Shari Barkin, William K. Warren Family Foundation Chair in Medicine and Professor of Pediatrics, Monroe Carell, Jr. Children’s Hospital at Vanderbilt University

9:00 Opening Remarks
David M. Klurfeld, National Program Leader, Human Nutrition, Agricultural Research Service, U.S. Department of Agriculture
Sandra Hassink, Medical Director, Institute for Healthy Childhood Weight, American Academy of Pediatrics
Jamie Bussel, Program Officer, Robert Wood Johnson Foundation
SESSION 1: THE ROLE OF EPIGENETICS IN PEDIATRIC OBESITY- CONCEPTUAL OVERVIEW

Moderated by Matthew Gillman, Harvard Pilgrim Health Care Institute

9:30 Fundamentals of Epigenetics
Robert Waterland, Baylor College of Medicine

9:50 Conceptual Model of Epigenetic Influence on Obesity Risk
Andrea Baccarelli, Harvard School of Public Health

SESSION 2: ETIOLOGY AND CAUSAL INFERENCE

Moderated by Karen Lillycrop, University of Southampton

10:10 Epigenetic Mechanisms for Obesity Risk
Jacob Friedman, University of Colorado, Denver

10:30 The Role of Disparity in the Origins of Obesity Risk
Linda Adair, University of North Carolina

10:50 Fathers’ Early Contribution to the Birth of the Child: the Role of Paternal RNAs
Stephen Krawetz, Wayne State University

11:10 Maternal Influences on Offspring’s Epigenetics and Later Body Composition
Caroline Relton, Newcastle University

11:30 Q & A with Participants

12:00 p.m. Break for Lunch

SESSION 3: OPPORTUNITIES FOR INTERVENTION AND PREVENTION

Moderated by Leann Birch, University of Georgia

1:00 Developmental Plasticity – Sensitive Periods and Risk of Obesity
Karen Lillycrop, University of Southampton

1:20 Maternal Health and Diet’s Effect on Offspring’s Metabolic Functioning
Kevin Grove, Novo Nordisk

1:40 Early Infant Rapid Weight Gain and the Epigenetics of Leptin
Marie-France Hivert, Harvard University

2:00 Therapies to Reverse Metabolic Disturbances Arising as a Consequence of Developmental Programming
Mark Vickers, University of Auckland

2:20 Panel Discussion with Speakers
Moderated by Leann Birch, University of Georgia

2:40 Break

3:00 The Microbiome and our Genome
Karen E. Nelson, J. Craig Venter Institute

3:20 The Epigenetics of the Microbiome
Meredith Hullar, Fred Hutchinson Cancer Research Center
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<th>Time</th>
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<td>3:40</td>
<td>Toxic Stress and Its Role in Childhood Obesity</td>
<td>Antonio Convit, Nathan Kline Institute for Psychiatric Research</td>
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<td>4:00</td>
<td>Panel Discussion with Speakers</td>
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<td>4:30 pm</td>
<td>Concluding Remarks</td>
<td>Shari Barkin, Monroe Carell, Jr. Children’s Hospital at Vanderbilt University</td>
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<td>Day 2</td>
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<td>8:50 am</td>
<td>Welcome and Summary from Day 1</td>
<td>Shari Barkin, Monroe Carell, Jr. Children’s Hospital at Vanderbilt University</td>
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<td><strong>SESSION 4: REAL WORLD APPLICATION</strong></td>
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<td>9:00</td>
<td>Early Exposure Events and Obesity-Related Outcomes</td>
<td>Aryeh Stein, Emory University</td>
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<td>9:20</td>
<td>Messages to Women about Epigenetics and Childhood Obesity</td>
<td>Sarah Richardson, Harvard University</td>
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<td>9:40</td>
<td>Theory to Policy</td>
<td>Matthew Gillman, Harvard Pilgrim Health Care Institute</td>
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<td>10:00</td>
<td>Theory to Clinical Practice</td>
<td>Shari Barkin, Monroe Carell, Jr. Children’s Hospital at Vanderbilt University</td>
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<td><strong>SESSION 5: DATA GAPS AND FUTURE DIRECTIONS</strong></td>
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<td>Facilitated Discussion on Data Gaps and Future Research</td>
<td>Invited Speakers from Days 1 and 2</td>
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<td>11:00</td>
<td>Facilitated Discussion on Opportunities and Challenges in Epigenetics Research</td>
<td>Judith Hall, University of British Columbia</td>
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<td>11:30</td>
<td>Chair’s Summary and Final Thoughts</td>
<td>Shari Barkin, Monroe Carell, Jr. Children’s Hospital at Vanderbilt University</td>
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<td>12:00 pm</td>
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EARLY ORIGINS OF OBESITY
The Role of Epigenetics and Opportunities for Intervention

The model presented is intended to highlight the workshop objectives, rather than to be fully comprehensive. All levels of the internal and external environment interact with each other in a dynamic manner.
Planning Committee on Understanding the Dynamic Relationship between Biology, Environment, and Early Childhood Development on Risk of Obesity

Shari Barkin, M.D., M.S.H.S.
William K. Warren Family Foundation Chair in Medicine
Professor of Pediatrics
Monroe Carell, Jr. Children’s Hospital
At Vanderbilt University

Leann L. Birch, Ph.D.
William P. (Bill) Flatt Professor
Department of Foods and Nutrition
The University of Georgia

Esa Davis, M.D. M.P.H.
Assistant Professor
Center for Research on Health Care
Department of Medicine
University of Pittsburgh Medical Center

Stephen R. Daniels, M.D., Ph.D.
Chairman and Pediatrician-in-Chief
Department of Pediatrics
University of Colorado
School of Medicine

Matthew W. Gilliam, M.D.
Professor of Medicine and Director, Obesity Prevention Program, Department of Population Medicine
Harvard Medical School and the Harvard Pilgrim Health Care Institute
Professor of Nutrition
Harvard School of Public Health

Debra Haire-Joshu, Ph.D.
George Warren Brown School of Social Work-Public Health
Washington University in St. Louis

Karen A. Lillycrop, Ph.D.
Professor of Epigenetics
Centre for Biological Sciences, Faculty of Natural and Environmental Sciences
University of Southampton
Speaker Biosketches

**Linda S. Adair, Ph.D.** is a Professor of Nutrition in the School of Public Health at the University of North Carolina. Dr. Adair is a biological anthropologist interested in maternal and child nutrition. Her theoretical orientation comes from human biology and she is interested in how human populations respond to nutritional stresses. She is currently working on a large-scale longitudinal survey of women and children in the Philippines. This work involves exploration of patterns and determinants of growth from infancy through young adulthood; the long-term consequences of fetal and early child-growth patterns; the development of chronic disease risk factors in adolescents and young adults; and determinants of women’s nutritional status through the life cycle. She also collaborates with other Department of Nutrition faculty in the study of (1) gene-environment interactions as determinants of health and nutritional status; (2) feeding, parenting styles and growth of African American infants; (3) factors affecting postpartum maternal to child transmission of HIV and of maternal and child nutritional status in Malawi as well as nutrition projects in rural South Africa and China. She teaches international nutrition, advanced methods of nutritional epidemiology and the doctoral seminar. She received her B.S. in biological sciences in 1971 from the State University of New York at Stony Brook and Ph.D. in biological anthropology from the University of Pennsylvania in 1980.

**Andrea Baccarelli, M.D., M.P.H., Ph.D.** is the Mark and Catherine Winkler Associate Professor of Environmental Epigenetics in the Department of Environmental Health and Department of Epidemiology at the Harvard School of Public Health. His research focuses on identifying molecular and biological factors reflecting the impact of environmental exposures on cancer risk, with particular interest in epigenetics. Epigenetic marks, including DNA methylation, histone modifications, and non-coding RNAs, modify chromatin structure and gene expression without changing the underlying DNA sequence. Unlike genetic mutations, which represent rare events with permanent consequences on genes, epigenetic changes are reversible and responsive to environmental influences. Using a highly quantitative pyrosequencing-based approach for DNA methylation analysis, he has been examining the effects on DNA methylation of a variety of environmental carcinogens, including particulate air pollution, airborne benzene, metals, pesticides, dioxin-like compounds, and persistent organic pollutants, which are known to be relevant to cancer etiology.

**Shari Barkin, M.D., M.S.H.S.** received her medical degree from the University of Cincinnati Medical College and completed a Robert Wood Johnson Clinical Scholars fellowship at UCLA. The Barkin laboratory studies family-based community centered clinical interventions to improve health behaviors such as physical activity and nutrition in parent-young child dyads. The lab is focused on changing early body mass index trajectories in childhood, to prevent childhood obesity and later related adult chronic conditions. The interventions developed and tested apply the ecologic model that considers the child in the context of their family, and the family in the context of their community, considering how to pragmatically apply scientific discovery into potentially sustainable interventions that can improve the public’s health. A theme of the lab is the dynamic interaction between genetics, behavior, and environment at sensitive periods of childhood development. The lab applies a wide variety of techniques to address these complex problems, including qualitative and quantitative methodologies. The lab considers objective biologic measurements (such as fat mass and BMI), genetic measurements (genetic allelic risk scores, epigenetics), social measurements (social networks), and behavioral measurements (actigraphy changes over time in both parents and children, use of existing built environment to sustain healthy lifestyle behavior changes). She serves as the PI of the Growing Right Onto Wellness (GROW) Trial, a 7-year RCT to prevent childhood obesity funded by NHLBI and NICHD and serves on the Steering Committee for the Childhood Obesity Prevention and Treatment Research (COPTR) NIH Consortium. She is also in her second term on the Institute of Medicine’s Board of Children, Youth, and Families.
**Antonio Convit, M.D.,** is the Deputy Director of the Nathan Kline Institute and a professor of Psychiatry, Medicine, and Radiology at the NYU School of Medicine. Dr. Convit’s work focuses on understanding the impact of obesity-mediated metabolic disease on the brain. He also created the Banishing Obesity and Diabetes in Youth (BODY) Project, a public health program to help obese adolescents reduce their risk of type 2 diabetes and early cardiovascular disease. Dr. Convit is a native of Venezuela. He obtained his M.D. from the University of Chicago, Pritzker School of Medicine and trained in psychiatry at the New York University Medical Center.

**Jacob E. Friedman, Ph.D.,** is Professor in the Department of Pediatrics at the University of Colorado Denver. His primary focus is on understanding the role of early nutrition and the environment on molecular, endocrine, and epigenetic origins of childhood obesity and diabetes. This involved developing novel animal models of obesity (mouse, Non-Human Primate) together with invasive human clinical investigation in-vivo and in-vitro utilizing human skeletal muscle, adipose tissue, and more recently umbilical-derived mesenchymal stem cells (MSC) from infants born to obese women with and without Gestational Diabetes Mellitus (GDM). Dr. Friedman is currently PI, Co-PI, or Co-I on multiple NIH, ADA, and Gates Foundation funded basic, clinical, as well as large-scale epidemiological studies of pregnancy and obesity and maternal-fetal outcomes.

**Matthew Gillman, M.D., S.M.,** is Professor of Medicine at Harvard Medical School and the Harvard Pilgrim Health Care Institute, Professor of Nutrition at the Harvard School of Public Health, and Director of the Obesity Prevention Program in the Harvard Pilgrim Health Care Institute’s Department of Population Medicine. His research interests include early life prevention of chronic disease, including obesity, diabetes, cardiovascular disease, and asthma; individual and policy-level interventions to prevent obesity and its consequences; and childhood cardiovascular risk factors. He directs Project Viva, an NIH-funded cohort study of pregnant women and their offspring, focusing on effects of gestational diet and other factors on outcomes of pregnancy and childhood. Dr. Gillman also leads or participates in several other federally-funded studies of diet, activity, obesity, and cardiovascular risk in children and adults. He has served in leadership roles in the U.S. National Children's Study, the International Society for Developmental Origins of Health and Disease, the American Heart Association, and the American Academy of Pediatrics. He was a member of the Institute of Medicine Committee on Weight Gain during Pregnancy: Re-examining the Guidelines. He is an active teacher of medical students and mentor to research trainees. Formerly a primary care internist and pediatrician, Dr. Gillman’s current clinical work is in preventive cardiology among children.

**Kevin L. Grove, Ph.D.,** is a Senior Scientist in the Division of Diabetes, Obesity, & Metabolism, and Division of Reproductive & Developmental Sciences. He is also the Vice President of Obesity Research for Novo Nordisk in Seattle, WA. In the past 20 years in Oregon, Dr. Grove has developed an internationally recognized research program focused on how poor pregnancy health and nutrition programs offspring for high risk of metabolic and psychiatric diseases. His group also focuses on dietary and nutrient supplements that may prevent these health complications. Both of these programs extensively use nonhuman primate (NHP) models. Using these highly relevant and translational research models Dr. Grove has built international collaborations to understand the critical aspects of malnutrition during pregnancy, including both consumption of Western Style diets as well as the impact of under-nutrition. Dr. Grove received his B.S. in the Department of Animal Science at Washington State University in 1990, and his Ph.D. in Neuroscience from the College of Veterinary Medicine at the same university in 1994. He did his postdoctoral work at the Institute of Clinical Research of Montreal.
Judith G. Hall, M.D., M.Sc., is Clinical Geneticist and Pediatrician. She is currently Professor Emerita of Pediatrics and Medical Genetics at the University of British Columbia. Her research interests are human congenital anomalies, including neural tube defects, the genetics of short stature, mechanisms of disease such as mosaicism and imprinting, the natural history of genetic disorders, the genetics of connective tissue disorders such as arthrogryposis and dwarfism and monozygotic twins. She has contributed in many leadership roles, including Presidency of the American Society of Human Genetics and the American Pediatrics Society. Dr. Hall has served on numerous national and international committees and boards and has received many honors for her scientific contributions and lifetime achievements. Among her publications are summary reviews and articles that are considered classics, having introduced aspects of the new genetics. Dr. Hall advocated for folic acid supplementation, pediatric physician resources, the development of specific disease health guidelines, and research on rare genetic disorders and natural history. Dr. Hall trained at Wellesley College, the University of Washington School of Medicine, and Johns Hopkins Hospital.

Marie-France Hivert, M.D., MMSc., is an Assistant Professor in the Department of Population Medicine at Harvard Pilgrim Health Care Institute at Harvard Medical School. Dr. Hivert is a clinical investigator with primary focus on the etiology and primordial prevention of obesity and related co-morbidities, particularly type 2 diabetes and gestational diabetes. Her interests also include fetal metabolic programming mechanisms and the integration of genetics, epigenetics, and environmental factors contributing to obesity and related disorders. Dr. Hivert is currently involved in many international consortia investigating the genetics determinants of glycemic regulation during and outside of pregnancy. Dr Hivert completed her clinical training as an Endocrinologist in 2007 at the Université de Sherbrooke (QC, Canada). Dr. Hivert was awarded a Scholar Research Award from the Fonds de Recherche du Québec – Santé, a Clinical Scientist Award from the Canadian Diabetes Association, and the New Investigator Award from the Canadian Institutes of Health Research (CIHR). From CIHR, she also received the Maud Menten New Principal Investigator Award from the Institutes of Genetics in 2011. Dr. Hivert has initiated her research in primary prevention by conducting a trial of lifestyle intervention to prevent weight gain in young adults and her work led to upgrading the medical school curriculum at Université de Sherbrooke to allow better training in lifestyle counseling of future physician. Related to this expertise, Dr. Hivert is involved in the Physical Activity Committee at the American Heart Association. Dr. Hivert completed her postdoctoral fellowship at the Massachusetts General Hospital and a Master in Medical Sciences in the Scholars in Clinical Sciences Program at Harvard Medical School.

Meredith A. J. Hullar, Ph.D., is a Staff Scientist at the Fred Hutchinson Cancer Research Center. Her research interests include the role of the microbiome and diet in human health. Her research focuses on how the gut microbiome metabolizes dietary constituents and alters exposures that may influence health outcomes related to cancer. She uses a combination of dietary interventions and cross-sectional human population designs to study changes in the microbial community composition and functional genes associated with health outcomes. More specifically, she is interested in the role of the gut microbiome in obesity, the metabolism of phytochemicals by microbiota, and intermediary mechanisms of inflammation modulated by the gut microbiome. Dr. Hullar received her Ph.D. from Harvard University in 2000.

Stephen Krawetz, Ph.D., is Charlotte B. Failing Professor of Fetal Therapy and Diagnosis, Associate Director C.S. Mott Center for Human Growth and Development, and Director, Center of Excellence: Paternal Impact of Toxicological Exposure at Wayne State University School of Medicine, Department of Obstetrics and Gynecology and Center for Molecular Medicine and Genetics. Dr. Krawetz is well-recognized in the fields of Reproductive Genetics and Bioinformatics. Using human spermatogenesis as a model system, his primary research focus is directed towards understanding the long range genetic
mechanisms that dictate cell fate. His laboratory continues to implement and develop state-of-the-art technologies to determine how RNAs feedback to the genome to modulate the system. The spermatozoal RNAs delivered at fertilization may provide an essential component to early paternal genome reprogramming acting as genetic and epigenetic effectors. Dr. Krawetz received his Ph.D. in Biochemistry from the University of Toronto in 1983 and trained with Gordon Dixon at The University of Calgary as an AHFMR postdoctoral fellow.

Karen A. Lillycrop, Ph.D., is Professor of Epigenetics in the Centre for Biological Sciences at the University of Southampton, UK. Dr. Lillycrop’s research focuses on the effect of early life environment on the epigenome and long consequences for disease susceptibility. She showed for the first time in collaboration with Dr. Graham Burdge (Faculty of Medicine) that maternal nutritional constraint induces long term epigenetic changes in the regulation of key metabolic genes leading to persistent changes in phenotype. She is a founder member of the Epigen consortium, an international consortium investigating the role of epigenetic processes in the developmental origins of disease.

Karen Nelson, Ph.D., is president of the J. Craig Venter Institute (JCVI), where she has worked for the past 16 years. Prior to being appointed president, she held a number of other positions at the institute, including director of JCVI’s Rockville Campus, and director of human microbiology and metagenomics in the Department of Human Genomic Medicine at JCVI. Dr. Nelson has extensive experience in microbial ecology, microbial genomics, microbial physiology and metagenomics. Since joining the JCVI legacy institutes, Dr. Nelson has led several genomic and metagenomic efforts and the first human metagenomics study on fecal material derived from three individuals that was published in 2006. Additional ongoing studies in her group include metagenomic approaches to study the ecology of the gastrointestinal tract of humans and animals, studies on the relationship between the microbiome and various human and animal disease conditions, reference genome sequencing and analysis primarily for the human body, and other omics studies. Dr. Nelson received her undergraduate degree from the University of the West Indies, and her Ph.D. from Cornell University.

Caroline Relton, PGCE, Ph.D., is Professor of Genetics and Epigenetic Epidemiology at Newcastle University. Her primary research interest is the application of epidemiological approaches to improve our understanding of the role that epigenetic patterns may play in health and development. Ongoing work in Dr. Relton’s laboratory includes projects focusing on the role of epigenetic variation in obesity, type 2 diabetes and related co-morbidities; the role of epigenetic variation in women’s health through the menopause; determinants of DNA methylation variation in infants and children; the identification of epigenetic biomarkers of cognitive function; the role of DNA methylation in the pathogenesis of lung cancer; variation in epigenetic signatures during fetal development. Underpinning these projects is the methodological development of epidemiological tools to strengthen casual inference in the context of epigenetic studies.

Sarah S. Richardson, M.A., Ph.D., is the John L. Loeb Associate Professor of the Social Sciences at Harvard University. She is jointly appointed in the Department of the History of Science and the Committee on Degrees in Studies of Women, Gender, and Sexuality. A historian and philosopher of science, her research focuses on race and gender in the biosciences and on the social dimensions of scientific knowledge. Richardson’s research presses for scholarly reflection on the many developments underway in the present postgenomic moment. Her essay, "Maternal Bodies in the Postgenomic Order," discussed the implications of a prominent postgenomic research stream that situates the maternal body as a central site of epigenetic programming and transmission and as a significant locus of medical and public health intervention.
Aryeh D. Stein M.P.H., Ph.D., is Professor in the Hubert Department of Global Health of the Rollins School of Public Health, Emory University, with a joint appointment in the Department of Epidemiology. He is a member of the faculty of the Nutrition and Health Sciences program of the Division of Biological and Biomedical Sciences in the Laney Graduate School of Arts and Sciences. In his research, Dr. Stein utilizes critical periods of susceptibility to nutritional deficits and surfeits (such as war-induced famine or migration) to study the role of nutrition over the life course (prenatal, childhood, adulthood) on the development of adult chronic disease. He has secondary interests in the methodologies of dietary assessment and program evaluation. He is currently working with CARE and ICDDR,B in the design and implementation of a novel approach to program evaluation in Bangladesh, with the COHORTS investigative team on the analysis of data from birth cohort studies in Brazil, Guatemala, India, Philippines and South Africa, with investigators from South Africa on the extension of the Birth to Twenty study to the next generation and with the Young Lives investigators to study the consequences through adolescence of variation in growth in childhood.

Mark H. Vickers, MsC, Ph.D., is an Associate Professor and Senior Research Fellow in the Liggins Institute at the University of Auckland. Dr. Vickers’ research focus is on the effect of alterations in early life nutrition on the later health and wellbeing of offspring with a particular focus on the development of obesity and the metabolic syndrome. Dr. Vickers has established a number of preclinical models utilizing the paradigm of altered early-life nutrition to examine the mechanistic basis of programming during critical periods of developmental plasticity. He also investigates the potential for reversibility of developmental programming via both nutritional and pharmacologic interventions and was one of the first to show that developmental programming was potentially reversible with interventions in the early life period via the adipokine leptin. Dr. Vickers original work on developmental programming was named the most cited paper of the decade in the American Journal of Physiology: Endocrinology and Metabolism for 2001-11. He has published over 90 peer-reviewed papers and 6 book chapters in the field of early life origins of adult disease and is on the Editorial Board of a number of journals in this area.

Robert Waterland, Ph.D., is Associate Professor of Pediatrics and Molecular & Human Genetics, Baylor College of Medicine. His research aims to understand how nutrition during prenatal and early postnatal development affects individual susceptibility to various adult-onset chronic diseases. Dr. Waterland’s group focuses on nutritional influences on developmental epigenetics as a likely mediating mechanism. The Waterland group is increasingly interested in whether maternal obesity and nutrition before and during pregnancy affect developmental epigenetics in the hypothalamus and, consequently, body weight regulation in her offspring.
Workshop Reading List

Session 1


Session 2


Session 3


Session 4


Mansfield B, Guthman J. Epigenetic Life: Biological Plasticity, Abnormality, and New Configurations of Race and Reproduction. *In press* *Cultural Geographies*. 
SESSION 1
DNA methylation and body-mass index: a genome-wide analysis


Summary

Background Obesity is a major health problem that is determined by interactions between lifestyle and environmental and genetic factors. Although associations between several genetic variants and body-mass index (BMI) have been identified, little is known about epigenetic changes related to BMI. We undertook a genome-wide analysis of methylation at CpG sites in relation to BMI.

Methods 479 individuals of European origin recruited by the Cardiogenics Consortium formed our discovery cohort. They typed their whole-blood DNA with the Infinium HumanMethylation450 array. After quality control, methylation levels were tested for association with BMI. Methylation sites showing an association with BMI at a false discovery rate q value of 0·05 or less were taken forward for replication in a cohort of 339 unrelated white patients of northern European origin from the MARTHA cohort. Sites that remained significant in this primary replication cohort were tested in a second replication cohort of 1789 white patients of European origin from the KORA cohort. We examined whether methylation levels at identified sites also showed an association with BMI in DNA from adipose tissue (n=635) and skin (n=395) obtained from white female individuals participating in the MuTHER study. Finally, we examined the association of methylation at BMI-associated sites with genetic variants and with gene expression.

Findings 20 individuals from the discovery cohort were excluded from analyses after quality-control checks, leaving 459 participants. After adjustment for covariates, we identified an association (q value ≤0·05) between methylation at five probes across three different genes and BMI. The associations with three of these probes—cg22891070, cg27146050, and cg16672562, all of which are in intron 1 of HIF3A—were confirmed in both the primary and second replication cohorts. For every 0·1 increase in methylation β value at cg22891070, BMI was 3·6% (95% CI 2·4–4·9) higher in the discovery cohort, 2·7% (1·2–4·2) higher in the primary replication cohort, and 0·8% (0·2–1·4) higher in the second replication cohort. For the MuTHER cohort, methylation at cg22891070 was associated with BMI in adipose tissue (p=1·72×10⁻⁵) but not in skin (p=0·882). We observed a significant inverse correlation (ρ=0·005) between methylation at cg22891070 and expression of one HIF3A gene-expression probe in adipose tissue. Two single nucleotide polymorphisms—rs8102595 and rs3826795—had independent associations with BMI.

Interpretation Increased BMI in adults of European origin is associated with increased methylation at the HIF3A locus in blood cells and in adipose tissue. Our findings suggest that perturbation of hypoxia inducible transcription factor pathways could have an important role in the increased weight in people.

Funding The European Commission, National Institute for Health Research, British Heart Foundation, and Wellcome Trust.

Introduction Obesity and its associated comorbidities constitute a major and growing health problem worldwide.1 Therefore, understanding the mechanisms that affect body-mass index (BMI)—the most widely used measure of obesity—and any downstream effects is an important health priority. BMI is a complex phenotype determined by lifestyle (eg, physical activity), environmental factors (food availability and intake), and genetic factors.2 In the past few years, a major effort to identify genetic determinants of BMI through genome-wide association studies has shown that more than 30 single nucleotide polymorphisms (SNPs) are associated with BMI, which together explain about 1-5% of interindividual variation in BMI.

DNA methylation is the reversible and heritable attachment of a methyl group to a nucleotide. The most common form of DNA methylation occurs at the 5’ carbon of cytosine in CpG dinucleotides, creating 5-methylcytosine. CpG dinucleotides are often located in CpG islands (clusters of CpG sites) within the promoter region or first exon of genes, or upstream from genes within CpG island shores (DNA regions within 2 Kb of CpG islands) or shelves (within 2 Kb of shores).4 DNA
methylation plays a part in transcriptional regulation of genes and miRNAs, control of alternative promoter usage, and alternative splicing.

Both genetic and environmental factors can affect the extent of DNA methylation. In view of the range of potential downstream functional outcomes of this epigenetic change, an effect on DNA methylation could impact the impact of both genetic and environmental factors on a phenotype. Alternatively, epigenetic changes caused by a phenotype can mediate its downstream effects by changing gene expression.

Unlike genome-wide association studies of genetic variants, progress in systematic analysis of DNA methylation has hitherto been hampered by an absence of analogous platforms to study epigenetic phenomena. However, the newly developed Infinium Human-Methylation450 array (Illumina, San Diego, CA, USA) assays about 485,000 methylation sites spanning 99% of genes in the Reference Sequence database, with an average of 17 CpG sites per gene region. The array has been validated and consistently detects CpG methylation changes. We used this array for a large-scale analysis of methylation patterns in whole-blood DNA in relation to BMI.

Methods

Participants

479 white individuals who had been recruited by the Cardiogenics Consortium formed our discovery cohort. They either had a history of myocardial infarction (n=241; recruited from four centres: Leicester, UK; Lübeck, Germany; Regensburg, Germany; and Paris, France) or were healthy blood donors (n=238; recruited in Cambridge, UK). Genome-wide SNP genotypes had been previously obtained for all participants with the Human Quad Custom 670 array (Illumina, San Diego, CA, USA) and genome-wide gene expression data obtained for monocytes and derived macrophages with the HumanRef-8 v3 Beadchip array (Illumina, San Diego, CA, USA). For our primary replication cohort, we used data for 339 unrelated white patients of French origin who had venous thrombosis recruited into the MARseille THrombosis Association (MARTHA) cohort. These patients had been genotyped with the Human 610/660W-Quad arrays (Illumina, San Diego, CA, USA).

We analysed methylation sites that showed a significant association in the primary replication cohort in a second replication cohort of 1789 white participants from Germany who had been recruited for the KORA (Cooperative Health Research in the Region of Augsburg) F4 survey. Genome-wide genotyping was done for KORA F4 with the Affymetrix 6.0 GeneChip array (Santa Clara, CA, USA). To investigate whether the association between methylation at H1F3A sites and BMI that we observed in blood DNA would also be seen in other tissues, we analysed data for white female individuals from the UK obtained as part of the Multiple Tissue Human Expression Resource (MuTHER) study. HumanMethylation450 arrays had been done for 635 subcutaneous adipose tissue biopsies and for 395 skin biopsies. The adipose tissue samples came from 249 twin pairs (93 monozygotic and 156 dizygotic twins) and 137 singletons. Skin samples came from 108 of the 249 twin pairs (44 monzygotic and 64 dizygotic) and 179 singletons. The collection and processing of the biopsy samples in the MuTHER study have been described previously. In addition to the methylation arrays, genome-wide genotype data (obtained with a combination of HumanHap300, HumanHap510, and 1M-Duo and 1·2M-Duo Illumina arrays; Illumina, San Diego, CA, USA) and genome-wide expression profiles in adipose tissue (obtained with the IlluminaHT-12 v3 array; San Diego, CA, USA) were available for the MuTHER participants. All individuals provided written informed consent to participate in the primary studies and to allow DNA analysis of their samples.

Procedures

Details of the methylation assay done for the discovery cohort and the quality checks that were undertaken are given in the appendix (p 2). Methylation is described as a β value, which is a continuous variable ranging between 0 (no methylation) and 1 (full methylation). In any one sample, a probe with a detection p value (a measure of an individual probe’s performance) of more than 0·05 was assigned missing status. If a probe was missing in more than 5% of samples, we excluded it from all samples. We excluded 830 probes on this basis. To avoid spurious associations, we also excluded probes containing genomic sites where variation is already known according to the HumanMethylation450 annotation files or the InfiniumHD Methylation SNP list that had a minor allele frequency of more than 1%, leaving 351,099 probes. Before analysis, methylation values were corrected for background values and then normalised with SWAN in the R Package minfi. We used the array annotations provided by Illumina (version 1·1) to assign probes to their corresponding genes. We used the same Illumina HumanMethylation450 array in the replication cohorts and in the MuTHER samples, following similar experimental procedures. We did post-array processing in a similar way for all studies and normalised methylation values before analysis with SWAN for the two blood replication cohorts and by quantile normalisation for the MuTHER study samples.

Statistical analysis

BMI was not normally distributed in the discovery cohort and therefore was transformed on the log scale. Regression analysis of log-transformed BMI with methylation level at each probe was adjusted for age, sex, smoking status, methylation array batch, and centre. Adjustment for centre also adjusted for whether patients

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**Genetic Epidemiology**

(C Gieger PhD, Helmholtz Zentrum München-German Research Center for Environmental Health, Neuenberg, Germany; INSERM, UMR_S 1062, Aix-Marseille University, Marseille, France (Prof P-E Morange MD); Division of Epidemiology, Dalla Lana School of Public Health, University of Toronto, Toronto, ON, Canada (F Gagnon PhD); German Centre for Cardiovascular Research, Munich Heart Alliance, Munich, Germany (Prof P Deloukas); Department of Haematology, University of Cambridge, Cambridge, UK (Prof W H Ouwehand); National Health Service Blood and Transplant, Cambridge, UK (Prof W H Ouwehand); Department of Twin Research and Genetic Epidemiology, King’s College London, London, UK (T D Spector PhD); William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK (Prof P Deloukas); and Princess Al-Jawhara Al-Brabim Centre of Excellence in Research of Hereditary Disorders, King Abdulaziz University, Jeddah, Saudi Arabia (Prof P Deloukas).

Correspondence to: Prof Nilesh J Samani, Department of Cardiovascular Sciences, University of Leicester, Glenfield Hospital, Groby Road, Leicester LE3 9QP, UK njs@le.ac.uk

See Online for appendix

For the InfiniumHD Methylation SNP list see http://support.illumina.com/downloads/infinium_hd_methylation_snp_list.html

For the R Package minfi see http://www.bioconductor.org/packages/release/bioc/html/minfi.html
had had myocardial infarction. Chip assignment was not associated with BMI and was therefore not included in the model. For models in which the dependent variable (BMI in this case) has been log transformed, the β coefficients from the regression analysis can be interpreted as the change in the dependent variable by 100ξ(coefficient) for an increase in one unit in the independent variable. Therefore, we present β coefficients as percentage change. A correction for genomic control (A=1-0.092) was applied (appendix p 11). We estimated q values for false discovery rates\(^2\) and associations with a false discovery rate q value of 0·05 or less were taken forward for replication.

We did sequential replication for the MARTHA and KORA cohorts with linear regression analysis of log-transformed BMI adjusted for age, sex, smoking status, and array batch. We assessed significance after Bonferroni correction.

In the MuTHER cohort, to account for family structure, we fitted a linear mixed effects model for log-transformed BMI with the lme4 package in R. We adjusted the model for age, array batch, and smoking status (fixed effects), and for family identification number and zygosity (random effects). We used the likelihood ratio test for model identification number and zygosity as random effects and exclusion of sex). We also used the same model to analyse the association between methylation level or BMI with individual blood cell counts in the discovery cohort. We did power calculations with powerreg in Stata (version 12.1).

### Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. PD and NJS had full access to data for the discovery cohort, D-AT to data for the MARTHA cohort, CG for the KORA cohort, and PD for the MuTHER cohort. NJS had the final responsibility for the decision to submit for publication.

### Results

20 individuals from the discovery cohort (two who had had myocardial infarction, 18 healthy blood donors) were excluded from analyses after quality-control checks of the methylation array data (appendix p 2), leaving 459 participants (table 1). As reported by others\(^2\) at a genomic level, methylation at CpG dinucleotides in our discovery cohort had a bimodal distribution, with the most frequent level of methylation occurring at a β value of 0·0–0·05 with a second, slightly lower peak at 0·90–0·95

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Discovery cohort (Cardiogenics)</th>
<th>Primary replication cohort (MARTHA; n=339)</th>
<th>Second replication cohort (KORA; n=1789)</th>
<th>MuTHER cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Individuals who had had myocardial infarction (n=239)</td>
<td>Healthy blood donors (n=220)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>55·2 (6·8)</td>
<td>55·2 (6·8)</td>
<td>43·8 (14·2)</td>
<td>60·9 (8·9)</td>
</tr>
<tr>
<td>Men</td>
<td>202 (85%)</td>
<td>125 (57%)</td>
<td>74 (22%)</td>
<td>871 (49%)</td>
</tr>
<tr>
<td>Body-mass index (kg/m(^2))</td>
<td>28·3 (4%)</td>
<td>25·9 (3·6)</td>
<td>21·2 (4·4)</td>
<td>28·1 (4·8)</td>
</tr>
<tr>
<td>Ever smokers</td>
<td>185 (77%)</td>
<td>89 (40%)</td>
<td>145 (43%)</td>
<td>1003 (56%)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>174·5 (8·7)</td>
<td>172·5 (9·1)</td>
<td>166·6 (7·7)</td>
<td>167·8 (9·2)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86·5 (15·8)</td>
<td>77·2 (12·5)</td>
<td>67·5 (14·4)</td>
<td>79·4 (15·1)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>130·5 (15·8)</td>
<td>127·5 (9·1)</td>
<td>166·6 (7·7)</td>
<td>167·8 (9·2)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>77·8 (10·9)</td>
<td>NA</td>
<td>76·1 (9·9)</td>
<td>78·6 (9·4)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>10 (4%)</td>
<td>11 (6%)</td>
<td>6 (2%)</td>
<td>163 (9%)</td>
</tr>
<tr>
<td>Methylated of cg22891070(^1)</td>
<td>0·434 (0·110, 0·189–0·910)</td>
<td>0·453 (0·09, 0·211–0·740)</td>
<td>0·473 (0·118, 0·127–0·823)</td>
<td>0·515 (0·131, 0·154–0·906)</td>
</tr>
<tr>
<td>Methylated of cg27146050(^1)</td>
<td>0·159 (0·05, 0·134–0·495)</td>
<td>0·328 (0·047, 0·193–0·495)</td>
<td>0·315 (0·042, 0·130–0·458)</td>
<td>0·380 (0·057, 0·179–0·202)</td>
</tr>
<tr>
<td>Methylated of cg16672562(^1)</td>
<td>0·389 (0·116, 0·071–0·952)</td>
<td>0·409 (0·101, 0·157–0·745)</td>
<td>0·454 (0·125, 0·107–0·795)</td>
<td>0·428 (0·126, 0·091–0·900)</td>
</tr>
</tbody>
</table>

Data are mean (SD), n (%), or mean (SD, range). NA=not available. *From subset of participants who had also provided adipose tissue samples. \(\beta\) values.

Table 1: Characteristics of participants in the studied cohorts
In a previous study (in which the Illumina HumanMethylation27 Bead Chip, the precursor of the HumanMethylation450 Bead Chip, was used), a robust association between current smoking and methylation at the cg03636183 locus in F2RL3 had been shown and replicated. As a form of overall validation of our discovery analysis, we examined the association of current or ever smoking with methylation at this site in our dataset. We recorded a similarly highly significant association (p=3·8 × 10⁻³³) between methylation at cg03636183 and smoking, with reduced methylation in smokers (appendix p 10).

The distribution of p values in the discovery cohort from regression of methylation level at each site and BMI is shown in figure 1. The quantile–quantile plot for expected versus observed χ² values is shown in the appendix (p 11). Five probes achieved a false discovery rate q value of 0·05 or less, including individual probes in CLUH on chromosome 15 and KLF13 on chromosome 17 (appendix p 3), and three probes in HIF3A on chromosome 19 (table 2). We excluded the possibility that these probes showed cross-reactivity for several CpG sites.

We took these five probes forward for analysis in our primary replication cohort (MARTHA). Although methylation level for the probes in CLUH and KLF13 were not associated with BMI in this cohort (appendix p 3), all three HIF3A probes were significant after Bonferroni correction for multiple testing (table 2). We further tested the association of these three probes in our second replication cohort (KORA). All three probes were significantly associated with BMI, although the association was weaker than for the other cohorts (table 2).

The three identified HIF3A probes (cg22891070, cg27146050, and cg16672562) are neighbouring probes in intron 1 of the gene (figure 2). Methylation levels at cg22891070, cg27146050, and cg16672562 are all highly correlated with each other (R²=0·89–0·95 in the discovery cohort). The three probes are flanked by others that had nominally significant associations with BMI in the discovery cohort (cg05286653: p=2·37 × 10⁻⁴; cg12068280: p=4·89 × 10⁻³) that did not meet our false discovery rate q value threshold of 0·05 or lower. Overall, there are probes for 25 CpG sites in HIF3A on the array, and the results for all the probes are shown in the appendix (p 4).

Methylation at CpG sites in the other members of the

<table>
<thead>
<tr>
<th>Position</th>
<th>Discovery cohort (Cardiogenics)</th>
<th>Primary replication cohort (MARTHA)</th>
<th>Second replication cohort (KORA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg22891070</td>
<td>46801562 4.00 × 10⁻⁴ 3·6% (2·4–4·9)</td>
<td>3.65 × 10⁻⁸ 2·7% (1·2–4·2)</td>
<td>6·69 × 10⁻⁹ 0·8% (0·2–1·4)</td>
</tr>
<tr>
<td>cg27146050</td>
<td>46801557 4·82 × 10⁻⁴ 7·8% (5·1–10·4)</td>
<td>5.09 × 10⁻⁸ 6·2% (3·8–10·4)</td>
<td>2·18 × 10⁻⁹ 2·1% (0·7–3·4)</td>
</tr>
<tr>
<td>cg16672562</td>
<td>46801672 5·36 × 10⁻⁷ 3·2% (2·0–4·4)</td>
<td>3·47 × 10⁻⁸ 2·1% (0·7–3·5)</td>
<td>0·011 0·7% (0·2–1·3)</td>
</tr>
</tbody>
</table>

The significance threshold after Bonferroni correction for multiple testing in the primary replication cohort is 0·01 and in the second replication cohort is 0·016. BMI=body-mass index. *λ corrected. †The β coefficients from the association analysis have been converted into percentage change in BMI for every 0·1 unit increase in methylation β value.

Table 2: Association between methylation at sites in HIF3A on chromosome 19 in whole-blood DNA and BMI in the discovery and replication cohorts.

*Figure 1: Manhattan plot showing the distribution of p values of the association of methylation probes with body-mass index in the discovery cohort.

The red dots indicate probes that fall within KLF13 (chromosome 15), CLUH (chromosome 17), and HIF3A (chromosome 19).*
hypoxia inducible transcription factor family (HIF1A [13 probes], EPAS1 [38 probes], and ARNT [17 probes]) was not associated with BMI (data not shown).

Because the DNA used in our methylation analysis is derived from a mixture of different white blood cell types, methylation in the HIF3A probes could vary between different white cell populations, and the correlation with BMI could simply be a result of varying proportions of these cell types in individuals with different BMIs. Therefore, using cg22891070 as an exemplar, we examined the association of methylation level of this probe with the number of each cell type in the discovery cohort using a linear mixed effects model. Additionally, we tested for an association between number of each cell type and BMI. We recorded a weak positive correlation (p=0.019) between methylation at cg22891070 and lymphocyte count that did not survive correction for multiple testing. We recorded no associations with other cell types (appendix p 5). Furthermore, adjustment for lymphocyte, monocyte, and neutrophil counts did not substantially attenuate the association between methylation at cg22891070 and BMI (p=0.04×10^{-7}).

We also examined the association of DNA methylation at HIF3A with the two individual components of BMI—height and weight—in the discovery cohort. Methylation at cg22891070 was significantly associated with weight (p=5.2×10^{-7}) but not with height (p=0.78). In exploratory analyses of the population-based KORA cohort, we did not find an association between methylation at cg22891070 and other characteristics associated with BMI, such as physical activity (p=0.955) or type 2 diabetes mellitus (p=0.680).

For the three significant sites in HIF3A, overall methylation β value in the discovery cohort ranged from 0.18 to 0.90 for cg22891070, from 0.14 to 0.52 for cg27146050, and from 0.07 to 0.95 for cg16672562 (appendix p 12). β values were similar in the replication cohorts (table 1). The correlation between methylation level at cg22891070 in blood DNA and BMI for the discovery cohort, and the change in methylation level at cg22891070 by quintile of BMI (and vice versa) are shown in the appendix (pp 13–14). Every 0.1 increase in methylation β value for cg22891070 was associated with a 3.6% higher BMI in the discovery cohort (table 2). For a person in the discovery cohort with the mean BMI (27 kg/m^2), this 3.6% increase equates to a 0.98 kg/m^2 higher BMI on average. The increase in BMI was greater in participants who had had myocardial infarction (4.6%, 95% CI 2.9–6.3) than in the blood donors (2.3%, 0.4–4.1). The percentage changes in BMI in the replication cohorts for a 0.1 increase in methylation were smaller than in the discovery cohort (table 2), and in KORA was equivalent to a 0.22 kg/m^2 higher BMI on average.

In the MuTHER cohort, methylation level at the three HIF3A sites was strongly associated with BMI in adipose tissue but not in skin (table 3). The range of methylation β values was narrower in both tissues than in blood DNA (table 1). However, it was narrower in adipose tissue than in skin, which means that a reduced range cannot be a reason for why an association was not observed in skin. The direction of the association between methylation in HIF3A in adipose tissue and BMI was the same as that in blood, but the percentage change was greater.

We could analyse whether methylation at the HIF3A locus was correlated with HIF3A gene expression for the MuTHER adipose dataset, because genome-wide expression profiles were available. We recorded a weak (β value −0.025, SE 0.008) but significant (p=0.005) inverse correlation between methylation at cg22891070 and one (ILMN_1663015) of five HIF3A gene-expression probes on the array (appendix p 6). Although we had genome-wide expression data from monocytes and macrophages for the discovery cohort,10 expression of HIF3A was below detectable levels in these cells so we could not directly examine whether variation in methylation level at cg22891070 is associated with expression of the gene in blood cells.

Because DNA sequence variation can be associated with methylation level, we looked for an association between SNPs within 1 Mb of cg22891070 and methylation at this probe, using the genome-wide SNP data available for the discovery cohort (appendix p 15). Two SNPs, rs8102595 and rs3826795, with an R² between them of 0.006 (D'=1), had independent associations with methylation at cg22891070 (table 4). rs8102595 had a stronger association than did rs3826795 (table 4).

Table 3: Association between BMI and methylation at sites in HIF3A in adipose tissue and skin DNA in the MuTHER cohort

<table>
<thead>
<tr>
<th>Adipose tissue (n=635)</th>
<th>Skin (n=395)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p value</td>
<td>Percentage change in BMI</td>
</tr>
<tr>
<td>cg22891070</td>
<td>1.72×10^{-7}</td>
</tr>
<tr>
<td>cg27146050</td>
<td>9.27×10^{-11}</td>
</tr>
<tr>
<td>cg16672562</td>
<td>5.01×10^{-11}</td>
</tr>
</tbody>
</table>

Data in parentheses are 95% CIs. BMI=body-mass index. *The β coefficients from the association analysis have been converted into percentage change in BMI for every 0.1 unit increase in methylation β value.

Figure 2: Location of methylation probes associated with body-mass index and SNPs affecting methylation levels of these probes in the HIF3A locus

Vertical black lines represent exons. The arrow indicates direction of transcription. The three methylation sites in intron 1 showing an association with body-mass index are shown in red. The two SNPs showing an association with methylation levels at these probes are shown in blue. The green blocks represent the position of CpG islands in this locus. SNP=single nucleotide polymorphism.
rs8102595 is located 3·8 kb and rs3826795 1·2 kb upstream of cg22891070 (figure 2). Associations between these SNPs and methylation at cg22891070 were also highly significant in the replication cohorts (table 4). Furthermore, the same associations were recorded in both adipose tissue and skin in the MuTHER cohort (table 4). Genetic variation in rs8102595 accounted for 6·4% of the variation in methylation at cg22891070 in the whole-blood DNA in the discovery cohort, 9·9% in the MARTHA cohort, and 4·8% in the KORA cohort. This genetic variation also accounted for 14·3% of variation in methylation at cg22891070 in adipose tissue and 21·8% in skin in the MuTHER study.

In view of the association between the two SNPs and methylation at cg22891070, we next tested for association with BMI in the discovery and other cohorts, but observed no consistently significant association (appendix p 7). However, the power of these analyses was low (appendix p 7). Therefore, we also tested for associations between these SNPs and indices of body mass in the publicly available GIANT consortium datasets.3 We found no significant association of either SNP with BMI (rs8102595: n=123 791, p=0·15; rs3826795: n=123 847, p=0·25; appendix p 8).

### Discussion

We have identified and replicated a specific association between BMI and methylation of HIF3A in whole-blood DNA. We recorded the same association in DNA from adipose tissue, which is of high relevance to bodyweight and obesity, implying that it is biologically relevant. Although some preliminary reports are available of HIF3A expression in liver and adipose tissue and is associated with increased energy expenditure and weight loss,35 in the hypothalamus, HIF signalling (primarily via EPAS1) has a role in glucose sensing and regulation of energy balance and weight by affecting expression of pro-opiomelanocortin.36

Although HIF3A has not been investigated as thoroughly as the other α subunits in this context, it has been shown to have a role in the cellular response to glucose and insulin, and functions as an accelerator of adipocyte differentiation.38,39 Furthermore, siRNA inhibition of HIF3A in Hep3B cells significantly downregulates mRNA expression of ANGPTL4,40 which could have a role in acquired obesity.41

The cross-sectional nature of our analysis means that we cannot assign a cause–effect association directly from the association we observed between HIF3A methylation and BMI. Previous studies42–44 have shown that DNA methylation at cg22891070 is subject to much alternate splicing, leading to at least seven variants with differing targets.31 The induction of target genes by HIF3A binding to ARNT is generally weaker than is that evoked by HIF1A and EPAS1 binding to ARNT.45,46 Furthermore, especially in situations in which the amount of ARNT could be limiting, at least some isoforms of HIF3A seem to hinder the response to hypoxia by sequestering ARNT and restricting its binding to HIF1A and EPAS1.45,46

Although the main focus on HIF has been its role in cellular and vascular response to changes in oxygen tension during normal development or pathological processes (eg, cardiovascular disease and cancer47), compelling and increasing experimental data suggest that the HIF system also plays a key part in metabolism, energy expenditure, and obesity.47–50 Specifically, targeted disruption of either HIF1A or ARNT in adipocytes in transgenic mice is associated with reduced fat formation and protection from obesity and insulin resistance induced by high-fat diets.51 Similarly, systemic use of an antisense oligonucleotide to HIF1A for 8 weeks in mice with diet-induced obesity substantially suppresses HIF1A expression in liver and adipose tissue and is associated with increased energy expenditure and weight loss.52 In the hypothalamus, HIF signalling (primarily via EPAS1) has a role in glucose sensing and regulation of energy balance and weight by affecting expression of pro-opiomelanocortin.53

<table>
<thead>
<tr>
<th>rs8102595</th>
<th>rs3826795</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of effect allele</td>
<td>Frequency of effect allele</td>
</tr>
<tr>
<td>β (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Discovery (Cardiogenics)</td>
<td>0.10</td>
</tr>
<tr>
<td>Primary replication cohort (MARTHA)</td>
<td>0.10</td>
</tr>
<tr>
<td>Second replication cohort (KORA)</td>
<td>0.09</td>
</tr>
<tr>
<td>MuTHER cohort: adipose tissue</td>
<td>0.10</td>
</tr>
<tr>
<td>MuTHER cohort: skin</td>
<td>0.10</td>
</tr>
</tbody>
</table>

The β values are from an additive model and are a unit change in methylation per copy of the effect allele. *G. †C.
obese adolescents identified five regions that showed differential methylation levels of obesity has been explored, without definitive findings. One study of overweight or pattern in human adipose tissue after a 6-month exercise intervention. Although further intervention. Another study showed significant changes in genome-wide methylation DNA methylation. We have shown that BMI is associated with methylation of Ours is the first large-scale genome-wide analysis of the association between adult BMI and validation is necessary, these studies show that DNA methylation can be dynamic and could also affect whether weight changes in response to lifestyle and dietary measures.

Sequence variation can affect levels of methylation at individual sites (methylation quantitative trait loci). To investigate directionality of the association between HIF3A methylation and BMI, we searched for genetic variants that associate with HIF3A methylation to establish whether these variants also associate with BMI in turn. We identified significant independent associations between genotypes at two SNPs—rs8102595 and rs3826795, upstream of HIF3A—and methylation at one of our identified HIF3A probes, cg22891070. However, we identified no association between these variants and BMI in our cohorts or in the large GIANT genome-wide association meta-analysis of BMI which included more than 123,000 individuals. Our analysis of GIANT data had more than 95% power to detect an association for both SNPs if one existed (appendix p 8). These findings suggest that the association between increased methylation and higher BMI is not causal. Furthermore, the finding that methylation in HIF3A in skin was not associated with BMI, despite a strong methylation quantitative trait locus for cg22890170 in this tissue, also indicates the absence of causal directionality. Therefore, our findings suggest that increased methylation at the HIF3A locus is a result of increased BMI.

An alternative possibility is that the association between methylation at HIF3A and BMI is due to a confounding factor which affects both variables. However, we did not observe the association between HIF3A methylation and BMI in skin. Furthermore, we did not observe any association with other characteristics associated with BMI, such as physical activity or diabetes.

The mechanism by which increased BMI could lead to rises in HIF3A methylation is unknown. Obesity predisposes individuals to obstructive sleep apnoea, which is associated with intermittent hypoxia. In turn, hypoxia activates HIF signalling. Therefore, chronic upregulation of HIFs in response to obstructive sleep apnoea could result in secondary changes in methylation of the HIF genes. However, the association of methylation level at the HIF3A locus showed a linear correlation across the range of BMI levels, and increased methylation was not confined to obese individuals (appendix p 13). Furthermore, the association of BMI with variation in methylation was specific to HIF3A and was not noted for HIF1A and EPAS1.

We identified a significant inverse association between HIF3A methylation and HIF3A expression in adipose tissue. The association was only recorded with one of five HIF3A expression probes on the genome-wide expression array (appendix p 6), suggesting that the effect of methylation could be transcript-specific. In this context, we note that all three CpG sites at the HIF3A locus that were associated with BMI are situated within regions of open chromatin as identified by formaldehyde-assisted isolation of regulatory elements (FAIRE) in H1-hESC cells and K562 cells, suggesting that these sites lie in a regulatory region. However, two of the expression probes analysed (ILMN_1663015 and ILMN_1687481) are reported to tag the same HIF3A transcript (appendix p 6), and the reason for the discrepant findings for these two probes is unclear. Therefore, further work needs to be done to confirm the effect of methylation on expression and any transcript specificity. However, our finding supports the possibility that even if the association between increased methylation of HIF3A and BMI is secondary, an alteration in HIF signalling as a result of obesity-induced HIF3A methylation could still have an important role in some of the deleterious downstream effects of the disorder.

Although we recorded significant associations between increased HIF3A methylation in blood DNA and increased BMI in three different cohorts, the strength of the association varied substantially across the different cohorts. The gradient of the relation between methylation at HIF3A and BMI was four-times steeper in the discovery cohort than in the second population-based replication cohort (KORA), despite a similar distribution of methylation values. Whether this difference represents an element of winner’s curse or reflects other variation in the characteristics of the cohorts (including the presence of disease in some) is unclear. Even in the discovery cohort, we noted a difference in the level of association between the individuals who had had myocardial infarction and the healthy blood donors. The strength of the association in the blood donors was similar to that in the MARTHA cohort, which comprised patients with deep vein thrombosis, suggesting that the variation is not entirely related to disease status. Therefore, further
studies are needed to identify factors that affect HIF3A methylation and modulate the association between BMI and HIF3A methylation in whole-blood DNA. Further work is also necessary to deduce the timing of the variation in methylation at the HIF3A locus in relation to BMI and whether it is dynamic or not.

Blood is readily accessible for DNA analyses. By contrast with genetic analyses, a challenge of epigenetic analyses is that circulating leucocytes—the source of DNA in blood—are composed of several different cell subtypes that could each show cell-type specific variation in DNA methylation patterns. To an extent, as we have shown, this variation can be assessed and statistical adjustment done. Perhaps a more fundamental issue for the epigenetics community is whether analysis of blood DNA methylation is worthwhile and can reflect changes in relevant tissues for a phenotype. In this regard, our finding of an association between BMI and specific HIF3A methylation sites in both blood and adipose tissue DNA supports the use of whole-blood DNA methylation profiling for identification of relevant epigenetic changes and provides a rationale for other studies of this type.

We used a strict sequential replication design to avoid the penalty of multiple testing for confirmation of the association of probes identified in the discovery cohort. We also started with a fairly small discovery cohort. Therefore, we recognise that we have probably missed associations between methylation of other genes and BMI. Meta-analyses of the datasets used in our study together with other datasets could yield additional insights into epigenetic changes associated with BMI.

In summary, we have reported a novel association of increased BMI in adults of European origin with increased methylation at the HIF3A locus in relation to BMI and whether it is dynamic or not.

Contributors
KJD, CPN, PD, and NJS conceived the study. JE, CH, FC, AHG, WHO, HS, and NJS were responsible for recruitment and phenotyping of the discovery (Cardiogenics) cohort. LT and EM generated methylation array data for the discovery cohort. KJD and CPN analysed data for the discovery cohort, supervised by JRT. DA, P-EM, FG, and D-AT provided data from the primary replication cohort (MARTHA) and did analyses. SW, HG, MW, AP, and GC provided data from the second replication cohort (KORA) and did analyses. JKS, TDS, and PD provided data from the MuTHER cohort and did analyses. KJD, CPN, and NJS wrote the report. All authors reviewed the report and provided comments.

Declaration of interests
We declare that we have no competing interests.

Acknowledgments
This work was done as part of the Cardiogenics Project, which is funded by the European Union (LSHM-CT 2006-037593). The MARTHA project was supported by a grant from the Program Hospitalier de Recherche Clinique, and the methylation array typing was funded by the Canadian Institutes of Health Research (grant MOP 86466) and the Heart and Stroke Foundation of Canada (grant T6484). Statistical analyses of the MARTHA datasets were done in the C2BIG computing centre (UPMC, Paris, France), which is funded by the Fondation pour la Recherche Médicale and Région Ile de France. The KORA study was initiated and financed by the Helmholtz Zentrum München—German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research and by the State of Bavaria. The MuTHER study was funded by a programme grant from the Wellcome Trust (081972/Z/07/Z), and receives support from the National Institute for Health Research BioResource Clinical Research Facility and Biomedical Research Centre based at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London. CPN is funded by the National Institute for Health Research Leicester Cardiovascular Biomedical Research Unit, and this work comes under the portfolio of translational research supported by this unit. DA was supported by a PhD grant from the Région Ile de France (CORDDIM). FG holds a Canada Research Chair. JE, FC, HS, D-AT, and NJS collaborate under a Fondation Leducq Grant (2UCVD02). TDS is a European Research Council Senior Investigator and is holder of an ERC Advanced Principal Investigator award. PD is supported by the Wellcome Trust core grant to the Wellcome Trust Sanger Institute (098051), which funded DNA methylation analysis for MuTHER. NJS holds a chair funded by the British Heart Foundation and is a National Institute for Health Research Senior Investigator. We thank the staff at the genotyping facilities at the Wellcome Trust Sanger Institute for sample preparation, quality control, and typing for the Cardiogenics and MuTHER cohorts.

References


Epigenetic Mechanisms Affecting Regulation of Energy Balance: Many Questions, Few Answers

Robert A. Waterland

Departments of Pediatrics and Molecular & Human Genetics, Baylor College of Medicine, USDA/ARS Children’s Nutrition Research Center, Houston, Texas 77030; email: waterland@bcm.edu

Keywords

obesity, DNA methylation, programming, metabolic imprinting, food intake, energy expenditure

Abstract

Extensive human and animal model data show that nutrition and other environmental influences during critical periods of embryonic, fetal, and early postnatal life can affect the development of body weight regulatory pathways, with permanent consequences for risk of obesity. Epigenetic processes are widely viewed as a leading mechanism to explain the lifelong persistence of such “developmental programming” of energy balance. Despite meaningful progress in recent years, however, significant research obstacles impede our ability to test this hypothesis. Accordingly, this review attempts to summarize progress toward answering the following outstanding questions: Is epigenetic dysregulation a major cause of human obesity? In what cells/tissues is epigenetic regulation most important for energy balance? Does developmental programming of human body weight regulation occur via epigenetic mechanisms? Do epigenetic mechanisms have a greater impact on food intake or energy expenditure? Does epigenetic inheritance contribute to transgenerational patterns of obesity? In each case, significant obstacles and suggested approaches to surmounting them are elaborated.
INTRODUCTION

Although the current obesity epidemic is arguably the single greatest nutrition-related problem in the developed world, its fundamental causes remain poorly understood. Individual genetic variation is certainly important but does not appear to explain the increasing worldwide prevalence of obesity in recent decades. The escalating public health burden of obesity together with the general failure of therapies aimed at achieving long-term weight loss in obese adults are motivating increasing consideration of obesity as a developmental disease (2, 31). Indeed, extensive human epidemiologic and animal model data indicate that environmental influences during critical ontogenic periods affect the developmental establishment of energy balance pathways, determining lifelong susceptibility to obesity (50, 58, 101). Epigenetic processes, which stably regulate gene expression potential and exhibit plasticity to environment during development, are a prime candidate mechanism to explain such “developmental programming” of body weight regulation (101). Accordingly, there has recently been extraordinary interest in the role of epigenetic dysregulation in human obesity (32, 85, 88).

Rather than summarize knowledge in this field, the goals of this review are to highlight outstanding research questions and current progress toward answering them, and elaborate the greatest obstacles and suggested approaches by which they may be overcome. Our long-term goal is to determine if environmental exposures—including nutrition—during critical periods of development can affect epigenetic mechanisms involved in energy balance regulation, thereby modulating a person’s lifelong risk of obesity. Hence, rather than survey the accumulating literature illustrating epigenetic correlates of obesity, this review focuses on the potential for early environment to affect developmental epigenetics of body weight regulation.

IS EPIGENETIC DYSREGULATION A MAJOR CAUSE OF HUMAN OBESITY?

Epigenetics is the study of mitotically heritable alterations in gene expression potential that occur without alterations in DNA sequence (43). Epigenetic modifications are a fundamental mechanism of cellular differentiation, in which pluripotent progenitor cells become increasingly committed toward and then morphologically and functionally diverge into diverse cell types during
mammalian development. Several molecular mechanisms regulate epigenetic development, including methylation of cytosines in CpG dinucleotides (i.e., cytosine followed by guanine), various posttranslational modifications of the amino terminal “tails” of the histone proteins that package DNA into nuclear chromatin, autoregulatory transcription factors, and noncoding RNA (for excellent reviews on epigenetic mechanisms, see 14, 43, 75). On the one hand, the inclusion of histone modifications on this list remains controversial. Although they certainly play a role in developmental processes and are widely viewed as an epigenetic mechanism, it remains unclear whether histone modifications have the definitive ability to convey information through mitosis (38, 69). On the other hand, autoregulatory transcription factors that bind to and trans-activate their own promoters constitute a classical and bona-fide epigenetic mechanism (92) that is often omitted from recent authoritative reviews on mammalian epigenetics. None of these individual mechanisms functions in isolation; these and potentially other layers of epigenetic modifications are sequentially established during embryonic, fetal, and early postnatal development and function synergistically to maintain cell type–specific regional chromatin conformations that regulate transcriptional competence. Of the known epigenetic mechanisms, DNA methylation stands out; once established during development, cell type–specific patterns of CpG methylation are mitotically inherited via semiconservative replication and are highly stable in differentiated cells and tissues (14). CpG methylation is also stable during tissue collection and storage and can be assayed in minute quantities of DNA. For these reasons, the vast majority of epigenetic studies pertaining to developmental programming of obesity have focused on DNA methylation.

Animal model and human data show clearly that epigenetic dysregulation can cause obesity. The best-characterized animal model is the agouti viable yellow (A(v)) mouse. The murine agouti gene, normally expressed only in hair follicles, encodes a paracrine-signaling molecule that regulates fur pigmentation. The A(v) mutation resulted from the spontaneous transposition of a retrotransposon upstream of agouti, causing profound epigenetic dysregulation; isogenic A(v)/a mice exhibit stochastic and systemic interindividual variation in DNA methylation at A(v), which in turn regulates variable degrees of ectopic agouti expression. Owing to its structural similarity to agouti-related protein (Agrp), agouti protein binds antagonistically to the melanocortin 4 receptor, causing hyperphagic obesity (109). Genetically identical A(v)/a mice can therefore range from yellow and obese to lean and brown, vividly illustrating epigenetically mediated obesity (99). Mice produced by somatic cell nuclear transfer (cloning) provide another example. Relative to isogenic mice conceived naturally, those produced by cloning are slightly heavier at birth and later develop obesity (90). Cloning requires a somatic cell to be epigenetically reprogrammed to a totipotent state; the adult-onset obesity of cloned mice, therefore, likely results from subtle aberrations in this epigenetic reset. Prader-Willi syndrome is a human neurodevelopmental syndrome characterized by insatiable hyperphagia and obesity, among other symptoms. Although most often caused by a genetic deletion of a specific region of chromosome 15, a subset of “sporadic” Prader-Willi cases is caused by aberrant DNA hypermethylation and epigenetic silencing of the same chromosomal region (34), providing clear evidence that epigenetic dysregulation can cause obesity in humans as in mice.

Despite these compelling examples, and extensive investigations in recent years, it remains unclear whether epigenetic dysregulation contributes meaningfully to the current worldwide obesity epidemic. This ostensibly slow progress can be attributed to several major obstacles. The first is that epigenetic mechanisms are, by nature, largely cell-type specific. In genetic studies of obesity, one can sample an individual’s DNA from any easily biopsiable tissue, such as peripheral blood. In most cases, however, it cannot be assumed that epigenetic marks in peripheral blood are correlated with those in other tissues. Hence, epigenetic studies of obesity will in most cases wish to obtain DNA (and potentially other cellular components) from tissues directly implicated in energy
Figure 1
Conceptual framework linking genetics, environment, epigenetics, and obesity. In addition to the direct pathways and interactions by which interindividual genetic variation and environment affect risk of obesity (pathways a, b, c, and d), interindividual epigenetic variation can also affect obesity risk (pathway e). The strong effect of genetic variation on epigenetic mechanisms (pathway f) and the potential for reverse causality (pathway g) complicate studies attempting to demonstrate that epigenetic variation causes human obesity. Developmental programming of obesity via epigenetic mechanisms is indicated by pathway g–e. Adapted from Reference 99 with permission.

balance regulation, requiring more invasive studies. The second major obstacle, which again distinguishes epigenetic from genetic association studies, is that epigenetic marks are inherently malleable. Hence, although epigenetic correlates of obesity may be easily identified, these may represent a consequence rather than a cause of obesity. The third major obstacle is that in outbred populations (such as humans and some animal models) genetic variation is a major determinant of interindividual epigenetic variation (72). One particularly relevant example is the recent report (54) of a “DNA methylation atlas” of two tissues (muscle and fat) in three breeds of pigs; breed (i.e., genetics) was the major driver of methylation differences, which, coincidentally, correlated with differences in adiposity. In another study, interindividual variation in DNA methylation at the human fat mass and obesity-associated (FTO) gene was found to be largely determined by local haplotype (6). Also, a recent study of human abdominal subcutaneous adipose tissue (22) identified 149 methylation quantitative trait loci (meQTLs), i.e., methylation variants highly associated with neighboring single-nucleotide polymorphisms (SNPs). Hence, in human populations, interindividual epigenetic variation must be studied in the context of genetic variation. The complexity of studying the contribution of epigenetic dysregulation to human obesity is portrayed in Figure 1 (98).

Perhaps due to the difficulty of overcoming these obstacles, most of the strongest recent studies fail to provide compelling evidence that epigenetic dysregulation is a common cause of human obesity. For example, recent studies have identified associations between site-specific methylation in peripheral blood DNA and concurrent obesity either at candidate genes (41) or using genome-scale profiling (96). Similarly, methylation of long-interspersed elements (LINE-1) (as a proxy for whole-genome methylation) in visceral adipose tissue of obese individuals was inversely related to concurrent prevalence of metabolic syndrome (94). The reliance on measurements in peripheral blood (in the first two studies), unclear direction of causality of these associations, and potential for genetic confounding, however, makes these results difficult to interpret. Causality has been explored in a recent series of prospective studies by linking responsiveness to supervised weight loss to locus-specific DNA methylation in peripheral blood at baseline (60, 62). The physiological
significance of these predictive methylation marks is unclear, however, since it is unknown whether the variants in peripheral blood indicate systemic epigenetic differences. Also, it is possible that both the methylation variants and responsiveness to the weight loss program are determined by underlying genetic variation. Of course, given the extremely small proportion of overall variance in human obesity that is explained by locus-specific genetic variation (19), one might argue that relatively robust relationships between site-specific DNA methylation and subsequent weight loss could not possibly be mediated by genetics. If we assume, however, that DNA methylation is, like obesity, a complex polygenic trait, then strong associations between locus-specific DNA methylation and various measures of human obesity could indeed be genetically mediated.

To eliminate genetic confounding, several groups are studying pairs of monozygotic (MZ) twins discordant for obesity. One study (83) measured methylation at 11 differentially methylated regions of imprinted genes in salivary DNA from 16 pairs of adult MZ twins discordant for body mass index (BMI) but found no correlations between intrapair BMI differences and intrapair differences in DNA methylation. Another study (112) did find significant positive correlations between twin-twin differences in adiposity and DNA methylation at the promoter of the gene encoding serotonin transporter (SLC6A4) in peripheral blood leukocytes. Although these within-twin-pair correlations cannot be explained by genetics, as the authors noted, the direction of causality and the physiological relevance of SLC6A4 methylation in peripheral blood DNA remain to be determined.

One promising approach to gain insights into the importance of epigenetic dysregulation in human obesity is to study human metastable epialleles (MEs). At MEs, establishment of DNA methylation occurs stochastically in the very early embryo, resulting in interindividual variation in epigenetic regulation (70) that is neither genetically mediated nor tissue specific (100, 102). One of the best-characterized MEs is the murine A<sup>v</sup>y allele described above. At birth, isogenic A<sup>v</sup>y/a mice with differential DNA methylation at A<sup>v</sup>y appear indistinguishable (Figure 2a); the effects of differential epigenetic regulation at A<sup>v</sup>y are not fully apparent until adulthood (Figure 2b). Nonetheless, one could have taken a drop of blood from each of these newborn mice and, by measuring DNA methylation at the A<sup>v</sup>y locus, predicted with absolute certainty which one would become yellow and obese. Although the adult-onset obesity of A<sup>v</sup>y/a mice is caused by paracrine effects of aberrant agouti expression in the hypothalamus (109), the epigenetic lesion causing this misexpression is detectable in peripheral blood DNA at birth! Analogously,
Table 1  Two classes of interindividual epigenetic variation

<table>
<thead>
<tr>
<th></th>
<th>Metastable epialleles</th>
<th>Cell type–specific differentially methylated regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue specificity</td>
<td>Little/none</td>
<td>Tissue- or cell type–specific</td>
</tr>
<tr>
<td>Developmental establishment</td>
<td>Early embryo</td>
<td>Fetal and early postnatal</td>
</tr>
<tr>
<td>Studying association with obesity</td>
<td>Relatively simple</td>
<td>Difficult, especially in humans</td>
</tr>
<tr>
<td>Genomic prevalence</td>
<td>Rare</td>
<td>Widespread</td>
</tr>
<tr>
<td>Likely role in human obesity</td>
<td>Occasional</td>
<td>Extensive</td>
</tr>
</tbody>
</table>

*Adapted from Reference 99.

discovery of human MEs that affect the regulation of genes involved in body weight regulation may someday enable prospective studies to test the hypothesis that interindividual epigenetic variation causes human obesity. Using a genome-wide screen for systemic interindividual variation in DNA methylation, a small set of candidate human MEs was recently identified (103); work is currently underway to identify MEs associated with human disease, including obesity. Interestingly, the limited data available suggest that a CpG island (CpG-dense region) in the 3' end of the human proopiomelanocortin (POMC) gene may be an ME (48).

**IN WHAT CELLS/TISSUES IS EPIGENETIC DYSREGULATION MOST IMPORTANT FOR ENERGY BALANCE?**

Although human MEs may be discovered that explain some interindividual variation in body weight regulation, the cell type–specific nature of most epigenetic regulation means that, in most cases, studies in easily accessible tissues such as peripheral blood will not be sufficient to test causal hypotheses about the epigenetic etiology of obesity (Table 1). Hence, rigorous studies in appropriate animal models offer some of the best opportunities to advance our understanding of epigenetic mechanisms in body weight regulation. The first obvious question is: What cell or tissue types should be studied? Most studies thus far have focused on white adipose tissue (WAT) (because of its ability to act as an endocrine organ regulating energy balance) and hypothalamus (given its central role in energy balance regulation) (for review, see 99). For example, several studies have characterized cell type–specific epigenetic regulation of the leptin (LEP) gene in adipocytes compared to other cell types (57, 67, 84). Also (as discussed below), animal model studies of developmental programming of body weight regulation have focused on epigenetic alterations induced in either hypothalamus or adipose tissue by early nutritional exposures. Even in these tissues, however, major questions remain unanswered. For example, most studies of epigenetic regulation in the hypothalamus have studied whole hypothalamus. This approach has limitations, considering its complex cell type–specific and region-specific epigenetic specialization. The two principal cell types in neural tissue are neurons and glia, each comprising approximately half of the cells in most adult brain regions. Previous studies have documented important epigenetic differences between neuronal and nonneuronal brain cells (42, 55, 81). In addition to the gross dichotomy of neurons and glia, another level of hypothalamic heterogeneity is its distinct functional regions, termed hypothalamic nuclei. In particular, the arcuate nucleus of the hypothalamus (ARH) integrates peripheral signals such as leptin and insulin and, via diverse neuronal projections, relays anabolic/orexigenic or catabolic/anorexigenic signals to other hypothalamic nuclei including the paraventricular, ventromedial, and lateral hypothalamus (27, 80). The specialized functions and gene expression patterns of the hypothalamic nuclei are maintained by epigenetic mechanisms.
By integrating genome-scale DNA methylation data in the mouse hypothalamus at both the cell type–specific and region-specific levels, it was recently shown that early-postnatal increases in DNA methylation in neurons within the ARH are strongly associated with genes regulating neuronal differentiation (53), providing the first insights into epigenetic regulation within specific cell types within a specific hypothalamic nucleus. Although this represents important progress toward disentangling the epigenetic complexity of the hypothalamus, we still have far to go. For example, even within one hypothalamic nucleus (such as the ARH) there are various types of neurons (and various types of glia), each likely to be epigenetically specialized.

Regarding epigenetic studies in adipose tissue, an important but often neglected point is that WAT likewise constitutes a heterogeneous mixture of cell types. Many studies seem to assume that when genomic DNA is isolated from WAT, this represents predominantly adipocyte DNA. Indeed, it is sometimes stated that WAT is primarily composed of adipocytes (16). Given their large size, adipocytes do take up most of the volume in WAT, but in epigenetic studies the number of cells (i.e., the contribution to the total pool of DNA) is what matters. Widely various estimates of the cellular composition of WAT have been published over the past few decades. Just several years ago, however, two studies by the same group—using completely different methods—arrived at remarkably consistent estimates. In human WAT isolated surgically or by aspiration, adipocytes constituted only about 20% of the cells, quantitated either by multicolor flow cytometry following collagenase digestion (86) or by whole mount histology and triple-fluorescence staining (26). According to these solid data, stromal-vascular cells [composed mainly of mural cells, fibroblasts, and leukocytes (26)] are actually the predominant cell type in human WAT. Hence, to understand how epigenetic regulation of adipocytes (relative to their role as endocrine cells) contributes to energy balance regulation, it is inappropriate to study whole WAT [as many studies have done (see 99)].

Other than hypothalamus and WAT (and, as described below, the liver), a variety of tissues with important roles in body weight regulation remain largely unexplored from an epigenetic perspective. In addition to the widely recognized metabolic control of energy balance centered in the hypothalamus (27), it has been argued that cortico-limbic systems orchestrating cognition and reward may in fact contribute more importantly to obesity predisposition in the current environment of food abundance (8, 49). The involvement of some of these brain regions (including the neocortex, hippocampus, amygdala, nucleus accumbens, and ventral tegmental area) in energy balance is only recently being elaborated; future studies should consider the potential for individual epigenetic variation in these regions to affect cognitive control of food intake. Humans are born with a substantial depot of brown adipose tissue (30), which can expend excess calories to produce heat. Although most is lost by mid childhood, there is a recent resurgence of interest in the potential for residual brown adipose tissue to play a meaningful role in adult energy balance (37). Epigenetic mechanisms are likely involved in the process of shutting down the production of brown adipose tissue during childhood; understanding these may lead to effective interventions to prevent or treat human obesity. Comprising the largest single component of adult resting metabolic rate (108) and indispensable for physical activity, skeletal muscle plays a central role in energy balance. Interestingly, some of the earliest data supporting a role for DNA methylation in development was the demonstration that in vitro treatment of various (non-muscle) cells with the DNA-demethylating agent 5-azacytidine can induce differentiation toward contractile muscle cells (91). The classical transcription factor cascade that establishes myogenic fate is reinforced by epigenetic mechanisms at multiple levels, including DNA methylation, histone modifications, and noncoding RNA (79). Surprisingly few investigations, however, have considered whether epigenetic regulation (and dysregulation) in skeletal muscle contributes to obesity. Given its roles in endocrine regulation of satiety (e.g., ghrelin and glucagon-like peptide 1) and mediation of...
nutrient uptake, the gut plays a central role in energy balance. However, despite a long-standing recognition of the likely importance of epigenetic mechanisms in gastrointestinal development (97), this remains a relatively understudied area. One interesting review (61) recently proposed the novel hypothesis that early diet affects the establishment of the gut microbiome, which in turn produces metabolic by-products (including folate and butyrate) that induce permanent changes in epigenetic regulation in the developing gut. Clearly, we have a great deal to learn about how epigenetic dysregulation in various organ systems contributes to body weight regulation.

DOES DEVELOPMENTAL PROGRAMMING OF HUMAN BODY WEIGHT REGULATION OCCUR VIA EPIGENETIC MECHANISMS?

Developmental programming of energy balance occurs when nutritional exposures during critical ontogenic periods affect the establishment of body weight regulatory mechanisms, with permanent consequences for obesity risk (31). Classic studies of survivors of the Dutch famine at the end of World War II (74) showed that famine exposure during prenatal or early postnatal development has opposing effects on risk of obesity in adulthood. More recently, comparisons of siblings born before and after maternal bariatric surgery have provided compelling evidence that maternal obesity before and during pregnancy promotes obesity in offspring (47, 82). These observations in humans are supported by vast experimental data in diverse animal models (64, 101). Induced alterations of epigenetic gene regulation are now widely viewed as the leading candidate mechanism to explain developmental programming of obesity (32).

Despite an accumulation of loosely supportive evidence, however, the data substantiating this hypothesis remain somewhat sparse. Various factors, including the complexity of the causal pathway, the long time period between initial exposure and adult outcomes, and the potential for reverse causality conspire to make it particularly challenging to test for mediation via epigenetic alterations. According to the mechanistic construct of metabolic imprinting proposed 15 years ago (101), if induced epigenetic alterations indeed serve as a primary imprint that mediates the persistent effects of early nutrition on later risk of obesity, they should (a) be present directly after the imprinting period and in adulthood and (b) be measurable in vitro (ideally, at the level of specific cell types). Additionally, in human observational studies, the potential for genetic confounding must be considered.

Several recent human studies have tested whether maternal obesity before and during pregnancy affects establishment of DNA methylation in the offspring. A study of 319 newborns (59) reported no correlations between maternal prepregnancy BMI and methylation at LINE-1 repetitive elements in either placenta or cord blood DNA. In a smaller study of 57 mother/infant dyads (29), maternal prepregnancy BMI was positively correlated with DNA methylation at the promoter of the peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A) gene in umbilical cord of the infant. (Together, these studies suggest that such associations are likely to be locus specific.) More recently (25), DNA methylation was measured at several candidate genes in cord blood and chorionic villus of infants born to mothers with or without gestational diabetes mellitus (GDM). Interestingly, infants of mothers with GDM showed decreased methylation in both tissues at the imprinted mesoderm specific transcript (MEST) gene and the gene encoding glucocorticoid receptor [nuclear receptor subfamily 3, group C, member 1 (NR3C1)]. Although these positive findings are generally consistent with the hypothesis that maternal obesity (and/or GDM) affects epigenetic development in the human fetus, there are several caveats; epigenetic changes in placenta clearly cannot serve as a mechanism to sustain physiological changes in the soma, the persistence of the methylation changes in peripheral blood was not evaluated, and it is unclear if these indicate systemic effects that could plausibly affect body weight regulation. One
recent study indirectly examined the issue of effect persistence by prospectively relating neonatal DNA methylation in umbilical cord to measures of adiposity at age 9 (33). Methylation at a specific CpG site in the retinoid X receptor alpha (RXRA) gene was both inversely correlated with maternal carbohydrate intake in early pregnancy and positively correlated with the children’s adiposity at age 9. Most impressively, similar results were obtained in a second independent cohort. Although these findings do suggest a causal pathway from maternal diet to offspring DNA methylation to offspring adiposity, it will be important to determine if RXRA methylation in umbilical cord at birth indeed predicts that in physiologically relevant somatic tissues later in life.

Given the ability to conduct controlled experiments and obtain tissues of greatest interest, animal model studies provide an important complement to the human data. Several years ago, in A\(^\text{v}\) mice it was shown that maternal obesity causes transgenerational amplification of obesity (106); supplementation with a promethylation dietary supplement prevented this effect, suggesting potential mediation via induced alterations in DNA methylation. The protective effect of methyl supplementation was recently corroborated in a different model of maternal obesity. Adult offspring of dams fed a high-fat diet before and during pregnancy exhibited higher body weight, behavioral changes, and altered gene expression and DNA methylation in specific brain regions (12). Many of these changes were prevented if the mothers received a dietary methyl supplement in addition to the high-fat diet.

The rodent small litter (SL) model is a classic paradigm for studying persistent effects of early postnatal overnutrition (3); rats and mice suckled in SLs remain heavier and fatter throughout life. This model was recently used to test the hypothesis that the persistent effects of early postnatal overnutrition are mediated in part via induced DNA methylation changes in skeletal muscle (56). As an appropriate test for a primary imprint (101), mice were studied both directly after weaning on postnatal day 21 (P21) and in adulthood (P140). Skeletal muscle of SL mice had lower insulin receptor substrate 1 (Irs1) expression at both ages, and higher methylation at specific CpG sites in the Irs1 promoter at P140 (56). However, there were no group differences in Irs1 promoter methylation at P21; clearly, detecting epigenetic changes in adulthood does not necessarily indicate that they were induced directly by and persisted since the exposure. Another recent study of SL mice (52) tested whether their persistently altered body weight regulation is mediated by changes in DNA methylation in the hypothalamus. DNA methylation was measured at 24 candidate loci, both shortly after weaning (P25) and in adulthood (P180). Subtle but consistent alterations in DNA methylation were found at four loci at both ages, providing the first evidence that early postnatal overnutrition induces persistent epigenetic alterations in the hypothalamus. Since whole hypothalamus was studied, however, it is impossible to rule out that these changes reflect shifts in the cellular makeup of the hypothalamus rather than persistent differences within specific subpopulations of cells.

Recent studies suggest that maternal obesity during pregnancy may induce epigenetic changes affecting adipogenic potential in the offspring. In one study (9), offspring of overfed rat dams were cross-fostered at birth (so exposure to maternal obesity was limited to fetal development only). Both at P21 and P100, the stromal-vascular fraction from WAT of rats born to overfed dams exhibited increased adipogenic potential, and alterations in DNA methylation were detected in whole WAT at one age. Most interestingly, enhanced expression and reduced DNA methylation at the gene encoding the adipogenic transcription factor zinc finger protein 423 (Zfp423) (9) were independently corroborated in fetal offspring of mouse dams fed a high-fat diet (111), suggesting a potential causal pathway for how maternal obesity during pregnancy might enhance adipogenesis in the offspring.

Given the central role of the liver in lipid and carbohydrate metabolism, many investigators have examined whether induced epigenetic alterations in the liver may contribute to developmental
programming of obesity. In offspring of rats fed a high-fat diet during pregnancy and lactation, methylation of the cyclin-dependent kinase inhibitor 1A (Cdkn1a) promoter region was reduced in hepatic DNA at P2 but not at P27 (23). Another study compared wild-type (a/a) offspring born to either obese yellow or lean pseudoagouti A/y/a dams (51). The male offspring of obese mothers had elevated body weight and deranged hepatic lipid metabolism into adulthood. Additionally, they exhibited changes in hepatic expression of some genes and DNA methylation changes at others. Given the lack of coordination between the methylation and expression changes, and that methylation was not also assessed at weaning, these data do not provide strong evidence that hepatic DNA methylation changes act as a primary imprint to perpetuate persistent effects of exposure to maternal obesity. Interestingly, however, among the most strongly upregulated genes (51) were the major urinary proteins, in which, more than 20 years ago, long-term consequences of early environment on epigenetic regulation were demonstrated in liver of mice derived from nucleocytoplasmic hybrids (76). A related study in a sheep model employed embryo transfer at seven days of gestation (E7) to study the persistent effects of embryonic exposure to maternal obesity during the periconceptional period only (66). Although there were widespread alterations in hepatic gene expression in the offspring at P90, no associated changes in DNA methylation were detected.

These studies (most of which were published in just the past few years) provide many meaningful insights. A conceptual weakness affecting them all, however, is that there seems to be a tendency to neglect the fact that epigenetics is just one of several interrelated developmental mechanisms that potentially underlie developmental programming (101). In the murine hypothalamus, for example, the suckling period is a critical time for the establishment of DNA methylation patterns that distinguish diverse hypothalamic cell types (53). This same postnatal epoch, however, is also a critical period for formation of the neuronal projections from the ARH to other hypothalamic nuclei, establishing the “wiring” that is so critical for lifelong integrative regulation of energy balance (10). Any early postnatal nutritional exposure that can affect epigenetic development within specific cell types in the hypothalamus, therefore, will likely also cause permanent alterations to its neuroanatomic architecture, with consequent effects on intercellular signaling, hypothalamic function, and gene expression. One could postulate analogous interdependence pertaining to almost any organ system relevant to body weight regulation (Figure 3). For example, when examining epigenetic changes in adipocytes in response to maternal obesity, we must ask

Figure 3
The interdependency of epigenetic and morphological development means that these processes must be studied in an integrated fashion.
whether maternal obesity also affects the establishment of the vascular network in WAT. Similarly, studies examining the effects of early postnatal overnutrition on epigenetic development in myocytes should consider whether the exposure coordinately affects myofiber formation and skeletal muscle innervation. These examples raise fascinating research questions. For example, is intracellular epigenetic development guided by inductive intercellular interactions or vice versa? Is cell type–specific epigenetic development instructive to or permissive of associated morphological development? Answering these complex questions will in many cases require collaborative interdisciplinary efforts. But without a doubt, understanding environmental effects on development will require epigenetic outcomes to be studied in the context of other developmental processes that are being coordinately impacted.

Another outstanding question pertaining to developmental programming of obesity is, what are the specific inputs that lead to alterations in epigenetic regulation? The dietary methyl donor supplementation paradigm (12, 17, 102) assumes that providing an excess supply of methyl groups leads to DNA hypermethylation at specific loci simply by mass action. In most of the epidemiologic and animal model studies relevant to developmental programming of obesity, however, the exposures are not obviously related to one-carbon metabolism, and inputs at the molecular level are largely unexplored. It has been proposed that the obesogenic effect of maternal obesity during fetal development is mediated by hyperglycemia (68). In a prospective study of women with and without GDM, however, the greatest predictor of high BMI in the offspring at age 9 was high maternal prepregnancy BMI; no effect of maternal GDM was found (13). Animal models afford opportunities to improve our understanding of exactly how effects of maternal obesity during pregnancy and early postnatal overnutrition are transduced to affect developmental epigenetics, but these must be carefully characterized to determine their appropriateness. For example, a recent study showed that offspring born to obese \( A^v / a \) dams are growth restricted in utero (4); since infants born to obese women tend to have elevated birth weight (15), these data indicate that the \( A^v \) mouse is not generally an apt model in which to understand programming effects of maternal obesity in humans.

Inextricably linked to the question of exactly how early environment affects developmental epigenetics is the question of when these effects occur. Metabolic imprinting occurs only during a specific ontogenic period of susceptibility—the critical window (101); defining the critical window for candidate phenomena will help narrow the list of potential underlying mechanisms. Ontogenic periods when epigenetic mechanisms are undergoing establishment or maturation comprise potential critical windows when environment can affect these processes (104). Various critical windows are implicated in developmental programming of body weight regulation, including early embryonic (66), fetal (4, 47), and early postnatal development (3, 87). Detailed understanding of the developmental changes in epigenetic regulation occurring in specific cell and tissue types during these epochs will help identify candidate genes and gene regions most susceptible to induced alterations in epigenetic regulation. Performing such studies in both animal models and humans, when possible, will be necessary to determine if critical windows for epigenetically mediated metabolic imprinting are conserved across species. For example, early postnatal life is a critical window for cell type–specific epigenetic development in the murine hypothalamus (52, 53). Based on neuroanatomic developmental milestones, this period in the mouse is generally believed to be comparable with late fetal development in the human brain (36). It is currently unknown, however, when cell type–specific epigenetic regulation is established in the human hypothalamus.

Overall, although substantial progress has been made in recent years, more sophisticated studies will be required to rigorously test the hypothesis that developmental programming of body weight regulation occurs via induced epigenetic alterations. In human studies, if epigenetic marks will be measured in peripheral blood (or other easily accessible tissues), correlational analyses should
initially be conducted (using cadaver tissues, for example) to determine if the epigenotype in peripheral tissues is correlated with that in tissues in which the gene is expressed and thought to regulate energy balance. In both human and animal model studies, quantitative measurement of epigenetic marks (such as DNA methylation) both directly after the exposure and later in life is critical to document a persistent epigenetic change (which may serve as a primary imprint) (101). To the extent possible, determining whether epigenetic changes within a tissue reflect induced alterations in intracellular epigenetic regulation or induced shifts in cellular composition will require studying separately isolated cellular subpopulations. Finally, programming mechanisms should be studied in a holistic framework integrating epigenetic and anatomic development.

**DO EPIGENETIC MECHANISMS HAVE A GREATER IMPACT ON FOOD INTAKE OR ENERGY EXPENDITURE?**

Positive energy balance requires excess energy intake and/or deficient energy expenditure. Although the debate has raged for some time as to which component is more important in human obesity, a compelling argument was recently put forth that, in the current environment of high food availability, increasing energy expenditure (via physical activity) is likely to be more effective at combatting obesity than reducing energy intake (39). For decades, studies of developmental programming of body weight regulation have focused almost exclusively on satiety and mechanisms regulating food intake (21, 44). Except for a handful of animal model studies (7, 45, 95), the potential for early environment to affect development of pathways that regulate physical activity has been largely neglected. Now, in two completely different animal models, studies published in the past year (4, 52) provide strong evidence that developmental programming of obesity may be mediated primarily via induced alterations in spontaneous physical activity. In the SL mouse model of early postnatal overnutrition, the persistently elevated weight and adiposity of SL mice were associated not with increased food intake but rather with decreased spontaneous physical activity (52). Likewise, the obesogenic effect in offspring of obese $A^v/a$ mice (106) was found to be mediated by a persistent decrease in spontaneous physical activity (4). Interestingly, in both models the effect on physical activity was specific to female offspring.

These results suggest a completely new area of research: exploring epigenetic and other developmental mechanisms involved in establishing individual lifelong propensity for physical activity. More than 15 years ago, Rowland proposed that each person has an “activity-stat,” a physiological set point that regulates the individual’s level of spontaneous physical activity (78). Unfortunately, compared to our understanding of central regulation of food intake, we still know relatively little about the neurobiology of voluntary physical activity (28). In support of the activity-stat construct, recent twin and association studies have documented a considerable genetic component to human physical activity (5, 20), with heritability estimates ranging from ~0.3 to 0.8. Because genetic factors clearly contribute to regulation of physical activity, it is likely that interindividual epigenetic variation does also, suggesting a testable causal pathway in which early environment affects epigenetic mechanisms involved in the establishment of the activity-stat, with lifelong consequences for risk of obesity. Whereas a few candidate genes have been identified in human genetic studies (20), this hypothesis initially will be most easily tested in animal models. Physical activity is typically dichotomized as either voluntary exercise or spontaneous physical activity (SPA). In rodents, running wheel activity is the universal model for voluntary exercise (28), and home cage activity is viewed as indicative of SPA (46). Although there are neurobiological differences in the regulation of SPA and voluntary exercise, common neural pathways contribute to both (46). SPA appears to be primarily regulated in the hypothalamus (by key molecular players including orexins, agouti-related peptide, and neuromedin U) (46). The lateral hypothalamus likewise plays a pivotal role in
the regulation of voluntary exercise (77), but dopaminergic signaling (particularly in the nucleus accumbens and hippocampus) is also important (28). It will be fascinating to determine whether early environmental influences on the epigenetic regulation of candidate genes within specialized brain regions leads to permanent alterations of the activity-stat in experimental animal models.

DOES EPIGENETIC INHERITANCE CONTRIBUTE TO TRANSGENERATIONAL PATTERNS OF OBESITY?

In addition to their definitive mitotic heritability, epigenetic marks can in some cases be meiotically heritable and hence conveyed from one generation to the next. Whereas transgenerational epigenetic inheritance is well documented in plants, fungi, and worms (35), evidence in mammals is relatively sparse. However, the phenomenon of genomic imprinting (in which alleles are epigenetically marked and expressed in a parent-of-origin-specific manner) (35) and transposon-associated epialleles such as \( A^T \) (63) and \( Axin^{Fused} \) (71) do provide clear examples of transgenerational epigenetic inheritance in mammals. [The reader is referred to several excellent reviews on this topic (18, 35, 73).] Accordingly, there has been growing interest in the possibility that transgenerational epigenetic inheritance is contributing to the epidemic of obesity and metabolic syndrome in developed countries (1, 110). Two points, however, merit special emphasis here. The first is that not all transgenerational phenomena are epigenetically mediated. Transgenerational epigenetic inheritance requires the transmission of epigenetic information across generations. Just as the gene was initially postulated as the fundamental basis for genetic inheritance, the sine qua non of epigenetic inheritance is a specific epigenetic mark that is present both in the parental germ line and in the offspring (Figure 4a). Given the multiple biological safeguards against this (18), transgenerational effects of environmental exposures (particularly through the maternal lineage) can in many cases more parsimoniously be explained by recapitulation (i.e., the reoccurrence of an established pattern or characteristic). The most intuitive example is transgenerational recapitulation of language (Figure 4b); we generally speak the same language as our parents not because of

Figure 4

Transgenerational epigenetic inheritance must be distinguished from recapitulation. (a) Documenting epigenetic inheritance requires evidence that a specific epigenetic mark is actually inherited from the parental germ line to the offspring. Here, hypermethylation (black) of specific CpG sites in the haploid sperm genome is present both in the early embryo and subsequently in somatic tissues of the offspring. (In this example, the sperm hypermethylation is intended to indicate an environmentally induced epigenetic change, not the result of genomic imprinting or allele-specific methylation.) (b) Recapitulation (for example, the early acquisition of language) describes transgenerational reoccurrence of phenotypic traits and is distinct from transgenerational epigenetic inheritance. [Adapted from Elementary CORE Academy Clip Art (available in the public domain: http://www.usu.edu/coreacademy/Materials/ClipArt)].
genetic or epigenetic inheritance, but rather because of recapitulation. In rats, maternal caregiving behavior affects offspring postnatal development in a nongenetic fashion; female offspring suckled by more nurturing mothers (i.e., more licking, grooming, and nursing) become nurturing mothers themselves (107). Interestingly, this is associated with epigenetic changes in the hippocampus of the offspring, suggesting epigenetically mediated transgenerational recapitulation of behavior but not epigenetic inheritance. In another relevant example, transgenerational amplification of obesity occurs when \( A^{\gamma} \) is passed through the female germ line (106). This does not occur via epigenetic inheritance at \( A^{\gamma} \) (105); rather, female offspring of \( A^{\gamma} \) dams are “programmed” in utero for reduced physical activity and elevated adiposity (4). Hence, although potentially involving epigenetic mechanisms within each generation, this feed-forward transgenerational cycle does not constitute epigenetic inheritance.

Transgenerational effects transmitted through the male germ line are more often viewed as providing evidence of epigenetic inheritance. Indeed, several molecular mechanisms including DNA methylation, various proteins (including prions), and diverse classes of RNA molecules could plausibly mediate male germ-line epigenetic inheritance (35). However, the second point deserving emphasis is that in outbred populations (such as humans) it is possible that paternal environment selects for specific haploid sperm genomes (73). The altered physiological milieu of an obese father, for example, could drive clonal selection during sperm development (or even favor specific sperm genotypes in terms of motility or other factors affecting fertility). In this regard, it is noteworthy that many animal models purporting paternal transgenerational epigenetic inheritance of metabolic phenotypes have utilized either outbred (11, 65, 93) or hybrid strains (24). It will be important to test whether these effects are also observed in inbred populations of animals. Moreover, the widespread availability of genome-wide sequencing now makes it possible to directly test the sperm selection hypothesis. Given the complexity of these issues, a recent expert opinion piece (35) concluded, “there are not yet any clear-cut studies that would unambiguously demonstrate the transgenerational epigenetic inheritance of environmentally induced effects” (p. 232). Hence, although plausible, much work will be required to convincingly demonstrate that epigenetic inheritance plays an important role in human obesity.

CONCLUSIONS

The exquisite system for regulation of energy balance is established just once in each individual’s life. In addition to the instructions laid down in the genetic blueprint, environmental influences during critical ontogenic periods determine the outcome of this process, with permanent consequences for body weight regulation. Given their importance in orchestrating and stabilizing cellular differentiation, epigenetic mechanisms must play a central role in maintaining the various physiological set points that conspire to promote obesity in our current environment of food surfeit. Despite the complexity of the compounded research obstacles elaborated here, there is considerable reason for optimism. Advances in epigenetic reprogramming (89), for example, illustrate the enormous potential to manipulate epigenetic processes to shape and potentially reshape developmental outcome. Although the challenges are daunting, developing a thorough understanding of the epigenetic mechanisms underlying energy balance regulation should eventually offer outstanding opportunities to devise effective approaches for the prevention and treatment of human obesity.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.
ACKNOWLEDGMENTS

I gratefully acknowledge Adam Gillum for his assistance in developing the figures. R.A.W. is supported by NIH grant 1R01DK081557 and USDA CRIS #6250-51000-055.

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SESSION 2
The presence, role and clinical use of spermatozoal RNAs

Meritxell Jodar¹,², Sellappan Selvaraju¹,²,³, Edward Sendler¹,², Michael P. Diamond⁴† and Stephen A. Krawetz¹,²†*, for the Reproductive Medicine Network

¹Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI 48201, USA ²Center for Molecular Medicine and Genetics, C.S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, Detroit, MI 48201, USA ³National Institute of Animal Nutrition and Physiology, Bangalore 560030, India ⁴Department of Obstetrics and Gynecology, Medical College of Georgia, Georgia Regents University, Augusta, GA 30912, USA

*Correspondence address. Tel: +1-313-577-6770; Fax: +1-313-577-8554; E-mail: steve@compbio.med.wayne.edu

Submitted on April 5, 2013; resubmitted on May 20, 2013; accepted on May 23, 2013

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BACKGROUND: Spermatozoa are highly differentiated, transcriptionally inert cells characterized by a compact nucleus with minimal cytoplasm. Nevertheless they contain a suite of unique RNAs that are delivered to oocyte upon fertilization. They are likely integrated as part of many different processes including genome recognition, consolidation-confrontation, early embryonic development and epigenetic transgenerational inheritance. Spermatozoal RNAs also provide a window into the developmental history of each sperm thereby providing biomarkers of fertility and pregnancy outcome which are being intensely studied.

METHODS: Literature searches were performed to review the majority of spermatozoal RNA studies that described potential functions and clinical applications with emphasis on Next-Generation Sequencing. Human, mouse, bovine and stallion were compared as their distribution and composition of spermatozoal RNAs, using these techniques, have been described.

RESULTS: Comparisons highlighted the complexity of the population of spermatozoal RNAs that comprises rRNA, mRNA and both large and small non-coding RNAs. RNA-seq analysis has revealed that only a fraction of the larger RNAs retain their structure. While rRNAs are the most abundant and are highly fragmented, ensuring a translationally quiescent state, other RNAs including some mRNAs retain their functional potential, thereby increasing the opportunity for regulatory interactions. Abundant small non-coding RNAs retained in spermatozoa include miRNAs and piRNAs. Some, like miR-34c are essential to the early embryo development required for the first cellular division. Others like the piRNAs are likely part of the genomic dance of confrontation and consolidation. Other non-coding spermatozoal RNAs include transposable elements, annotated Inc-RNAs, intronic retained elements, exonic elements, chromatin-associated RNAs, small-nuclear ILF3/NF30 associated RNAs, quiescent RNAs, m-se-RNAs and YRNAs. Some non-coding RNAs are known to act as epigenetic modifiers, inducing histone modifications.
and DNA methylation, perhaps playing a role in transgenerational epigenetic inherence. Transcript profiling holds considerable potential for the discovery of fertility biomarkers for both agriculture and human medicine. Comparing the differential RNA profiles of infertile and fertile individuals as well as assessing species similarities, should resolve the regulatory pathways contributing to male factor infertility.

**CONCLUSIONS:** Dad delivers a complex population of RNAs to the oocyte at fertilization that likely influences fertilization, embryo development, the phenotype of the offspring and possibly future generations. Development is continuing on the use of spermatozoal RNA profiles as phenotypic markers of male factor status for use as clinical diagnostics of the father’s contribution to the birth of a healthy child.

**Key words:** spermatozoal RNA / embryogenesis / epigenetics modifiers / transgenerational epigenetic inherence / fertility biomarkers

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**Introduction**

Spermatogenesis is a highly regulated transcriptional, translational and posttranslational process. Transcription continues through the initial stages of spermiogenesis until development of the round spermatids. Those transcripts that are required to complete the transition to the spermatozoa are protected and maintained as ribonucleoproteins (RNPs). During this time, the majority of the cytoplasm with its RNA component is depleted as a cytoplasmic droplet, residual body (reviewed in RNPs). Those transcripts that are required to complete the transition to the spermatozoa are protected and maintained as ribonucleoproteins (RNPs). During this time, the majority of the cytoplasm with its RNA component is depleted as a cytoplasmic droplet, residual body (reviewed in RNPs). During this time, the majority of the cytoplasm with its RNA component is depleted as a cytoplasmic droplet, residual body (reviewed in RNPs).

The overall functional significance of many spermatozoal RNAs remains to be understood and their individual importance remains to be elucidated. Using the zona-free hamster oocyte/human sperm penetration assay, it has been established that sperm-specific transcripts (not present in the unfertilized oocyte) are transmitted to the oocyte upon fertilization (Ostermeier et al., 2004). They can also be translated into a functional protein as shown by the injection of the sperm borne PLCζ, (3-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta) transcript into the mouse oocyte, yielding a functional calcium oscillator (Sone et al., 2005). The distinctive landscape of non-coding RNAs that appears during the final stages of sperm maturation also strongly hints at their potential role in early post-fertilization and embryo development (Ostermeier et al., 2004; Liu et al., 2012). This has now been extended to the position that spermatozoal RNAs may epigenetically and transgenerationally affect phenotype (reviewed in Rando, 2012). These avenues remain to be explored.

A substantial number of spermatozoal transcripts appear compromised (Sendler et al., 2013), suggesting that they may simply be remnants echoing prior roles (Ostermeier et al., 2002). Even if only existing in mature sperm in this form, comparison of the differential transcript profiles between fertile and infertile patients has shown their utility as markers of fertility (reviewed in Waclawska and Kurpisz, 2012).

The primary focus of this review is to examine what a spermatozoal transcript profile may reveal with regard to the integrity of the spermatogenic pathway, characteristics of the mature sperm and their potential epigenetic and post-fertilization developmental functions. The potential
use of spermatozoal RNAs as biomarkers impacting human clinical diagnosis and agriculture will also be discussed.

**Methods**

Previously published human RNA-seq results (Krawetz et al., 2011; Sendler et al., 2013) and recent results from RNA sequencing of four representative sperm and two testes samples from a larger dataset were utilized. This set of sperm samples (D1–D4) were from four of the National Institutes of Health (NIH)/Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), Reproductive Medicine Network for Assessment of Multiple Intrauterine Gestations from Ovarian Stimulation dataset, whose partner withdrew from the study. Sperm and testes libraries were prepared for sequencing using the SEQR RNA Amplification kit (Sigma-Aldrich) and ENCORE® NGS Library System (NUGEN Technologies, Inc.). Amplification begins using 2 ng of total RNA for both sperm and testes samples. Library construction continues using 200 ng of amplified material. All libraries were paired-end sequenced using Illumina Hi-Seq 2500 and aligned using Novoalign (V2.08.01, NovoCraft Technologies Sdn Bhd), with distribution of reads shown as UCSC genome browser tracks, similar to that described (Sendler et al., 2013). The main figures in this review show sperm transcript characteristics of interest as portrayed by a single sample, while the supplementary figures show similarities among the four sequenced donors for each of these characteristics.

Genomic distribution (exonic, intronic and intergenic) of the large sperm RNA fraction was obtained from total sperm sample AS062 (GEO accession GSM721696) using Genomatix RegionMiner (Genomatix, v 3.0426). RT–PCR of specific intronic RNAs was performed using primer pairs that spanned the complete intron length in both sense and anti-sense orientations and were separately performed on both sperm and testes RNA in order to determine both the direction and relative abundance of these elements in sperm. Distribution of snc-RNA reads across transposable elements L1NE1 (Genbank accession M80343), variant forms of ERVL-MaLR (RepBase 16.09) and tRNAs were obtained from results of small RNA sequencing of sperm sample AS062 (GSM530234). The NCBI HomoloGene database (Coordinators, 2013) was mined to identify homologous human, stallion and bovine sequences. Gene abundance from stallion RNA-seq was based on the distribution the most abundant exon of mapped RNA sequence tags (Das et al., 2013). Literature was mined through i-HOP, PubMed, GoPubMed and GEPS (Dietze et al., 2009; Epple and Sherf, 2009; Leitner et al., 2009; Reavie, 2009).

**Spermatozoal RNAs-characterization and potential roles**

The most extensive sperm transcript profiling is available from human. RNA-seq has been performed for both small and large RNA fractions, with the latter examined as both total RNA and separate A⁺ and A⁻ fractions. The landscape of sperm RNAs evident from multiple normal individual samples has revealed a wealth of different classes of coding and non-coding transcripts (Fig. 1). Many relatively abundant RNAs that appear in all normal sperm samples are unannotated or of unknown function and appear uniquely in sperm, emphasizing the high level of complexity of the population of spermatozoal RNAs.

**Considerations**

A single spermatozoon contains ~50 fg of long RNA (>200 nt) and 0.3 fg of small non-coding (snc) RNA (<200 nt; Goodrich et al., 2013). This amount is ~200 times less than the quantity of RNA found in other cells (10 pg of long RNA and 1–3 pg of snc-RNAs), making somatic cell removal essential to resolve the unique pool of sperm transcripts. The absence of intact 28S and 18S sperm rRNA is often used to confirm the absence of somatic contamination (Johnson et al., 2011b; Goodrich et al., 2013). This was previously thought to be reflective of complete removal of rRNA with consequent enrichment of mRNA, but in fact was later resolved as the selective fragmentation of the majority of

![Figure 1](http://humupd.oxfordjournals.org/) Composition of spermatozoal RNAs. The distribution of the various classes of RNAs as determined by RNA-seq is shown. The most abundant class is ribosomal RNAs followed by mitochondrial RNA (mitoRNAs), annotated coding transcripts, small non-coding RNAs (snc-RNAs), intronic retained elements, Inc-RNAs and Transcribed regions of Unknown Coding Potential (TUCP), short expressed regions, transposable elements and annotated non-coding RNAs, including ssnr, ssnz, pri-miRNA, RNU.
sperm rRNAs (Ostermeier et al., 2002; Johnson et al., 2011b). To overcome these challenges the protocol to isolate spermatozoal RNA has undergone several revisions (Goodrich et al., 2013).

Resolution of the RNA population has been optimized with the use of Next Generation Sequencing (NGS) strategies. Typical RNA-seq library construction uses poly(A\(^+\)) selection to provide an enriched population of mRNAs excluding the otherwise overwhelming contribution of ribosomal and mitochondrial RNAs. While this provides an effective strategy to enrich for coding transcripts, it will exclude RNAs with short poly(A) tails and those that are not polyadenylated. In contrast, total RNA libraries are not subject to this limitation. This is an important consideration when characterizing sperm RNAs that have been observed to possess a large population of non-coding RNAs. However, sequencing the total population of RNAs comes at the cost of the increased representation of ribosomal and mitochondrial RNAs. Specific fractions of snc-RNAs (\(<200\) nt) are typically lost during library construction but can be recovered and sequenced with modified protocols and size selection (Krawetz et al., 2011).

### Coding RNAs

Both RNA-seq analysis in the human (Sendler et al., 2013) and RT–PCR of select bovine transcripts (Gilbert et al., 2007) suggests that the majority of coding RNA observed in sperm exists in a fragmented, or at the very least, atypical state. In contrast to equivalently selected testes RNA, RNA-seq profiles of the majority of poly(A\(^+\)) selected sperm transcripts exhibit a marked 3' bias. As illustrated in Fig. 2, this characteristic is indicative of transcript fragmentation in spermatozoal RNA. Ontological analysis of the relatively minor fraction of intact coding transcripts retained in sperm shows an enrichment of genes associated with male infertility, fertilization and early embryo development (Sendler et al., 2013). This is strongly suggestive of a functional role for these preferentially retained transcripts during the final stages of spermatogenesis or upon delivery. For example, \textit{INTS1} (Integrator complex subunit 1), involved in the transcription and processing of small-nuclear RNAs (snRNA) U1 and U2, is retained and appears by microarray analysis to increase after fertilization prior to zygotic genome activation (Vassena et al., 2011). \textit{INTS1} knockouts are embryonic lethal at the blastocyst stage.
stage (Hata and Nakayama, 2007). This is congruent with a potential role of this complex in the first steps of embryogenesis which could reflect the paternal contribution.

As in the above, RNA-seq also affords the ability to identify novel transcripts or isoforms. The production of transcript variants through the use of alternative promoters and splicing has been described in testes (Freiman, 2009). Interestingly, mature sperm display isoforms that are distinct in a variety of ways from those found in whole testes, indicating that these modifications arise only in the final transcriptionally active stages of spermatogenesis. This includes the sperm-specific isoform of PKM2 (Pyruvate kinase isozymes M1/M2), a key enzyme regulating glucose metabolism (Sendler et al., 2013). Further, approximately one quarter of the sperm transcripts show alternative sites of polyadenylation (APA), which maintain the integrity of the coding region, but exhibit an abbreviated 3′ untranslated region (UTR) (Fig. 3). This trait is common in testes (Liu et al., 2007) and may serve to modulate transcript stability, localization and/or transport. Additionally, this modification may impact translation by affecting the ability of different regulatory proteins and miRNAs to bind to the alternative UTR (Di Giammartino et al., 2011). It has recently been reported that Bromodomain testis-specific protein (BRDT) actively modulates APA in testes (Berkovits et al., 2012). Male mice lacking the first bromodomain of BRDT are infertile (Shang et al., 2007) and spermatozoal transcripts from such knockouts possess longer 3′ UTRs (Berkovits et al., 2012). This likely emphasizes the critical nature of APA observed in human sperm. Lastly, sperm RNA-seq has identified many examples of abundant predicted transcripts (such as ORFs) that are not observed in somatic cells and are of low abundance in testes. Together, variations in expression and form of coding RNAs found in sperm likely have a significant impact on both the regulation and function of this class of RNAs.

Small non-coding RNAs

It has been proposed that the germline genome is protected through paternal small non-coding miRNAs, siRNAs, piRNAs, qRNAs and repeat associated RNA mechanisms (reviewed in O’Donnell and Boeke, 2007; Bourc’his and Voinnet, 2010; Krawetz et al., 2011; Siomi et al., 2011). For function, the parental RNAs must be of sufficient quantity and quality to interact with their target for successful embryo development (Bourc’his and Voinnet, 2010). Recent sequencing of the small (18–24 nt) RNA population from multiple normal human donors has also shed light on the complexity of the snc-RNA population present in spermatozoa. The majority of sperm snc-RNAs correspond to four major classes: repetitive elements, transcription start sites (TSS)/promoter associated, piRNAs, and miRNAs, with other classes such as snRNAs, snorRNAs, mse-tsRNA and YRNAs representing a relatively minor portion (Krawetz et al., 2011). Additional snc-RNA sequencing reads correspond to unannotated regions of the genome and portions of coding and non-coding transcripts. Whether these short fragments serve a particular role, e.g. regulating their longer precursor elements, or are merely end-points of fragmentation is not yet known.

miRNAs

The most well-characterized non-coding sperm RNAs are miRNAs, which have been shown to modulate various stages of spermatogenesis (reviewed in Moazed, 2009). Along with siRNAs, these RNAs typically

Figure 3  Alternative polyadenylation of sperm transcripts. GIGYF2 encodes a protein that interacts with GRB10 and may be involved in the regulation of tyrosine kinase receptor signaling. The 3′ UTR region of GIGYF2 gene is highlighted (upper panel). RNA-seq (lower panel) of this specific region exhibits a truncated 3′ UTR in sperm (green). This contrasts with coverage extending over most of the UTR observed in testes (black). (See Supplementary data, Fig. S1 for more details.)
function to regulate expression by inhibiting or activating translation or targeting mRNAs for degradation usually by binding to a 3′UTR target sequence (reviewed in Gangaraju and Lin, 2009). They are typically transcribed by polymerase II as larger precursors that are then processed to an intermediate form by DROSHA (Ribonuclease 3) and DGR8 (DiGeorge syndrome critical region 8). These precursors are subsequently transported to the cytoplasm and further matured by Dicer, an RNase III endonuclease, to their mature 20–24 nt functional form. They are then incorporated into an Argonaute containing RNP forming RISC, RNA-induced silencing complex. Spermatogenic-specific Dicer or DROSHA knockouts arrest spermatogenesis (Hayashi et al., 2008; Korhonen et al., 2011; Wu et al., 2012), confirming their essential role.

While many miRNAs are conserved among different species, some are species-specific (Curry et al., 2009; Krawetz et al., 2011; Govindaraju et al., 2012; Peng et al., 2012; Das et al., 2013). The majority of mature spermatozoal miRNAs are also observed in testes (Landgraf et al., 2007), but most of their computationally predicted 3′UTR targets are absent in mature sperm (Krawetz et al., 2011). Recent studies suggest that some miRNAs act as transcriptional regulators by targeting other regions, e.g. promoters (Kim et al., 2008; Place et al., 2008). Perhaps in the transcriptionally quiescent sperm, they provide a signal for early embryonic histone replacement (Johnson et al., 2011a) or transcriptionally poise the genome for early embryonic expression or affect epigenetic modification (Khraiwesh et al., 2010). Support for this notion has been gained from the observation that more than 10% of all snc-RNAs map to histone-enriched TSS and promoters. These novel RNAs, termed quiescent RNAs (qRNAs), are similar to tiny RNAs (tiRNAs). They are associated with the TSS region but not enriched in GC regions or correlated with histone modifications (Krawetz et al., 2011). The tiRNAs derived from regions adjacent to TSS may indirectly modulate local chromatin states through other binding factors (Taft et al., 2011). However, the function of qRNAs remains to be established (Krawetz et al., 2011).

The most abundant sperm miRNA in the human is miR-34c (Krawetz et al., 2011). It has also been identified in stallion and mouse (Peng et al., 2012; Das et al., 2013), and has been shown to be essential for the first cleavage division in mouse zygotes (Liu et al., 2012). Except for miR-34c-5p, where we have a glimpse, their mechanism of action and functional role in spermiogenesis and/or fertility remain to be fully delineated (Curry et al., 2011; Krawetz et al., 2011; Govindaraju et al., 2012; Das et al., 2013). For example, in mouse testes, miR-34c expression is p53 independent (Bouhallier et al., 2010), whereas miR-34c targets p53 in cancer cells (Corney et al., 2007). This is somewhat in line with their ability to influence growth status during periods of rapid growth like oncogenesis (reviewed in Shvidasani, 2006; Croce, 2009; Luningschrorr et al., 2013). Spermatooza also contain several intact miRNA precursors (pri-miRNAs, 100–150 nt). Since the zygote has the capacity to process immature miRNAs (Liu et al., 2012), the potential role of the pri-miRNAs requires consideration. For example, pri-miRNA-181c is the most abundant immature miRNA in human spermatozoa. Predicted targets of this miRNA include those critical to early embryonic development and globally decrease at the 4–8 cell stage of human embryo development (Vassena et al., 2011; Sendier et al., 2013). One specific target of miR-181c is CARM1 (Coactivator-Associated aRginine Methytransferase 1), an embryonic stem cell pluripotency factor. CARM1 directly catalyzes the methylation of H3 arginine in the promoters of POU5F1 (POU domain, class 5, transcription factor 1) and SOX2 (Transcription factor SOX-2). This forms an active chromatin mark coinciding with induction (Xu et al., 2013). At the 2-cell stage overexpression of CARM1 in one of the blastomeres predisposes its derivatives to contribute to the pluripotent cells of inner cell mass (Torres-Paliffa et al., 2007). It is tempting to speculate that through the delivery by sperm miR-34c and pri-miR-181c, the division and partitioning of the targeted CARM1 are, respectively, ensured, thereby decreasing some pluripotency factors in one blastomere while pushing the other towards the trophoectoderm lineage.

piRNAs
Piwi-interacting RNA (piRNAs) are abundant in the mammalian male germline (reviewed in Girard et al., 2006) and their presence in spermatooza has been confirmed in several species (Krawetz et al., 2011; Kawano et al., 2012; Peng et al., 2012). They are typically organized in the genome as clusters that range up to 100 kb in size. piRNAs precursors are processed to their 23–32 nt mature form by a Piwi protein-dependent mechanism (reviewed in Ishizu et al., 2012). Although not clearly articulated, several functions have been proposed for this class of transcripts. These include regulation of RNA stability and epigenetic states as well as protecting the germline genome from transposition (reviewed in Aravin and Hannon, 2008; Gangaraju and Lin, 2009). During spermatogenesis, the activation of mobile transposable elements is suppressed by piRNAs. The absence of these regulatory RNAs can induce spermatogenic arrest (Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007). The piRNAs may act in a similar protective manner during early embryo development as the genome undergoes extensive demethylation and remethylation. They could protect genome integrity by binding to DNA and thus preventing the action of various classes of repetitive and transposable elements like SINE, LINE, MER and LTR at specific stages of embryogenesis (Krawetz et al., 2011).

Potentially novel classes of sperm-ncRNAs, sperm RNAs (spRs) -12 and -13, were recently identified in mouse spermatozoa. They are ~20 nt in length and are likely derived from additional processing of mature piRNAs (Kawano et al., 2012). This has defined yet another snc-RNA biogenesis pathway. These abundant spRs are maintained post-fertilization until the blastocyst stage, suggestive of their potential role in early embryo development perhaps to ensure genome integrity at this critical stage.

Confrontation and consolidation
The classes of spermatozoal RNAs described above may play an integral role in the confrontation and consolidation mechanism that has been described in plants and animals (Bourc’his and Voinnet, 2010; Goring and Indriolo, 2010; Krawetz et al., 2011; Miller and Iles, 2013). When the sperm and oocyte meet, it is necessary to ensure the compatibility of the genomic contributions of each parent to ensure that their combination will be conducive to embryonic development. During confrontation, the pairing of paternal RNAs, such as repeat associated spermatozoal RNAs, with complementary maternal repetitive elements, may activate or suppress their partner. Once compatibility between gametes is assured, the RNA-based information could then be transferred to a chromatized state, i.e. consolidation, likely by modifying the epigenome. Interestingly, snc-RNAs have been associated with heterochromatization, perhaps consolidation (reviewed in Lippman et al., 2004; Lippman and Martienssen, 2004). Examples of this surveillance pathway and its consequence are apparent when one considers the various outcomes that can occur when two different species or breeds are crossed to produce hybrid offspring. The
potential consequences include failure at fertilization, inappropriate embryo development or compromised fertility of the offspring. The latter outcome is best characterized by the mule (mare and donkey hybrid) which is infertile/sterile (Short, 1975). Similarly, blastomere formation is halted at the 8-cell stage when hybrid embryos are created by in vitro fertilization of a water buffalo (Bubalus bubalis) oocyte with bovine (Bos taurus) spermatozoa. This parallels a failure to undergo zygotic genome activation leading to developmental arrest (Patil and Totey, 2003). Perhaps this reflects an incompatible paternal contribution in which the specific mechanism necessary to activate or suppress elements necessary for embryo development is absent.

Transposable elements

A large proportion of human spermatozoal snc-RNAs map to repetitive elements. The most abundant repeat classes represented in mature spermatozoa are the various members of the, LTR, SINE/ALU and LINE families of transposable elements (Krawetz et al., 2011). The role that transposable elements may play in the germline and early embryo remains controversial (Beraldi et al., 2006; Georgiou et al., 2009; van der Heijden and Bortvin, 2009). For example, LINE1 has a dynamic activity during early embryo development. Interrupting this activity results in embryonic arrest at the 2- or 4-cell stage (Beraldi et al., 2006). This may reflect the disruption of LINE1-associated reverse transcriptase (Pittoggi et al., 2003; reviewed in Spadafora, 2008). At this stage, LINE1 transcription is thought to be mediated by polyurine enriched LINE1 RNA fragments. These fragments form a triple helix within several regions of LINE1 potentially serving as a scaffolding that alters the association of chromatin modifiers and the transcriptional machinery (Fadloun et al., 2013) thereby promoting their own expression. Perhaps the large number of LINE1 fragments observed in and delivered by sperm activates this feedback loop. The distribution of short reads derived from RNA-seq of human spermatozoal snc-RNAs over the length of LINE1 exhibits very high enrichment of specific fragments, with some being homopurine polymers or near polyurine in sequence (Fig. 4). A similar distribution of highly enriched fragments is observed for other transposable elements in sperm such as ERVL-MaLR, for which early embryo function is also noted (Kigami et al., 2003; Inoue et al., 2012) and for which sperm-delivered fragments may play a similar activating role. It remains to be determined whether the transcribed transposable elements found in sperm such as SINE/ALU, which are complementary to coding and/or regulatory regions, may modulate host gene expression in early embryogenesis, since such behavior has been observed in other developmental processes (Polak and Domany, 2006). It appears though, that far from being ‘junk’ or simply an unintended consequence of global genomic demethylation, perhaps the abundant repeat associated sperm RNAs modulate other regulatory elements in the early embryonic stages of development.

‘Other’ sperm RNAs

The distribution of poly(A\(^{+}\)) selected sperm and testes sequencing reads demonstrates that while ~10% of testes reads correspond to intergenic or intronic regions, more than two-thirds of sperm reads align to regions of unknown annotation or function (Sendler et al., 2013). Many of these unidentified yet prominent transcripts appear in all sperm samples from normal individuals and show little or no expression in other cell types. Although the majority of these RNAs have no known function, their abundance suggests that they may play a significant functional role. Whether this occurs during the final stages of sperm maturation, at delivery to the oocyte or during early preimplantation development remains to be determined. The observation that these transcripts correspond to genomic regions that retain histones, specifically H3K4me3, a histone modification correlated with transcriptional activity, suggests that sperm chromatin is uniquely structured to facilitate the transcription of...
these RNAs and that they are not artifacts (Hammoud et al., 2009; Sendler et al., 2013). Some classes of these elements with specific characteristics are described below.

**Intronic retained elements**

Non-coding RNAs contained within introns of coding mRNAs have been described in other systems (Hill et al., 2006). Both precursor miRNAs and snoRNAs often originate from these regions (Kiss et al., 1996; Lin et al., 2006; Li et al., 2007b). At least 200 distinctive non-coding sperm transcripts appear to be full-length introns specifically retained in sperm (Sendler et al., 2013). The mechanism by which they escape degradation after splicing remains to be defined. Interestingly, the corresponding mRNAs are often abundant in testes, while in sperm these coding segments show a marked reduced presence. Genes from which these intronic elements are derived do not classify into a distinct ontological category nor do they correlate with a specific pattern of early embryonic expression (Vassena et al., 2011). No evidence has been found to show that the intronic elements observed in sperm comply with the computationally predicted models for precursor elements of either sno- or mi-RNAs.

Figure 5 shows an example of the sperm transcript DNAH1 (Dynein heavy chain 1, axonemal) in which several introns are retained. Several abundant intron spanning RNAs are apparent. Examination by RT–PCR of three such elements found within transcripts, TRIM66 (Tripartite motif-containing protein 66), KAT8 (Histone acetyltransferase KAT8) and QRICH1 (Glutamine-rich protein 1), confirmed that they were transcribed in the same orientation as the transcript in which they are embedded. Interestingly, they are present in much higher levels in sperm than in testes (unpublished data). These observations suggest that they are retained in mature sperm perhaps as part of a separate regulatory mechanism. The recent suggestion that some intronic ncRNAs are specifically regulated by a drop in temperature (Heo and Sung, 2011) to target their host transcripts for rapid degradation is intriguing. Spermatogenesis is temperature-sensitive and this may act as a physiological monitor.

**Long non-coding RNAs**

Long non-coding RNAs (lnc-RNAs) range in size from ~200 to 10,000 nt and are scattered throughout the genome. They are generally classified as a function of their relative position to protein coding genes (reviewed in Ponting et al., 2009). This includes intronic or intergenic regions where strand orientation cannot be directly determined, exonic regions primarily derived from the reverse strand, or from pseudogenes and retrotransposons. Spermatogenesis is in part regulated through the action of lnc-RNAs (Nolasco et al., 2012) some of which are certain to be antisense. Specifically, the abundance of antisense transcripts in testes may add to the mechanisms strictly regulating expression and function during spermatogenesis (Lee et al., 2009a). Lnc-RNA mechanisms have been described to operate in somatic cells at both the transcriptional or posttranscriptional levels (reviewed in Mercer et al., 2009; Lee, 2012; Rinn and Chang, 2012). At the transcriptional level this is accomplished by promoting specific histone modifications. For example, HOTAIR (HOX transcript antisense RNA) can modulate transcription through chromatin structure by recruiting PRC2 (Polychrome Recruiting Complex) to the HoxD locus thereby repressively marking histone H3 (Tsai et al., 2010). Transcription can also be modulated through the interaction of an lnc-RNA with an associated promoter region as exemplified by DHFR (Dihydrofolate reductase). Transcription of this gene by an alternative promoter results in a regulatory transcript that targets the usual promoter via triplex formation repressing the expression of DHFR.
Lnc-RNAs can also function post-transcriptionally during splicing (reviewed in Yoon et al., 2012a). For example, MALAT1 (Metastasis-Associated Lung Adenocarcinoma Transcript 1) regulates the alternative splicing of a subset of transcripts through its interaction with splicing factors (Tripathi et al., 2010). Translation of RNAs can also be modulated through the interaction of lnc-RNAs with specific repressors (Yoon et al., 2012b) or by general interference with the translation initiation complex (reviewed in Kindler et al., 2005). Finally, a similar regulatory effect can be achieved by modifying RNA stability. This can be affected through sense–antisense pairing that protects the target from miRNA-mediated degradation (Faghihi et al., 2008).

A number of predicted lnc-RNAs (Trapnell et al., 2010; Cabili et al., 2011) correspond to both abundant poly (A⁺) and poly (A⁻) sperm transcripts. In some cases, the sperm lnc-RNAs appear to represent different isoforms than the predicted forms. This is not unexpected given that the list of lnc-RNAs was primarily derived from expression in somatic cells. In many cases, the sperm lnc-RNAs appear more abundant in sperm than in testes (Fig. 6). The function of such lnc-RNAs in sperm maturation, fertilization and early embryo development remains to be explored.

A specific class of lnc-RNAs overlaps coding transcripts and they are derived from the reverse strand (NAT: natural antisense transcripts). Several NAT have been described in mature spermatozoa (Ostermeier et al., 2005; Sendler et al., 2013). Roles in gene silencing, selective transcript editing, promoter inactivation and epigenetic modifications of the genome have been revealed for such RNAs (Lavorgna et al., 2004; Lapidot and Pilpel, 2006; Faghihi and Wahlestedt, 2009; Werner et al., 2009; Werner and Swan, 2010). For example, independent regulation of sense–antisense pairs of some nc-RNAs at specific stages of cellular development (Cawley et al., 2004; Katayama et al., 2005; Werner et al., 2007) results in the rapid processing of the longer transcripts into short ~23 nt fragments (Borsani et al., 2005; Carlile et al., 2008). There are several examples of abundant of 100–300 nt sperm RNAs that overlap either the coding or UTR portion of an otherwise low-expressed or absent transcript. A striking example is the antisense transcript that overlaps ARFGEF1 (Brefeldin A-inhibited guanine nucleotide-exchange protein 1). This transcript is an ADP-ribosylation factor, required for maintenance of Golgi structure and function (Manolea et al., 2008). The corresponding fragment is abundant in sperm and corresponds to the middle of the 5'UTR. The full-length transcript is present in testes but virtually absent in sperm (Fig. 7). It would appear likely that this may be one example of a processing mechanism in sperm perhaps targeting specific transcripts for rapid degradation and which may be achieved throughout spermatogenesis through a variety of means. In addition to the possible action of NATs in the physiology of spermatozoa, some NATs present in mature spermatozoa overlap genes involved in early embryo development (Ostermeier et al., 2005) suggesting that such antisense RNAs may also have a role during fertilization and in the first steps of embryogenesis (Li et al., 2002).

Specific classes of lnc-RNAs

A number of elements from previously identified nc-RNA classes are abundant in the longer fraction of sperm RNA. These include Chromatin-associated-(CAR) and some small-nuclear ILF3/NF30 associated-(snaR) RNAs. CARs are found associated with chromatin and may act in cis or in trans to influence genomic architecture or regulate gene expression (Rodriguez-Campos and Azorin, 2007; Mondal et al., 2010). Three intronic and three intergenic regions in sperm, which show substantial sequencing coverage, overlapped with CARs recently identified in testes (Fig. 6). Unique sperm lnc-RNAs isoforms. A 30 kb region of chromosome 3 containing a series of putative lnc-RNAs as identified by the Human Body Map lincRNA UCSC track (Trapnell et al., 2010; Cabili et al., 2011) is shown in upper panel. Although low-level expression of a number of identified lnc-RNAs is evident across this region in testes, a single highly expressed two exon RNA is observed in sperm (lower panel). Many junction reads, as measured by RUM (Grant et al., 2011) (box), confirm that these two exons are part of a single spliced transcript, which was not previously identified as a unique lnc-RNA isoform. (See Supplementary data, Fig. S3 for more details.)

Figure 6

Downloaded from http://humupd.oxfordjournals.org/ at The National Academies on February 9, 2015
identified in human fibroblast (HF) cells (Mondal et al., 2010). Perhaps a significant number of the unidentified lone elements in sperm are CARs and serve to aid the unique packaging requirements of the paternal genome. Several of the small NF90-associated RNAs (snaR) including snaR-G1 are also abundant. While their role remains uncertain, it is significant that snaR-G1 resides within the promoter region of the embryonic developmentally important human chorionic gonadotrophin (hCG1) (Parrott and Mathews, 2007). The level of this sperm snaR is elevated relative to that observed in testes, which is already at a level that is \( \approx 100 \) times that of somatic tissues. This is certainly suggestive of a prominent functional role for these transcripts in mature spermatozoa.

tRNA-derived snc-RNAs and YRNAs
The majority of mouse and human mse-tsRNAs (mature-sperm-enriched tRNA-derived small RNAs) correspond to specific cleavage products (Krawetz et al., 2011; Peng et al., 2012). They typically represent 5’ end fragments between the D-loop and anticodon loop (Peng et al., 2012). However, as illustrated in Fig. 8, unlike mouse, human-specific mse-tsRNAs are also derived from the 3’ region (Krawetz et al., 2011; Peng et al., 2012). While initial inspection suggested that the fragmentation of rRNAs and tRNAs is to ensure translational silence (Johnson et al., 2011b), perhaps some of the mse-tsRNAs are functional. For example, their action as a stress responder appears conserved as far back as bacteria. In response to stress, an abundant 5’ end fragment of Val-tRNA in Haloflexaz volcanii specifically targets and inhibits the translational machinery (Gebetsberger et al., 2012). This effect can be mimicked by the transfection of natural and synthetic tRNAs fragments (Ivanov et al., 2011). Knock-down of trF-1001, (a 3’ end tRNA-derived fragment from the Ser-TGA tRNA precursor) inhibits cancer cell proliferation (Lee et al., 2009b), suggesting a role in maintaining proliferation. Perhaps mse-tRNAs acts in a similar manner upon delivery to the oocyte (Peng et al., 2012).

A minor portion of the snc-RNAs corresponds to YRNAs. YRNAs are a small cytoplasmic RNAs (85–115 nt) associated with Ro protein forming a RNP complex. In humans, specific YRNAs fragments that bind the Ro RNP complex have been observed (Krawetz et al., 2011). It has been proposed that this RNP complex participates in a quality control pathway for misfolded small RNAs (Stein et al., 2005). After ultraviolet irradiation, bacterial YRNAs and Ro protein increase suggesting that Ro RNP complex could have a role in the recognition or repair of DNA damage (Chen et al., 2003). Similarly, this RNP complex could act in the first steps of embryogenesis to initiate repair.

Spermatozoal RNA as epigenetic modifiers
Sperm-specific RNAs can influence fertilization and early embryo development but may also epigenetically modify the phenotype of the offspring (reviewed in Cuzin et al., 2008; Lalancette et al., 2008a; Johnson et al., 2011a; Hamatani, 2012; Rando, 2012). Following somatic cell nuclear transfer, some pathological changes in the placenta and congenital defects in the fetus as well as in the offspring are observed (reviewed in Shiel et al., 1999; Lanza et al., 2000; Xu and Yang, 2003). These changes reflect inappropriate epigenetic reprogramming of the donor and recipient cells leading to aberrant inner cell mass and trophectoderm formation (Niemann et al., 2008). It is possible that this mechanistic perturbation reflects the absence of the early effects of paternal elements (Krawetz, 2005; Krawetz et al., 2011) as somatic cells lack spermatozoal specific RNAs (Krawetz et al., 2011) that are likely to be integral to this
pathway. This could involve targeting by epigenetic RNAs (Krawetz et al., 2011) that modify chromatin structure (Taft et al., 2011), e.g. through DNA methylation (Khraiwesh et al., 2010).

While the role of RNAs as modifiers of the epigenome altering gene expression is generally accepted, their transmission through the mammalian germline has been debated (Rassoulzadegan et al., 2006). The transgenerational epigenetic effect of paramutation is well established in the plant kingdom. Paramutation is the transfer of an epigenetic state to an unlinked homologous wild-type allele (paramutated allele) yielding a heritable phenotype in absence of an altered gene. Paramutation was first observed in maize (Brink, 1956) of the \( r1 \) gene that affects plant color. The most well-characterized paramutated gene in maize is \( b1 \), which employs the siRNA silencing pathway to modify methylation. At least three-repeat sequences upstream of the \( b1 \) gene are required to exceed a threshold to observe the effect conveyed by the siRNAs (reviewed in Arteaga-Vazquez and Chandler, 2010) for full penetrance and stability across generations. Paramutation in mammals seems to be reflected in complex processes like color, growth and disease, e.g. cardiac hypertrophy (Rassoulzadegan et al., 2006; Wagner et al., 2008; Grandjean et al., 2009). For example, while complete disruption of the mouse \( Kt \) (Mast/stem cell growth factor receptor \( Kt \)) gene was lethal, the heterozygote and paramutated animal presented a white tail and feet. Spermatozoa from heterozygote and paramutated progeny were enriched with truncated \( Kt \) RNA. Interestingly, microinjection of heterozygote RNAs or miRNAs that target \( Kt \) (miR-221, miR-222) into fertilized oocyte induced the heterozygote phenotype (Rassoulzadegan et al., 2006). Other miRNAs, with a paramutation function that display a transgenerational effect have been described. These include miR-1 and miR-124 that paramutate \( Cdk9 \) (Cyclin-dependent kinase 9) and \( Sox9 \) (Transcription factor SOX-9), respectively. Although, the mechanism remains unknown, transactivation through methyltransferases is being aggressively pursued.

Paramutation of human genes may reconcile familiar predisposition of some non-Mendelian genetic diseases. Transgenerational epigenetic effects could be a mechanism to confer increased competitiveness that allows the progeny to adapt to new environments to which the parents have been exposed. Alternatively, exposure to a toxic environment could hijack this response compromising the offspring. Transgenerational genetic effects occur when genetic factors in one generation affect the phenotype in the following generations without inheritance of the parental genetic factor. For example, daughters from genetically equal fathers but with a different \( Y \) chromosome differ in behavioral phenotype. This is remarkable, considering the low number of genes present on the \( Y \) chromosome (Nelson et al., 2010). On the one
hand, it may simply reflect the high level of recombination during spermatogenesis yielding diverse gamete genomes (Lu et al., 2012; Wang et al., 2012). On the other hand, the variability may reflect epigenetic and/or transcript sharing among the maturing sperm cells through the cytoplasmic bridges (Caldwell and Handel, 1991). Evidence for the latter is provided by the apparent unequal distribution of transcripts among each sperm (Wykes et al., 2000).

Several reviews are available that provide examples of transgenerational epigenetic effects in mammals (reviewed in Jirtle and Skinner, 2007; Curley et al., 2011; Rando, 2012). For example, the progeny of mice receiving a high-fat diet during pregnancy are at increased risk of obesity and metabolic disease with subsequent passage through the paternal lineage (Dunn and Bale, 2011). Exposure to endocrine disruptors in female rats during gonadal sex determination increases the incidence of F1 male infertility. Some consequences of transgenerational inheritance are reflected by changes in the pattern of male germ cell methylation (Anway et al., 2005). Perhaps spermatozoal non-coding RNAs known to regulate DNA methylation and chromatin structure are components of transgenerational epigenetic mechanisms (reviewed in Lee, 2012; Rinn and Chang, 2012).

Models in agriculture

Animal models including cattle (Adams and Pierson, 1995; Burns et al., 2005) and equine (Carnevale, 2008) have proved essential to developing various assisted reproductive techniques (Bavister, 2002) and for providing a framework to study human reproductive disease (reviewed in Matsunari and Nagashima, 2009). While many critical sperm transcripts are conserved among different mammals (mouse, sheep, cattle, horse, pig and human) some appear species-specific (Card et al., 2013; Das et al., 2013; Sendler et al., 2013). Several orthologous spermatozoal transcripts observed among human, bovine and stallion are presented in Table I.

<table>
<thead>
<tr>
<th>Transcript name (percentile ranking in human)</th>
<th>Transcript symbol</th>
<th>Reported function*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protamine 1 (0.99)</td>
<td>PRM1</td>
<td>Sperm DNA condensation</td>
<td>Cho et al. (2001) (reviewed in Miller et al., 2005; Oliva, 2006)</td>
</tr>
<tr>
<td>Cysteine-rich secretory protein 2 (0.99)</td>
<td>CRISP2</td>
<td>Sperm capacitation and sperm–egg interaction</td>
<td>Busso et al. (2007), Wang et al. (2004)</td>
</tr>
<tr>
<td>A-kinase anchor protein 4 (0.99)</td>
<td>AKAP4</td>
<td>Sperm motility</td>
<td>Miki et al. (2002) (reviewed in Turner, 2006)</td>
</tr>
<tr>
<td>Kinesin heavy chain isoform 5C (0.98)</td>
<td>KIF5C</td>
<td>Nucleocytoplasmic exchange activities during spermatogenesis</td>
<td>Mannowitz et al. (2010)</td>
</tr>
<tr>
<td>Family with sequence similarity 71, member D (0.95)</td>
<td>FAM71D</td>
<td>Functional role in sperm not reported Involved in sperm morphogenesis with likely role in genome stability, cell division, survival and/or proliferation</td>
<td>Platt et al. (2007), Kittler et al. (2007), Paulsen et al. (2009), Chia et al. (2010)</td>
</tr>
</tbody>
</table>

The transcripts were selected and compared based on the RNA-seq data from human (Sendler et al., 2013), bovine (Card et al., 2013) and stallion (Das et al., 2013). The top 5% of the transcripts of the RNA-seq data from human were compared with bovine (FPKM > 100) and stallion (FPKM > 40). Orthologous genes were identified with Genomatix RegionMiner: Search for orthologous GeneIDs module. Only stallion, bovine were compared for abundance, as these are the only species for which equivalent RNA-seq data are available.

occur at the 4–8-cell stage (reviewed in Telford et al., 1990; Memili and First, 2000). The centrosome of the developing embryo is maternally derived in mouse (Schatten et al., 1986), whereas in other mammals, including human, it is paternally derived (Sathananthan et al., 1991, 1997; Manandhar et al., 2005). In mouse, pig and human, the male pronucleus is rapidly demethylated following fertilization whereas in bovine, sheep and rabbit, demethylation is comparatively delayed (Fulka et al., 2004). In part, this may reflect the degree of sperm chromatin condensation (Beaujean et al., 2004). In mammals, early developmental failure or altered phenotype has been associated with the perturbation of demethylation following in vitro fertilization (Yoshizawa et al., 2010), ova-

lation induction (Shi and Haaf, 2002), embryo culture (Zaitseva et al., 2007) or somatic cell nuclear transfer (reviewed in Morgan et al., 2005; Ma et al., 2012).

Artificial insemination (AI) can be viewed as one of the most important techniques devised for the genetic improvement of animals. In the dairy sector, improving reproductive efficiency is 5–10 times more economically important than any of the other production parameters including milk production and carcass quality (Vwitbank, 1994). Unexpectedly, the genetic selection of animals for higher milk production has lowered fertility (Veerkamp et al., 2003). One of the factors influencing fertility in the herd is the quality of the semen. However, sires with equivalent measurable semen parameters may produce vastly different pregnancy rates (reviewed in Kastelic and Thundathil, 2008). Since a single bull is used to inseminate hundreds of females, the use of semen from subfertile or infertile animals can have devastating consequences for the dairy industry. Selection of bulls or semen samples-based primarily on pro-

gressive forward motility invariably does not equally yield bulls of high or equal fertility (Selvaraju et al., 2008). Efficient semen evaluation methods including cellular and molecular approaches are required to predict fertility potential of a bull with high reproductive efficiency. In this regard, the potential of sperm transcripts to provide a marker of sperm quality and embryonic development in farm animals is of considerable interest. The levels of specific sperm RNAs associated with sperm functional parameters (Bissonnette et al., 2009; Curry et al., 2011) and conception (Lalancette et al., 2008b; Arangasamy et al., 2011; Kasimanickam et al., 2012) have been explored (Table II). These studies have now been extended to miRNAs in bovine (Govindaraju et al., 2012), porcine (Curry et al., 2009, 2011) and stallion (Das et al., 2013). As in the human (Krawetz et al., 2011), they may have critical roles both in mature sperm or after delivery to the oocyte where they may regulate fertilization and/or early embryonic development.

### Biomarkers of human fertility

Infertility is a growing problem in contemporary society, affecting ~10–15% of reproductive aged couples (reviewed in Evers, 2002). The evaluation of observable semen parameters is well suited to diagnosing some obvious forms of male infertility. However, even when the sample is deemed suitable based on external characteristics, fertilization potential is still in question. Hence, there is significant need for additional markers of sperm fertility status. Differences in the levels of individual or transcript groups between infertile patients and fertile controls may provide a means to assess the fidelity of past spermatogenic events and/or potential post-fertilization success (reviewed in Antion and Krawetz, 2012).

Microarray analysis has identified altered mRNA profiles in infertile patients presenting suboptimal seminal parameters (Platts et al., 2007; Jodar et al., 2012; Montjean et al., 2012). These results identified some altered pathways allowing further insight into the pathogenic mechanisms involved in male infertility. For example, the ubiquitin–proteosome pathway is severely disrupted in teratozoospermic patients (Platts et al., 2007) and oligozoospermic patients a decrease in the transcripts involved in DNA repair and oxidative stress regulation has been observed (Montjean et al., 2012). Because of the relatively high cost of microarrays, the use of real-time PCR has been explored.

Proteome transcripts are among those most strongly associated with the different seminal parameters such as sperm concentration and motility (Lambard et al., 2004; Kempisty et al., 2007) as well as with sperm fertilization ability and embryo quality (Depa-Martynow et al., 2007, 2012; Steger et al., 2008; Jodar et al., 2012; Rogenhofer et al., 2013). This is likely reflective of the relative abundance of the protamines and their requirement for chromatin packaging. Although the spermatozoa contain a heterogenous population of transcripts, some transcript pairs are proposed to have a stable correlation of expression among different fertile individuals (Lima-

Souza et al., 2012). All of these reported RNA factors could provide a useful suite of fertility biomarkers, and are summarized in Table III.

Most of the high abundant transcripts in human sperm have a relationship with testicular function and spermatogenesis (Sendler et al., 2013). Microarray analysis of serially sectioned testes has produced transcript profiles from different stages of spermatogenesis (Chalmel et al.,

### Table II Mature spermatozoa RNAs associated with semen parameters and fertility in animals determined by RT–PCR.

<table>
<thead>
<tr>
<th>Study</th>
<th>Technique</th>
<th>Phenotype</th>
<th>Altered specific RNAs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bissonnette et al. (2009)</td>
<td>RTPCR</td>
<td>High motility bovine sperm fraction</td>
<td>↑TSSK6 and ADAMSP</td>
</tr>
<tr>
<td>Arangasamy et al. (2011)</td>
<td>RTPCR</td>
<td>High sire conception rate in bovine</td>
<td>↑CRISP2</td>
</tr>
<tr>
<td>Curry et al. (2011)</td>
<td>RTPCR</td>
<td>Low motility in porcine</td>
<td>↓CCT8</td>
</tr>
<tr>
<td>Hwang et al. (2012)</td>
<td>RTPCR</td>
<td>Altered morphology in porcine</td>
<td>↑miR let 7d and 7e</td>
</tr>
<tr>
<td>Kasimanickam et al. (2013)</td>
<td>RTPCR</td>
<td>Low porcine embryo cleavage after IVF</td>
<td>↑MYC, CYP19, ADAM2, PRM1 and PRM2</td>
</tr>
<tr>
<td>Ganguly et al. (2013)</td>
<td>RTPCR</td>
<td>High capacitated porcine spermatozoa</td>
<td>↓MYC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fertile males in bovine</td>
<td>↑Adiponectine and receptors ADR1 and ADR2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Motility impaired in bovine</td>
<td>↓PRM1</td>
</tr>
</tbody>
</table>

*Transcript abundance: ↑ increased and ↓ decreased.
In recent years, there has been speculation about the phenotype and health of the offspring born out of assisted reproductive technologies (ART) especially after IVF and ICSI (reviewed in Batcheller et al., 2011; Savage et al., 2011). The children born with the use of ART have been reported, by some, to have a higher risk to health (e.g. fertility disorders) when compared with those naturally conceived. However, considerable controversy remains as to the composition of the appropriate ‘control’ group that would permit such comparisons. The infertility associated with a genetic defect (reviewed in Matzuk and Lamb, 2008), if any, would also be carried to the next generation along with the increased

### Table III  Altered spermatozoa transcripts and pathways associated with human male infertility determined by microarray or RT–PCR.

<table>
<thead>
<tr>
<th>Study</th>
<th>Technique</th>
<th>Phenotype</th>
<th>Altered specific RNAs*</th>
<th>Altered pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lambard et al. (2004)</td>
<td>RTPCR</td>
<td>Low motility sperm fraction</td>
<td>† PRM1, eNOS and nNOS</td>
<td>Ubiquitin-proteosome pathway, apoptotic pathway and MAP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High capacitated spermatozoa</td>
<td>↓ MYC</td>
<td>kinase signaling</td>
</tr>
<tr>
<td>Wang et al. (2004)</td>
<td>RTPCR</td>
<td>Asthenozoospermic patients</td>
<td>† TPX1 and LDHC</td>
<td></td>
</tr>
<tr>
<td>Depa-Martynow et al. (2007)</td>
<td>RTPCR</td>
<td>IVF failure</td>
<td>↓ Fetilin beta, PRM1 and PRM2</td>
<td></td>
</tr>
<tr>
<td>Guo et al. (2007)</td>
<td>RTPCR</td>
<td>Oligozoospermic patients</td>
<td>↓ VASA</td>
<td></td>
</tr>
<tr>
<td>Jedrzeczak et al. (2007)</td>
<td>RTPCR</td>
<td>Azoospermic patients</td>
<td>↓ HILS1, TNP1, and TNP2</td>
<td></td>
</tr>
<tr>
<td>Kempisty et al. (2007)</td>
<td>RTPCR</td>
<td>Azoospermic patients</td>
<td>↓ PRM1 and PRM2</td>
<td></td>
</tr>
<tr>
<td>Li et al. (2007a, b)</td>
<td>RTPCR</td>
<td>Low motility sperm fraction</td>
<td>↓ CatSper2 and CatSper3</td>
<td></td>
</tr>
<tr>
<td>Platts et al. (2007)</td>
<td>Array</td>
<td>Teratozoospermic patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steger et al. (2008)</td>
<td>RTPCR</td>
<td>Infertile patients</td>
<td>Aberrant PRM1 / PRM2</td>
<td></td>
</tr>
<tr>
<td>Avendano et al. (2009)</td>
<td>RTPCR</td>
<td>Infertile patients</td>
<td>↓ Bcl2</td>
<td></td>
</tr>
<tr>
<td>Garrido et al. (2009)</td>
<td>Array</td>
<td>Infertile normozoospermic patients</td>
<td>↓ TRY1, GGF1 and CAB39L</td>
<td>Spermatozoa differentiation</td>
</tr>
<tr>
<td>Nguyen et al. (2009)</td>
<td>RTPCR</td>
<td>Cryptorchid male</td>
<td>↓ TPX1</td>
<td>Germ cell maturation and sperm tail formation</td>
</tr>
<tr>
<td>Ferlin et al. (2010)</td>
<td>RTPCR</td>
<td>Varicocele, oligozoospermic patients</td>
<td>↑ HSPA4, HSF1 and HSF2</td>
<td></td>
</tr>
<tr>
<td>Garcia-Herrero et al. (2011)</td>
<td>Array</td>
<td>ICSI failure</td>
<td>↑ HSP90</td>
<td>Testicular function, spermatogenesis and sperm physiology</td>
</tr>
<tr>
<td>Zheng et al. (2011)</td>
<td>RTPCR</td>
<td>Oligoasthenozoospermic patients</td>
<td>↓ BDNF</td>
<td></td>
</tr>
<tr>
<td>Depa-Martynow et al. (2012)</td>
<td>RTPCR</td>
<td>Low concentration, motility, morphology, fertilization ability, embryo quality</td>
<td>↓ PRM1 and PRM2</td>
<td></td>
</tr>
<tr>
<td>Jodar et al. (2012)</td>
<td>Array</td>
<td>Asthenozoospermic patients</td>
<td></td>
<td>Spermatid development and the ubiquinone biosynthesis pathway</td>
</tr>
<tr>
<td>Montjean et al. (2012)</td>
<td>RTPCR</td>
<td>Asthenozoospermic patients</td>
<td>↓ ANXA2, BRD2 and OAZ3</td>
<td>Spermatogenesis, sperm motility, DNA repair and oxidative stress regulation</td>
</tr>
<tr>
<td>Roggenhofer et al. (2013)</td>
<td>RTPCR</td>
<td>Low fertilization capacity (IVF and ICSI)</td>
<td>↓ PRM2, TPDS2L3, JMJDIA and NIPBL</td>
<td>Altered PRM1 / PRM2</td>
</tr>
</tbody>
</table>

*Transcript abundance: ↑ increased and ↓ decreased.

2012). These data, in conjunction with the profiles obtained from mature spermatozoa, will be instrumental in correlating perturbations during spermatogenesis and specific forms of male infertility. For example, using sperm transcript profiling, the initial effect of teratozoospermia was traced to the pachytene spermatocyte (Platts et al., 2007). Identification of these disruptions by profiling sperm transcripts rather than invasive testicular biopsy offers obvious benefits to the patient (Yatsenko et al., 2006). These novel techniques are expected to be useful in identifying the origins, prognosis and treatment of various forms of what was previously considered to be male idiopathic infertility.
risk of epigenetic disorders (Kobayashi et al., 2009) that could be mirrored by changes in the pattern of DNA methylation (reviewed in Savage et al., 2011; Feuer et al., 2013; Hart and Norman, 2013).

Maternally, it is well established that advanced age increases the risk of cytogenetic abnormalities that can manifest as Down syndrome (Hassold et al., 1984). With the ability of ART to ‘bypass’ some of the boundaries that have limited conception, studies are now beginning to suggest an association between increasing paternal age at conception and neurologic-al disorders like autism, with a noticeable effect between 30 and 39 years and a substantial effect at ≥ 50 years (Grether et al., 2009; Hultman et al., 2011; van Balkom et al., 2012). Recent data show that the autism spectrum of disorders are strongly associated with de novo mutations (Sanders et al., 2012) present in spermatozoa. This likely reflects the continuity of sperm production during the life of an adult male that arises from the ∼840 divisions from each stem cell that give rise to the mature spermatozoon. The resulting cumulative effect of mutations at each division (Kong et al., 2012) along with effects on chromatin integrity (Wyrobek et al., 2006) and methylation errors (Flanagan et al., 2006) may contribute to the growing prevalence of age-related effects as observed, e.g. autism.

Paternal factors are thought to underlie the etiology of infertility/sub-fertility in approximately half of the couples undergoing ART (Jarow et al., 2002). This may, to some extent, be reflective of the sperm transcript profile that often varies between infertile and fertile males (Platts et al., 2007; Garrido et al., 2009; Garcia-Herrero et al., 2010a; Jodar et al., 2012; Montjean et al., 2012). Thus, the use of the paternal transcriptome as a biomarker deserves consideration. Moreover, different transcript profiles have been suggested that may coincide with successful pregnancy in different fertility treatments (Garcia-Herrero et al., 2010b, 2011). This has further supported the notion (Ostermeier et al., 2002; Platts et al., 2007; Lima-Souza et al., 2012) of the potential use of the microarray strategy as a clinical diagnostic tool (Garrido et al., 2013). Perhaps, sperm transcript profiling of patients undergoing ART (Garcia-Herrero et al., 2011) will aid in identifying both specific paternal factors and pathways which are negatively affecting fertility outcomes (reviewed in Carrell, 2008). As costs continue to decline, it is very likely that sperm transcript sequencing will reach the clinic shortly. These data and their analysis provide several advantages compared with the microarray assays. These include a quantitative description of abundance.

Figure 9 The potential actions of spermatozoal RNAs during early embryo development. Spermatozoal RNAs are delivered to the oocyte acting during the first steps of embryogenesis. Some intact paternal mRNAs like INST1 could be translated by maternally machinery. On one hand, paternal mature miRNAs like mouse miR-34c are essential for the first cell division. On the other hand, primiRNAs like 181c, can be processed and thus activated by maternal DICER to their mature miRNAs regulating transcript stability, whereas others may target promoters. Interestingly, some non-coding RNAs act through triplex structures and perhaps are transcriptional regulators. For example, homopurine fragments of LINE1 provided by spermatozoa induce LINE1 transcription during the first divisions of the zygote. It has also been proposed that piRNAs, miRNAs and other potential RNAs may be the pathway to confrontation and consolidation.
immediate assessment of the fidelity of the information content and allele-specific expression reflective of Expressed Quantitative Trait Loci and hence genotype. This truly heralds the beginning of Male Personalized Reproductive Medicine.

**Conclusion**

Rapid development of RNA assay technologies including RNA-seq has detailed the specific presence of a wide variety of spermatozoal transcripts. Such transcripts appear to reflect both the past course of spermatogenesis, yet also include factors critical to fertilization and successful embryo development (Fig. 9). This likely includes a significant epigenetic component that is mediated by the action of sperm-borne RNAs on both genotype and phenotype of the offspring. An increasing number of comparative animal models will be of use in identifying key transcripts, many of which may be unique to sperm, and in elucidating their functional role. With this understanding of the differentiative history of the pool of spermatozoal transcripts, the outcome of ART, and perhaps in the future the ability to both treat underlying causes of infertility and ensure the birth of a healthy child, will be optimized.

**Supplementary data**

Supplementary data are available at http://humupd.oxfordjournals.org/.

**Acknowledgements**

The authors would like to thank Mr G. Johnson for his critical review of this manuscript and Mr R. Sanchez Giones and Mr Yitzchok Sendler for their assistance in the preparation of the illustrations. We apologize to others who were not able to include their work in this review. The authors would like to thank the members of RMN for their invaluable assistance and for providing some of the samples used to illustrate the properties of spermatozoal RNAs. Prerelease access to the SEQR Whole Transcription Amplification system from Sigma Chemical Corporation is gratefully acknowledged.

**Authors’ roles**

M.J., S.S. and E.S. analyzed the data, performed the literature searches and wrote the manuscript with M.P.D. and S.A.K. S.A.K. directed the data analysis, writing and editing of the manuscript. The authors alone are responsible for the content and writing of the paper.

**Funding**

This work was supported in part by National Institutes of Health (NIH)/Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Grant U10 HD039005. S.S. is supported by a Cutting-edge Research Enhancement and Scientific Training Award, Department of Biotechnology, Government of India. M.P.D. and S.A.K. are recipients of an EMD Serono grant to Wayne State University. Otherwise the authors report no conflicts of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NICHD or NIH.

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Complex population of spermatozoal RNAs


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Approaches for drawing causal inferences from epidemiological birth cohorts: A review

Rebecca C. Richmond, Aleef Al-Amin, George Davey Smith, Caroline L. Relton

Abstract

Large-scale population-based birth cohorts, which recruit women during pregnancy or at birth and follow up their offspring through infancy and into childhood and adolescence, provide the opportunity to monitor and model early life exposures in relation to developmental characteristics and later life outcomes. However, due to confounding and other limitations, identification of causal risk factors has proved challenging and published findings are often not reproducible. A suite of methods has been developed in recent years to minimise problems affecting observational epidemiology, to strengthen causal inference and to provide greater insights into modifiable intra-uterine and early life risk factors. The aim of this review is to describe these causal inference methods and to suggest how they may be applied in the context of birth cohorts and extended along with the development of birth cohort consortia and expansion of “omic” technologies.

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1. Introduction

Large-scale population-based birth cohorts recruit women during pregnancy or at birth over a defined time period and follow up their offspring through infancy and into childhood and adolescence. The longitudinal design of these cohorts is a key feature, providing the opportunity to monitor and model early life exposures in relation to developmental characteristics and later life outcomes, with prospective data collected at repeat follow-ups. Data are often collected on both parents and offspring and include information on demographic, socio-economic and lifestyle characteristics and environmental exposures obtained from questionnaires, clinic data for assessing health and development, and data from biological samples. Some cohorts have been designed as multipurpose resources, whilst others focus on specific health or exposure-related research questions. The size of birth cohorts varies considerably, from a few hundred individuals to over 100,000 in countries where population-based record linkage is possible.

A major focus of such studies is exposure to risk factors during early life developmental periods which can have important consequences for health and disease. The “Developmental Origins of Health and Disease” (DOHaD) hypothesis outlines how the risk of chronic disease in adult life is initially induced through biological programming of the foetus or infant in response to early environmental signals [1,2]. These responses include molecular, hormonal, metabolic or physiological changes which may have negative impacts on later health. Of particular interest is the data captured on maternal exposures acting during pregnancy, driven by the notion that the intra-uterine environment is a critical period for influencing offspring development and programming events [3,4]. Studies have reported associations between foetal growth, maternal nutrition, exposure to drugs, pollutants and hormones in-utero and a whole host of perinatal and later life offspring traits. The influence of postnatal factors has also been explored, including early life growth [5] and breastfeeding [6]. Of particular value are historical birth cohorts which can be used to study the influence of early life exposures on later disease [7]. As well as DOHaD, other aspects of research within lifecourse epidemiology may be investigated within the context of a birth cohort [8,9] and details of these can be found elsewhere [10].

An attractive feature of birth cohorts is the ability to obtain information on other family members, not only the mothers of the offspring, but sometimes fathers, siblings and grandparents. Family-based sampling can facilitate inter-generational studies of the influence of parental characteristics on a range of offspring outcomes and may aid in disentangling the genetic determinants of disease from environmental risk factors [11].

Increasingly, birth cohorts collect and store biosamples from their participants, which can be used to obtain genetic, epigenetic and metabolic profiles, and to measure biomarkers of environmental exposures such as smoking and pollutants. Biosampling allows the exploration of how social and environmental factors leave biological imprints, independent of or in combination with genetic background. The ‘omics’ revolution [12] offers the potential to explore putative mechanisms by which specific exposures convey disease risk, whereby identified molecules provide robust biomarkers of early life exposure or may act as intermediates in pathways between exposure and risk of later outcomes.

In addition to the wealth of data collected, longitudinal birth cohorts can offer more to observational epidemiology than other study designs because they allow for prospective time-ordering of the associations of interest i.e. with exposures preceding outcomes, which is useful for establishing causality. However, a key limitation to causal inference in epidemiological birth cohorts is potential confounding, leading to spurious observational associations [13,14]. Distinguishing causality from correlation is essential to identify key early life definable causes of ill health and disease and to uncover new mechanistic pathways for therapeutic intervention. A suite of methods has been developed in the last decade to minimise problems afflicting observational epidemiology and to strengthen causal inference. The aim of this review is to describe the causal inference methods that have been used to provide greater insights into modifiable intra-uterine and early life risk factors in the context of large epidemiological birth cohorts and to suggest how we may improve methodological approaches, especially in relation to the expansion of “omics” technologies.

2. Challenges of establishing causality in birth cohorts

Key problems of observational epidemiology which limit its ability to establish causal effects include: 1) reverse causation—where the outcome of interest affects the exposure; 2) confounding—the presence of common causes of the risk factor of interest and the outcome; 3) selection bias—when the study participants are selected in a manner that biases the effect estimate in an association; and 4) measurement error in the exposure, confounding factors or outcome. The characteristics of birth cohorts are such that some of these problems can be minimised. For example, their prospective study design means that there is no biased retrospective assessment and the likelihood of reverse causation is reduced due to the time-ordering of the exposure-outcome associations. These studies also allow for repeated measures to be taken at different time points and appropriate analytical techniques may be used to account for missing data, reducing the role of measurement error and selection bias [15,16].

Observational epidemiology undertaken in the context of a birth cohort generally relies on the assumption that confounding characteristics have been identified and measured with little or no error. However, confounders may be inadequately measured (residual confounding) or there may be unobserved factors (unmeasured confounding) [17] which can lead to spurious associations and conclusions about intra-uterine and early life risk factors [18,19]. Inconsistent findings between cohorts and randomised controlled trials (RCTs) highlight the methodological challenges in establishing robust causal links [13,20]. For example, in observational studies maternal vitamin C intake has been found to be associated with higher birth weight in the offspring [21]. However, large RCTs where pregnant women have been randomised to vitamin C supplements [22–24] have found no benefit of supplementation on birth weight. These conflicting findings are likely due to confounding in the observational association, as mothers with higher vitamin C intake tend to have lower rates of smoking and are from a higher socioeconomic background, which influence birth weight [25].

Other limitations introduced by the very nature of birth cohorts include the long time gap between outcomes and exposures, increasing the likelihood of confounding. Another implication of this time gap is the relevance of early life exposures experienced when the birth cohorts were established to contemporary cohorts. Finally, given the high correlation between maternal exposures and behaviours in pregnancy with those postnatally it is often difficult to tease apart intra-uterine from postnatal effects [26].

3. Classic epidemiological approaches for drawing causal inferences

Data collected on parents, offspring and other family members in epidemiological birth cohorts may be integrated in a suite of methods which minimise problems of confounding, strengthen causal inference and provide greater insights into modifiable early life risk factors. The strength of evidence obtained from these methods can be placed between observational associations and RCTs in the hierarchy of evidence for clinical guideline production. Table 1 includes a selection of large, well-established cohorts and the data available in these cohorts which may permit the application of the causal inference methods described in this review. Table 2 outlines each of the main causal inference methods, with examples and linked schematic diagrams in Fig. 1.

4. Randomised controlled trials

Well-conducted, large RCTs, where study participants are randomly allocated to a treatment to avoid potential confounding between
treatment and outcome, are the gold standard for estimating causal effects in population health. This is also the case in the setting of early life influences, for example with the randomization of women to different interventions in pregnancy. A number of RCTs of pregnancy and early life interventions originally set up to investigate short-term outcomes have been extended to follow up offspring at multiple ages. One example of a birth cohort nested within an RCT is the PROBIT trial [27,28]. This cluster-randomised controlled trial involved randomization to a breastfeeding promotion intervention which resulted in longer duration of any and exclusive breastfeeding and has been used to investigate the causal effect of breastfeeding on later health outcomes, including obesity, blood pressure, cognitive function and eating attitudes [29–33]. RCTs require large investment and their experimental nature means that they should be reserved for interventions that have strong support from observational epidemiology. In addition, for some exposures it is not possible or would be unethical to randomise participants and where RCTs are conducted, they are often done so in selected populations and so findings may not be generalizable.

5. Cross—cohort comparisons

Support for the initiation of the PROBIT trial came from observational studies which have shown breastfeeding to be protective against a wide range of later outcomes. However, not all of these associations persist in a randomised trial setting [29–31]. This discordance can be explained by the fact that the majority of observational studies have been conducted in higher-income countries where breastfeeding is strongly related to higher socio-economic circumstances, maternal non-smoking and healthy diet. The links between breastfeeding and these factors would generate non-causal observational associations between breastfeeding and health outcomes, and the ability to fully evaluate and statistically adjust for such confounding is limited. One way to circumvent this problem, without initiating an RCT, would be to compare associations between two or more populations in which the underlying confounding structures are markedly different. For example, if the associations found in higher-income countries are causal then one would expect them to be found in low- and -middle-income countries where breastfeeding is often not associated with socio-economic position [34]. An analysis of a UK-based cohort study, ALSPAC, and a Brazilian-based cohort study, the Pelotas 1993 Cohort, showed that the inverse association of breastfeeding with later offspring body mass index (BMI) and blood pressure found in higher income countries is not present in low- and middle-income countries. By contrast, a positive association with intelligence quotient (IQ) was found in both settings [34]. These findings have been validated by results of the PROBIT study, based in the middle-income country of Belarus [27,28]. The assumption about different confounding structures in different cohorts may not be correct and has to be thoroughly investigated. In addition, harmonisation of variables between cohorts is required in order to minimise the influence of statistical heterogeneity.

6. Negative controls

It is also possible to infer a causal effect by comparing an observed association between a particular exposure and an outcome with a negative control. A negative control situation is one that cannot involve the association between a particular exposure and an outcome with a negative control situation. A negative control situation is one that cannot involve the association between a particular exposure and an outcome with a negative control. A negative control may not be correct and has to be thoroughly investigated. In addition, for some exposures it is not possible or would be unethical to randomise participants and where RCTs are conducted, they are often done so in selected populations and so findings may not be generalizable.

7. Parental comparisons

A negative control design that is primarily used for exploring the extent to which associations of intra-uterine exposure might be causally related to offspring outcomes in later life is the parental comparisons approach. If there is a causal intra-uterine effect, one would expect a stronger maternal-offspring association than paternal-offspring association for the same exposure assessed at the time of pregnancy. Where associations are similar for both parents it is likely that there is confounding by genetic or shared environmental characteristics [11,18,36]. Proof of concept has been illustrated with maternal smoking in pregnancy which is strongly associated with lower offspring birth weight, whereas paternal smoking is only weakly associated. When both maternal and paternal smoking during pregnancy are taken into account, the former association is little attenuated whereas the latter association is essentially abolished, arguing for a biological effect of maternal smoking in pregnancy on offspring birth weight [18].

It has been hypothesised that maternal obesity and metabolic profiles related to this may, during pregnancy, programme the offspring for greater risk of obesity in later life [40,41]. This could result in inter-generational acceleration, with ever-increasing levels of obesity in the population [42]. Some parental comparison studies find stronger associations of maternal BMI than paternal BMI with offspring BMI [43–45], although these have often been of small sample size, with different sources and degrees of validity for BMI measures, and non-paternity for biological measures has generally not been taken into account [46]. Subsequent studies addressing these issues have found that maternal and paternal BMI relate very similarly to offspring adiposity [46–50], arguing against a major specific effect of the intra-uterine environment and suggesting that the associations are driven by shared familial genetic or lifestyle characteristics.

Some evidence has been found which supports potential male-line transgenerational responses, invoking parent of origin, imprinting and epigenetic phenomena [51,52]. Maternal and paternal associations of similar magnitude may therefore be interpreted as showing intra-uterine maternal influences which are offset by these paternal pathways. However, it has been posited that the likelihood of such perfectly matched effects being produced by mechanistically distinct processes is low [18,33].

8. Sibling comparisons

It may be possible to compare outcomes within siblings who are concordant or discordant for early life exposures. Since familial
Table 1
Characteristics of a selection of birth cohorts described in this review and the data currently available which may permit causal inference.

<table>
<thead>
<tr>
<th>Birth cohort</th>
<th>Study description</th>
<th>Initial sample size</th>
<th>Data collection during pregnancy</th>
<th>Data on both parents</th>
<th>Prospective/retrospective</th>
<th>Follow-up</th>
<th>Frequency of follow-up</th>
<th>Methods of data collection</th>
<th>Biological samples</th>
<th>Dna extracted</th>
<th>Gwas data</th>
<th>Epigenetic data</th>
<th>Metabolomic data</th>
<th>Sibling data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avon Longitudinal Study of Parents and Children (ALSPAC) (UK)</td>
<td>Prospective pregnancy cohort study set up from 1991 to 1992 in the south west of England and the surrounding areas</td>
<td>15,247 pregnancies (14,541 in initial recruitment, 706 added a later time)</td>
<td>Yes</td>
<td>Yes</td>
<td>Prospective</td>
<td>From around 8th gestational week to 21 + years postnatal for parents and between birth and 21 + years of age for child.</td>
<td>68 data collection timepoints for child. 19 data collection timepoints for mother</td>
<td>Questionnaires, clinical assessments, medical and educational records</td>
<td>In offspring (at birth and later ages), mothers (during pregnancy and postnatally) and fathers</td>
<td>8365 children, 8340 mothers, fathers in process</td>
<td>~10,000 mothers and offspring: ~2000 fathers</td>
<td>~7500 offspring (~7000 metabolochip and immunochip)</td>
<td>MeDIP in subsample</td>
<td>No</td>
</tr>
<tr>
<td>1958 British birth cohort (National Child Development Study) (UK)</td>
<td>Longitudinal study of all infants being born in England, Scotland and Wales in one week in March 1958. Initially a study on perinatal mortality, but later extended to lifelong tracing of participants.</td>
<td>17,416 births</td>
<td>No, some retrospective data from medical records and perinatal survey.</td>
<td>Yes</td>
<td>Prospective though some retrospective data about pregnancy.</td>
<td>Since 1958, 9 further 'sweeps' of all cohort members</td>
<td>Maternal interview at birth; obstetric data from medical records; parental interviews, medical examinations, school attainment and questionnaires in childhood; interviews and questionnaires for offspring (and parents) in adulthood. Biomedical assessment and blood collection at age 44-45. Data from medical records.</td>
<td>In offspring</td>
<td>~7500 offspring (-7000 metabolochip and immunochip)</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pelotas Birth Cohort Studies (1982) (Brazil)</td>
<td>One of three parallel longitudinal studies of all infants being born to mothers living in Pelotas in 1982, 1993 and 2004.</td>
<td>5914 births</td>
<td>No, though some retrospective data from perinatal survey</td>
<td>Yes, though limited for partners</td>
<td>Mostly prospective data collection. Retrospective data about pregnancy.</td>
<td>Between birth and 30 years</td>
<td>On entire cohort at age 2, 4, 22 and 30 years but more frequent follow-up of subsamples</td>
<td>Maternal questionnaire and offspring anthropometric assessment at birth; follow-up interviews, anthropometric assessments and questionnaires; some national record linkage.</td>
<td>In offspring</td>
<td>~4000 offspring</td>
<td>~3500 offspring</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>The Norwegian Mother and Child Cohort Study (MoBa) (Norway)</td>
<td>Prospective pregnancy cohort which enrolled women at week 17-18 in pregnancy from all over Norway between 1999 and 2008, with record linkage.</td>
<td>&gt;114,500 children born to &gt;95,000 mothers</td>
<td>Yes</td>
<td>Yes</td>
<td>Prospective</td>
<td>From pregnancy week 17-18 to date, 7 year and further follow-ups are being processed.</td>
<td>3 questionnaires during pregnancy, further questionnaires at 6 months, 18 months and 3 years.</td>
<td>Questionnaires and record linkage with Norwegian Patient Registry</td>
<td>In offspring (at birth), mothers (during pregnancy and at birth) and fathers</td>
<td>~70,000-95,000 offspring, mothers and fathers</td>
<td>~3000 mothers and children. 14,000 mothers and children and 11,000 fathers available in 2015</td>
<td>450 K DNA methylation data available in 1,000 mother-child pairs</td>
<td>Includes almost 18,000 pairs of siblings</td>
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<tr>
<td>Study</td>
<td>Type</td>
<td>Recruitment Period</td>
<td>Study Design</td>
<td>Follow-Ups</td>
<td>Data Collection</td>
<td>Sample Size</td>
<td>SNP Assays</td>
<td>Notes</td>
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<tr>
<td>Generation R (The Netherlands)</td>
<td>Population-based multi-ethnic birth cohort study</td>
<td>April 2002 and January 2006</td>
<td>Prospective</td>
<td>Early pregnancy to date</td>
<td>Data collection at age 10 years will be completed in 2015</td>
<td>9778 mothers and children</td>
<td>3 assessments in the prenatal phase, frequent follow-ups from birth to 48 months and data collection at focus visit at age 5-6 years</td>
<td>Observational assessments, parental and child interview and questionnaires, records of child health care centres, obstetric records</td>
<td>In mothers (during pregnancy), children (at birth and age 6) and fathers</td>
<td>Available on ~6500 offspring, 8,000 mothers and 5,000 fathers</td>
<td>450 K DNA methylation data available in 1,000 cord blood samples</td>
<td>Only if born during recruitment period.</td>
<td></td>
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<tr>
<td>The Danish National Birth Cohort (DNBC) (Denmark)</td>
<td>Prospective cohort study</td>
<td>1996 and 2003</td>
<td>Largely prospective with some retrospective data collection about medical history</td>
<td>From early pregnancy to date</td>
<td>Computer-assisted telephone interviews and 1 food questionnaire in pregnancy, further follow-ups at 6 months, 18 months, 7 years and 11 years. The plan is for the study to be lifelong. Follow-up took place once between 2003-2005 at around age 59 years.</td>
<td>92,274 mothers with a total of 100,418 pregnancies</td>
<td>GWAS data available on ~8000 mothers</td>
<td>In offspring (at birth) and mothers (during pregnancy)</td>
<td>-91,000 samples on mothers</td>
<td>450 K DNA methylation in ~1300 offspring of GOYA study on available soon</td>
<td>Only if born during recruitment period.</td>
<td></td>
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<tr>
<td>The Dutch Hunger Winter Families Study (The Netherlands)</td>
<td>Longitudinal cohort study of part of the population that was in the fetal stage at the time of the ‘Hunger Winter’ of 1944-45</td>
<td>Recruitment from 1996-1997 and mothers eligible if they intended to breastfeed and had given birth to a healthy singleton infant.</td>
<td>Largely retrospective</td>
<td>One follow up in adulthood</td>
<td>Telephone interview, questionnaires and clinical assessment</td>
<td>3307 live birth singletons at 3 institutions in famine-exposed cities in Western Netherlands were identified. 1075 (751 cases and 324 sibling controls) not lost to follow-up. Follow-up included once between 2003-2005 at around age 59 years.</td>
<td></td>
<td>In offspring</td>
<td>-1000 offspring</td>
<td>No</td>
<td>324 siblings of cases formed the control group for the study</td>
<td></td>
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</tr>
<tr>
<td>Promotion of Breastfeeding Intervention Trial (PROBIT) (Belarus)</td>
<td>Cluster-randomised control trial</td>
<td>1996-1997</td>
<td>Prospective though sometimes retrospective if participant missed one or more follow-up study visit.</td>
<td>From early post-partum to date. Data from age 16 years is currently being processed.</td>
<td>Polyclinic and home visits for observational assessment, interviews, parent and child-reported questionnaires and administered tests; data from routine check-ups abstracted from medical records, teacher-reported questionnaires</td>
<td>17,046 mother-infant pairs</td>
<td>450 K DNA methylation available on ~600 offspring</td>
<td>In offspring at age 11.5</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td></td>
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</tbody>
</table>
background will generally be similar for siblings, comparing outcome differences in relation to discordant exposures within sibships effective- ly “matches” on family characteristics, providing a stronger means of controlling for certain confounding factors [11]. Such study design have been used to show that gestational diabetes [54,55], gestational weight gain [56] and extreme BMI [57,58] are likely to be causally related to later offspring obesity and other metabolic outcomes [41], with findings being translated into long-term follow-up of participants in randomised controlled trials [59,60].

Again there are instances where this causal analysis method has pro- vided contrasting results in different studies. For example, sibling stud- ies have been used to explore whether the positive association between birth weight and later IQ [61] is causal. Whilst some studies suggest that birth weight differences within sibships are related to differences in intelli- gence, implying an intra-uterine effect [62,63], others show no evi- dence of association [64,65], arguing that the association observed in the population may be explained by factors such as family socioeco- nomic background.

It is important to bear in mind that, although sibling comparison es- timates will not be influenced by unmeasured familial confounders, there are notable limitations to this study design which may explain the discrepancy in findings [66]. Such estimates are more severely bi- ased by non-shared confounders than population-level comparisons [67] and are more sensitive to misclassification of the exposure and measurement error [66,68]. Use of a sibling comparison design also limits the population included, affecting power and demonstrating the need for large sample sizes to obtain robust causal evidence.

9. Mendelian randomization

Mendelian randomization (MR) is a method that utilises genetic vari- ants robustly associated with modifiable exposures to infer causality [69]. The MR design is analogous to an RCT [70] and creates a similar scenario by exploiting Mendel’s laws (segregation and independent assortment). Given these laws, at a population level genetic variants should not be associated with genetic or environmental confounding factors that can distort conventional observational studies. Analysing data according to genotype will therefore compare groups that differ by an on-average level of a modifiable exposure, not by a myriad of behavioural, social and physiological variables that may confound ob- servational associations [71,72]. In addition, in a genetic association the direction of causation is from genetic variation to the outcome, and not vice versa as disease processes do not alter germline genotype. Genetic variants are also subject to relatively little measurement error or bias and variants will generally be related to a modifiable exposure through- out life, avoiding attenuation by errors [73].

Where maternal genotype is taken to be a proxy for environmentally- modifiable exposures in pregnancy, this may provide unique insights into the causal nature of intra-uterine environment influences on later offspring outcomes [18]. For example, variation in MTHFR is associated with methylenetetrahydrofolate reductase activity and hence with circu- lating folate and homocysteine levels. Maternal MTHFR variants have been found to influence risk of neural tube defects (NTD) in off- spring [74], implying a causal effect of low maternal folate. These find- ings are consistent with the results of RCTs of maternal folate supplementation which is associated with reduced risk of offspring con- genital abnormalities [75,76]. In this example, the effect of maternal ge- notype on risk of NTD was greater than paternal or offspring genetic estimates, implying an independent maternal effect [74] which is con- sistent with the hypothesis that maternal folate intake is the exposure of importance.

Limitations of the Mendelian randomization approach have been outlined in detail elsewhere [77,78], and include low statistical power due to the small amount of variance in a trait explained by the genetic variant; population stratification, which may induce confounding when allele frequencies and disease risk differ according to the genetic ancestry of populations within the study; and pleiotro- py, where the genetic variant influences more than one post- transcriptional process and may affect the outcome via a pathway that is independent of the exposure. Methods may be implemented to address these limitations and extensions of the MR approach ap- plied to avoid them [77,78].

10. Non-genetic instrumental variable analysis

The use of genotype in MR studies is an application of instrumental variable (IV) analysis [79,80], which may be used to obtain an estimate for the magnitude of a causal effect. An IV is a variable that is associated with the outcome only through its robust association with the exposure, and therefore an IV will typically not be associated with factors that con- found the association of exposure and outcome. Examples of non- genetic instrumental variables include external factors which influence a population largely at random, such as the famine experienced in the Dutch Hunger Winter [39], climate conditions [81], or cigarette taxation [82]. However, in these cases the external or “exogenous” factor is gen- erally rare or of small effect. Another non-genetic IV which is more com- monplace is the phenotype of a family member in family-based studies, which may be used to proxy for own phenotype. For example, offspring anthropometry has been used as an IV for examining the causal effect of own anthropometry on mortality [83,84]. As offspring anthropometry is likely influenced by the same socio-economic, lifestyle and genetic con- founders as parental anthropometry, this method is used primarily to deal with reverse causation, under the assumption that offspring’s anthropometry will not be influenced by parent’s illness.

11. Triangulation of causal inference methods

The above causal inference methods have different underlying as- sumptions, strengths and limitations and an integration of different app- roaches to the same research question may be used to improve the identification and estimation of causal effects through the “triangulation” of findings. This may be done under the supposition that independent biases are unlikely to lead to the same result across a range of methodo- logical approaches. If causal effects are consistently estimated, the likeli- hood that they are unbiased is high. If they differ between the approaches, there is a further need to investigate whether the underlying assumptions for each approach have been violated. One example of trian- gulation has already been alluded to, which is the similarity in findings between a cross-cohort comparison study [34] and a randomised con- trolled trial investigating the effect of breastfeeding on offspring BMI, blood pressure and IQ [29–32]. Conventional multiple regression, paren- tal comparison, between-sibling analyses, Mendelian randomization, non-genetic instrumental variable and RCT studies have all been consis- tent in their findings of a causal effect of maternal smoking in pregnancy on offspring birth weight [85]. “Triangulation” methods have also been exemplified within single studies where two complimentary approaches have shown consensus on early life causal effects [45,54]. The approach of privileging a hypothesis which fits with the overall pattern of findings and knowledge across all informative sources is within the tradition of “inference to the best explanation” approaches to causal reasoning [86].

12. Consortia

One characteristic which all of the described causal inference methods have in common is that they are often underpowered and generally require large sample sizes. Therefore, as well as using triangu- lation, there is a need for independent replication of findings in order to avoid spurious conclusions in causal inference analysis. Cross-cohort analysis can improve power and statistical precision, and can provide high quality evidence on the causal effects of early life exposures on later health and disease. Collaboration is already evi- dent in some instances, with the pooling and harmonising of data to
<table>
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<td>Randomized control trial (RCT)</td>
<td>Subjects are randomly allocated to either exposure or control groups with assumption that there is no difference between the two groups except for the intervention they are receiving</td>
<td>Confounding, reverse causality, selection bias, loss-to-follow-up bias (using intention-to-treat analysis), measurement error</td>
<td>Gold standard for estimating causal effects. Any effect is very likely to be causal if study has large number and trial is reliably performed.</td>
<td>Generalizability may be questionable; impossible to unethical to randomize to certain exposures; can be expensive.</td>
<td>Association between maternal breastfeeding behaviour and childhood outcomes. 27–33</td>
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<td>Cross-cohort comparison</td>
<td>Associations are compared between two or more populations with markedly different confounding structures. If the observed association is causal, it should be present in both cohorts</td>
<td>Confounding, Selection bias</td>
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<td>Association between breastfeeding and IQ, obesity and blood pressure in two cohorts.</td>
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<td>Natural experiment</td>
<td>Empirical study approach where a population is exposed to an external event or intervention at a specific time point. Associations are then compared with a similar cohort who was not exposed. The assumption is that exposure is caused by quasi-random assignment</td>
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<td>Improves causal inference of intrauterine effect if exposures are measured in both parents at same time in pregnancy, and non-paternity is taken into account for phenotypic traits.</td>
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<td>Sibling comparison</td>
<td>Compares outcomes when siblings are discordant for an exposure. If causal then there will evidence of a difference in outcome in relation to discordant exposure levels within sibships.</td>
<td>Confounding</td>
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<td>Mendelian randomization (MR)</td>
<td>MR is the use of a genetic variant robustly associated with an exposure/risk factor of interest as an instrumental variables to test and estimate the causal effect of that exposure/risk factor with a disease or health related outcome.</td>
<td>Confounding, reverse causality, selection bias, measurement errors, generalizability</td>
<td>Genetic instruments are not subject to confounding from environmental or lifestyle factor, are not influenced by the outcome, do not change over time and are measured with high accuracy.</td>
<td>Low power, lack of instrumentation, pleiotropy and linkage disequilibrium, population stratification, non-linear associations, developmental canalisation. True exogenous factors are generally rare or of small effect. When exposure in one family member is used as IV for exposure in another family member, residual confounding is likely.</td>
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<td></td>
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address research questions on environmental exposures \cite{87,88} and genetic associations \cite{89–91}. There are several examples of birth cohort collaborations, including CHICOS (http://www.chicosproject.eu/the-project/management/), EAGLE (http://www.copsac.com/content/eagle-consortium), EGG (http://egg-consortium.org/) and ENRIECO (http://www.enrieco.org/) \cite{92} and a tool for accessing information

![Schematic diagrams outlining the main causal inference methods. MR = Mendelian randomization.](image)
on each birth cohort has been made available at http://www.birthcohorts.net [93]. Also of importance in this field is the inclusion of birth cohort studies from low- and middle-income countries [94, 95], where variation in environmental exposures, health outcomes and confounding structures may be used to improve causal inference [34]. To date, collaborations have been used to replicate findings from causal inference analysis in multiple cohorts, including parental comparisons [96] and Mendelian randomization [97].

13. New data

As has already been mentioned, an attribute of many birth cohorts is their biological sampling which includes the collection of blood, urine and hair samples. New technologies permit genotyping and profiling of methylation, metabolites and biomarkers of environmental exposures, and open up new avenues for exploring underlying causal pathways. Of particular value is the collection of serial samples from the same individuals in some birth cohorts, which allows assessment of change in molecular measures over time.

13.1. Genetics

As shown in Table 1, many birth cohorts now have genome-wide data available on a large number of individuals, including both offspring and parents. These may be used in Genome Wide Association Studies (GWAS), where associations between a wide range of phenotypes and genetic variants across the genome are determined in a hypothesis-free approach. More recently, an innovative method utilising genome-wide data in mothers and offspring has been developed which allows the delineation of maternal-specific influences on offspring outcomes [98]. The ability to identify many robust genotype-phenotype associations is of merit for Mendelian randomization which has classically involved the use of a single variant to proxy for a particular modifiable exposure. GWAS has uncovered a host of genetic variants which explain an increasing proportion of the variance in a trait and may act as a stronger instrument for improving the precision of causal estimates [99]. The use of genetic scores, created by adding up the total number of risk alleles a person has, offers particular promise in this regard [100,101]. However, as the function of a variant identified in GWAS is often unknown, the assumption that it will only influence the outcome through its direct effect on the exposure is difficult to assert. Nonetheless strategies exist for assessing potential pleiotropy [72,99].

Building on the success of GWAS and the availability of cost effective and robust technologies is the use of “omics” within population health science. This is largely concerned with understanding how gene regulatory mechanisms or gene products interact with the environment to influence health-related outcomes and is useful for investigating the molecular pathways that may underpin causal effects. Of particular utility are large-scale epigenetic and metabolomic scans for formulating novel hypotheses on biological processes. However, in contrast to germ-line genetic variation, epigenetic and metabolomic signatures are largely phenotypic, and are subject to the same problems of confounding and reverse causation which afflict conventional epidemiology [53,102,103] (Fig. 2). The extension of causal inference approaches is therefore of particular relevance in determining causal associations between “omic” markers and a range of exposures and outcomes [77].

14. Epigenetics

Epigenetic mechanisms are involved in regulating gene activity which creates phenotypic variation without altering the underlying DNA code. Epigenetics is a potentially major mechanism by which environmental factors can affect physiological function and disease risk. In particular, DNA methylation has become increasingly integrated into population-based studies as a potential modifiable indicator of the underlying biological changes.

Epidemiological approaches can be used to identify whether epigenetic processes are involved in mediating the association between various risk factors and common complex disease [104,105]. Longitudinal cohort studies that make use of multiple time points are useful for investigating how the epigenome changes over time, as a result of varying exposures, and how this contributes to disease development [106]. In particular, there is considerable interest in the role of epigenetic mechanisms in DOHaD as epigenetic states are often established in early development [107–109]. This makes birth cohorts with sample collection from pregnant women and offspring at birth of particular value for providing insights into the temporal relationship between early life

![Diagram outlining the interplay between genomics, other “omics” and environmental factors in relation to disease or health-related outcomes. GWAS = Genome-wide association study.](image-url)
exposures and epigenetic changes [110–112], which may then predict later health-related outcomes [113–115].

It is important to bear in mind that epigenetic profiles can be influenced by technical or genetic factors, cellular and tissue heterogeneity, time-varying artefacts and stochastic changes. These sources of noise threaten the detection of biological signals and the ability to infer causality from associations [53,103]. Careful study design, data collection and control of sources of variability are therefore required, as are methods which will contribute to the identification of predictive epigenetic biomarkers and modifiable targets for intervention [102,116,117].

Many of the approaches already listed to address causality in conventional epidemiological settings can also be used to interrogate causality in associations involving epigenetic changes. For example, maternal smoking in pregnancy has been shown to be associated with DNA methylation in newborns and the finding of no paternal associations highlights the prominent intra-uterine influence of maternal smoking on offspring DNA methylation at birth [118] and at later ages in the offspring [119]. Mendelian randomization analysis has also been used in the context of epigenetic epidemiology to investigate the causal effect of maternal red blood cell folate on genome-wide methylation in infant cord blood [120], using the previously described MTHFR genotype as an instrument. However, further work is needed to investigate whether the identified methylation changes mediate the influence of intra-uterine exposures on developmental outcomes, for example in a “two-step Mendelian randomization” framework [77,102,116,117].

15. Metabolomics

Metabolomics is a technology involving the measurement of metabolites which likely act as intermediates in biological pathways. An advantage of using metabolites as intermediate phenotypes is that they are more proximal to biological pathways than downstream phenotypes or clinical endpoints [121], boosting the statistical power to detect associations [122,123]. Metabolites are also useful in birth cohorts when disease endpoints have not yet been reached.

However, as metabolites are influenced by both genetic and environmental factors and by disease processes, they too are prone to the limitations of observational study. Once an association between a metabolite and a trait has been observed, the next challenge is to distinguish causal effects, with potential implications for clinical outcomes and disease pathogenesis, from non-causal associations, which may have potential implications for biomarker discovery [12,124]. Different statistical methodologies may be used to construct a causal framework involving metabolites, and to dissect causal relationships [125]. This framework also suggests the usefulness of “triangulating” causal inference methods in the domain of high-dimensional molecular data as an exploratory tool to infer causal relationships.

16. Summary

This review has outlined a suite of causal inference methods including cross-cohort comparisons, negative control studies, sibling studies, Mendelian randomization analysis and instrumental variable techniques. These methods make use of the wide range of data available in epidemiological birth cohorts in order to establish causal links between early life influences and a range of developmental and health outcomes. Such methods have often been shown to produce the same conclusions regarding causal effects as randomised controlled trials, which are not always feasible or ethical, and may be used to inform on interventions. Strengthening causal inference is also an important step in “omics” research for distinguishing causal molecular pathways that may underpin causal effects of early life exposures on complex traits and diseases.

The methods for causal inference described enhance capability to interpret conventional observational associations, though some discrepancies in findings between studies highlight their limitations, in particular their lack of power in small samples. An integration or “triangulation” of different approaches to the same research question may be used to improve the identification and estimation of causal effects in observational data. In addition, cross-cohort analysis and the independent replication of findings can improve power and statistical precision and provide more high-quality evidence for causality. This may be enabled with collaboration amongst different birth cohorts and the dissemination and harmonisation of techniques through the established consortia.

Conflicts of interest statement

None declared.

Acknowledgements

RCR, GDS and CLR are members of the MRC Integrative Epidemiology Unit (IEU) funded by the UK Medical Research Council (MC_UU_12013) and the University of Bristol. RCR is funded by a Wellcome Trust 4-year PhD studentship (Grant Code: WT083431MF).

GDS and CLR are partially supported by the ESRC (RES-060-23-0011) “The biosocial archive: transforming lifecourse social research through the incorporation of epigenetic measures”. GDS’s work is supported in part by the European Research Council grant DEVHEALTH 269874.

References


A paternal environmental legacy: Evidence for epigenetic inheritance through the male germ line

Adelheid Soubry1)*, Cathrine Hoyo2), Randy L. Jirtle3)4) and Susan K. Murphy2)

Introduction

A number of animal and human studies demonstrate that periconceptional and in utero developmental maternal exposures to a variety of environmental factors affect the risk for disease development in subsequent generations. Early observations of intrauterine exposure to maternal malnutrition as a determinant for type two diabetes in the offspring led to Barker’s “thrifty phenotype hypothesis” [1]. This theory has now been extended to reflect a wider scope of exposures, and is called the “Developmental Origins of Health and Disease” (DOHaD) hypothesis. DOHaD concepts include exposures to environmental chemicals and toxins, use of medicines, infections, nutritional status, and other stressors in pre-pregnancy, during in utero development, and during the first years of life (reviewed by [2]). Poor health outcomes in children associated with harmful maternal exposures include congenital abnormalities [3], obesity, and insulin resistance [4], cardiovascular diseases [5], behavioral disorders [6], and potentially even cancer [7]. Following a landmark study by Waterland and Jirtle [8] using the agouti viable yellow (A<sup>vy</sup>) mouse model, the biological mechanisms underlying these associations in humans are now proposed to involve alterations in the epigenome, including DNA methylation, histone modifications, and transcription of non-coding RNAs. During the development of gametes, DNA methylation is

Keywords:
- developmental origins of health and disease (DOHaD); environment;
- epigenetics; imprinted genes; offspring; paternal exposures; spermatogenesis;
- transgenerational effects

DOI 10.1002/bies.201300113

1) Epidemiology Research Group, Department of Public Health and Primary Care, Faculty of Medicine, KU Leuven, Leuven, Belgium
2) Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC, USA
3) Department of Oncology, McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI, USA
4) Department of Sport and Exercise Sciences, Institute of Sport and Physical Activity Research (ISPAR), University of Bedfordshire, Bedford, Bedfordshire, UK

*Corresponding author:
Adelheid Soubry
E-mail: adelheid.soubry@hotmail.com

Abbreviations:
- A<sup>vy</sup>, agouti viable yellow; DMR, differentially methylated region; DNMT, DNA (cytosine-5)-methyltransferase; DOHaD, developmental origins of health and disease; IGF2, insulin-like growth factor 2; PAHs, polycyclic aromatic hydrocarbons; PCP, pentachlorophenol; ROS, reactive oxygen species; SGP, slow growth period.
uniquely regulated. Primordial germ cells undergo a nearly complete epigenetic erasure, followed by reprogramming of DNA methylation patterns in a sex-specific manner, such as at imprinted genes [9–11]. Imprinted genes are characterized by parent-of-origin dependent monoallelic expression; their functional haploid state being controlled by differentially methylated regions (DMRs) [12, 13]. The establishment of inherited imprint methylation marks at these DMR sites during gametogenesis is essential [9, 14, 15], and their aberrant methylation is associated with infertility and several chronic disorders [16–20]. Hence, paternal influences on the formation of epigenetic marks during spermatogenesis, and their impact on the health of the offspring are important biological endpoints to investigate. Human epidemiologic studies covering two or more generations are difficult to conduct, and only a few have provided evidence for the inheritance of epigenetic information through the male germ line [21, 22]. Nevertheless, a significant number of epidemiologic studies report unexplained father-child effects from various occupational or other environmental exposures. Although DNA damage or mutations are often suggested or assumed as the biological background for these harmful outcomes, the literature does not always provide sufficient evidence for an exposure-related mutagenic effect. An increasing number of animal experiments confirm that the offspring’s epigenetic profile and health status is influenced by paternal preconceptional insults, such as exposures to endocrine disruptors or toxins [23], ionizing radiation [24], and nutritional status [25, 26]. Hence, an additional (or sole) epigenetic component responding to the environmental insult cannot be excluded. In this essay, we explore paternal exposures to various pollutants and lifestyle-related conditions, and their potential effect on the health status of future generations. We discuss the accumulating evidence that epigenetic mechanisms are important in the transfer of information from one generation to the next through the male germ line.

Do paternal exposures to environmental toxins promote transgenerational epigenetic changes?

Fathers occupationally exposed to high levels of carcinogenic substances may not only endanger themselves, but also their children. The realization that paternal occupational exposure to chemical substances can affect the integrity of spermatogenesis, and potentially result in the transmission of carcinogenic defects to the children, was initially reported by Fabia and

Box 1
Animal models: Evidence for transgenerational epigenetic effects from paternal exposures to environmental toxins.

Animal models indicate that male exposure to pesticides or other harmful chemicals causes defects in the gametes and abnormal development of the offspring. Insecticides, such as chloropyrifos, affect sperm quality, and pregnancy outcomes in mice [52]. The fungicide vinclozolin induces fertility problems and abnormalities in rats for at least four subsequent generations. Interestingly, this effect was most pronounced in males and was correlated with altered DNA methylation patterns in the germ line [23, 53]. Reproductive consequences of vinclozolin appear at relatively low doses; lower than the dose defined by the US Environmental Protection Agency (EPA) as “no observed adverse effect level” (NOAEL) [54]. Chronic exposures to low doses of vinclozolin not only affect male fertility, but also affect the levels of mRNA in mice testes [54]. Similarly, very low doses of a herbicide used worldwide, Simazine, administered during pregnancy did not elucidate measurable toxicity in the mother, but adversely affected normal development and reproductive activity of male offspring, accompanied with changes in expression of several genes in the testes [55]. These results on low-dose exposures are concerning, especially given the fact that toxicological classifications are generally based on mutagenic or other non-epigenetic tests. Another report on the commercially available pesticide Roundup reveals that low dose exposure during prepuberty in rats negatively affects fertility and causes overproduction of ROS in the testis [56]. Increased levels of ROS potentially cause cellular damage. Unbalanced ROS have been linked with impaired spermatogenesis, DNA damage and epigenetic alterations that ultimately increase risk of development of diseases [57, 58]. Pollutants such as plasticizers (e.g. phthalates) and heavy metals (e.g. lead) also stimulate testicular ROS generation, resulting in (at least) impaired spermatogenesis [59–61]. Plastic-derived endocrine compounds bisphenol-A (BPA), bis(2-ethyl-hexyl)phthalate (DEHP) and dibutyl phthalate (DBP) and other toxins such as the hydrocarbon mixture JP-8 (or jet fuel) cause permanent changes to DNA methylation in F3 generation animals, testis or pubertal abnormalities, and several adult onset pathologies [62, 63]. Another study in mice confirmed the concept that environmental toxins induce transgenerational epigenetic changes in sperm DNA. Adult males treated with methoxychlor, an insecticide, showed a decrease in methylation at the paternally imprinted Dlk1/Meg3 (Delta-like, drosophila homolog1/maternally expressed gene3) gene, and an increase in methylation at the maternally imprinted gene Peg1/Mest (paternally expressed gene1/mesoderm specific transcript), Snrpn (small nuclear ribonucleoprotein polypeptide N), and Peg3 (paternally expressed gene3) in sperm. Interestingly, administration of methoxychlor in pregnant mice seemed to encompass the erasure of the methylation marks and the beginning of the methylation resetting within imprinted genes in sperm of offspring over two generations [64]. Although the mechanisms have not yet been elucidated, these observations suggest that environmentally induced epigenetic defects can survive transgenerational reprogramming.
with an increased risk of congenital malformations [40] and cancer in the offspring [29, 41]. However, it is not always clear to what extent the potential environmental effect is related to the father only, especially in a domestic or agricultural context. If the outcome varies by gender of the parent it is easier to separate paternal from maternal influences. In a case-control study of families residing in industrially or agriculturally polluted regions of the Yangtze River, an association was reported between high concentrations of PCP in the father’s urine and unexplained spontaneous abortions; while similar significant associations were not found for urinary PCP in mothers [42]. A recent meta-analysis, focusing on case-control and cohort studies where information on preconceptional exposures of both parents was available, provided evidence for increased risk of childhood brain cancer if the father was exposed to pesticides through occupational activity or the use of household or garden pesticides; maternal exposures were also linked to the incidence of childhood cancers, but cancer sites were different [43]. Not all studies show an association between paternal exposure to pesticides and health outcomes in the offspring; and many miss the assessment of exposure-risk gradients. Additionally, the evaluation of broad classes of pesticides may dilute the potential effect(s) [44]. Hence, there is a need for studies that better define and quantify the exposures to the different categories of biocides used at home or through occupation. Organophosphates and organochlorides (or dioxins) are likewise health burdens on a large scale, but they are still used as pesticides and as flame retardants. High concentrations of some of these organophosphate chemicals in house dust, such as tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and triphenyl phosphate (TPP), are associated with decreased semen quality [45]. Whether flame retardants present in furniture and clothing also affect the offspring remains unanswered. Exposure to other contaminants, such as bisphenol A (BPA), phthalates, heavy metals, and other toxic compounds through maternal exposures are known to affect pregnancy outcomes and the offspring’s health potentially by altering the epi-genome; however, the consequences of paternal exposures to the male germ line and offspring are unknown.

The striking consequences of involuntarily human exposures to high concentrations of chemicals, such as during war, confirm that paternal chemical exposures before conception can affect the offspring’s health. “Agent orange”, a mixture of chemicals including herbicides and dioxin, used by the US Army in the Vietnam war, not only caused devastating disabilities and birth defects in the Vietnamese population, it also affected the offspring of exposed US soldiers. A meta-analysis conducted by Ngo et al. [46] describes a strong association between congenital malformations in offspring, and the exposure of veterans and Vietnamese; with an overall estimated relative risk of 1.95 (95% CI: 1.6–2.4) and 3.0 (95% CI: 2.2–4.1), respectively. In a subsequent meta-analysis, a twofold increased risk of spina bifida was reported in children from agent orange-exposed Vietnam veterans [47].

Both genomic and epigenetic pathways have been suggested to explain the transmissible effects of environmental contaminants, including sperm DNA mutations, genomic instability, suppression of germ-cell apoptosis, and imprinting errors [48]. However, most epidemiologic studies do not include evidence for these mechanisms, and many assume that the (only) mechanistic underlying cause is a genetically inherited mark. Since epidemiologists, environmental toxicologists and molecular biologists have just begun to explore these questions through interdisciplinary research, yet undiscovered epigenetic effects from occupational or environmental exposures through the paternal germ line will undoubtedly be revealed in the future. An interesting study performed in people who migrated from agricultural areas to urban settings in India showed that having a malformed or aborted child is associated with high DNA damage and high reactive oxygen species (ROS) levels in semen [49]. In the same population, high seminal ROS was also found in men who fathered children with retinoblastoma (personal communication with Rima Dada). We know from animal models that high ROS in testes is related to epigenetic changes in sperm. Hence, it is possible that paternal occupational...
exposure to pesticides may have affected some genetic and epigenetic characteristics of the sperm through altered ROS, and ultimately increased the risk for disorders in the offspring. Further investigation in this and other populations is necessary to confirm our hypothesis. Noteworthy is the study of Warmlander et al. [50] on the skeletal phenotypes of Ancient Californian Indians and their use of bitumen more than 2,000 years ago. Bitumen (tar) is a mixture of PAHs that was used in Indian manufacturing techniques, from the making of leak-free water baskets to the sealing of fishermen’s canoes. Skeletal analyses revealed an association between the increase in use of bitumen over centuries and a decrease in population stature, reflecting a decline in health conditions. Although caution is warranted when drawing conclusions from these ancient data, since the exact exposure levels are unknown, a gender-related decline in cranial volume was observed over multiple generations; the effect appears to be stronger in males [50]. If the current evolving technology makes it possible ultimately to determine PAH levels in these archeological specimens, and if next-generation sequencing technologies are included to perform (epi) genome-wide analyses, we may be able to decipher the effects of environmental changes in the past on human adaptation. Recent research on ancient bison bones indicates that DNA methylation patterns are faithfully retained along with nuclear DNA over evolutionary timescales [51]; making these ancient samples ideal tools to explore the role of environmentally induced epigenetic modifications and their effects on evolution. Research on animal models shows that toxin-induced epigenetic changes are measurable in the germ line and can survive several generations. Epigenetic effects from different harmful chemicals in animals are summarized in Box 1.

**Do paternal exposures to low dose ionizing radiation promote transgenerational epigenetic changes?**

Ionizing radiation induces germ line genomic instability and may have adverse effects on the offspring [48]. Men who received radiation treatment for childhood cancers have an elevated sperm DNA fragmentation index, and are at increased risk of having fertility problems when compared to controls [65]. Transgenerational effects from paternal exposure to radiation through occupation, airport scans, medical treatment and diagnosis, and other man-made sources of radiation presently remain mostly unknown. An unsolved epidemiologic finding relates to the Sellafield case. Public concerns in the 1980s prompted the UK government to investigate the excess of malignant

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**Box 2: Animal models: Evidence for transgenerational epigenetic effects from paternal exposures to ionizing radiation.**

High doses of ionizing radiation in mice, administrated before mating, cause an accumulation of DNA double strand breaks in somatic cells of the offspring, which is accompanied by global hypomethylation, changes in the levels of methyltransferases, and altered microRNA expression [24, 87]. An acute gamma-irradiation of male mice destabilizes the sperm genome and F1 brain genome, indicating a transgenerational instability triggered by a certain threshold dose of acute paternal irradiation [88]. Koturbash et al. [87] speculate that sperm cells damaged by radiation may interfere with the epigenetic programming of the fertilized egg, causing genomic instability and potential carcinogenesis in the progeny of the exposed parent. Additionally, an epigenetic bystander effect occurs in the cells of unirradiated organs of rats after cranial radiation with high doses. These changes included loss of global DNA methylation, altered levels of miRNAs, and downregulation of DNA methyltransferases and methyl CpG binding protein 2 (Mecp2) [89, 90]. Since these epigenetic effects occur in organs that neighbor the exposed tissues, it is possible that germ cells may also be affected through a bystander process, thereby causing heritable defects in the offspring. The same research group investigated the effects of chronic low dose radiation exposure on somatic cells in an in vivo murine model, and found that it induced epigenetic changes, such as genome-wide hypomethylation; while acute low dose administration showed no direct measurable effects [91].

Bernal et al. [92] recently showed, with the use of the agouti viable yellow (Avy) mouse model, that paternal exposure to doses of X-rays used in diagnostic CT-scans (0.7–7.6 cGy) alters the epigenome in the offspring. The offspring were irradiated at implantation stage, while an effect of paternal irradiation on the epigenome of the offspring has not yet been determined. The results of this study demonstrate that low doses of X-rays induce dose- and sex-dependent increases in DNA methylation at the Avy locus, causing a significant shift in the coat color distribution of the offspring from yellow to brown [92]. Dietary antioxidants taken during pregnancy negate the radiation-induced increase in DNA methylation, indicating that low doses of ionizing radiation increase DNA methylation at the Avy locus in part through the generation of ROS. Persistent induction of ROS as a response to radiation exposure has been suggested earlier [93], and free radical injury can profoundly alter DNA methylation levels [58, 94]. Thus, events such as exposure to X-rays during early pregnancy may alter the cellular redox state in pluripotent stem cells, determining the ultimate methylation status at the Avy locus at birth. Once the utero-placental circulation is established, dietary antioxidants may reduce the abundance of highly reactive ROS, and reduce the epigenetic consequences. Although this is speculative, this intriguing possibility needs to be investigated.
diseases in children living in the vicinity of the Sellafield nuclear plant. A population-based analysis confirmed the high incidence of leukemia and lymphoma in the young residents of Seascale, the village near the Sellafield plant, when compared to those in national registries and surrounding areas [66]. In a cohort study of children attending school at Seascale, an increased rate of leukemia and other cancers was observed among children born in Seascale, but not in children who moved to the village after birth [67].

A case-control study indicated that children of fathers working at the nuclear plant at the time of conception had a three times higher risk of developing leukemia or non-Hodgkin’s lymphoma before the age of 25. Interestingly, the same study suggested a preconceptional dose–response relationship [68]. An independent case-control analysis confirmed the association between excess risk of childhood leukemia and lymphoma in the area when paternal radiation exposure occurred at the time of conception, but not when radiation exposure occurred three to six months before conception [69].

These studies ultimately led to “Gardner’s Hypothesis”, which proposes a causal relationship between paternal exposure to ionizing radiation and cancer risk in the offspring [68, 70]. Gardner’s hypothesis has been widely criticized, and was ultimately rejected [71–73]; in part, because after a comparison with other studies, such as those on the atomic bomb survivors in Japan, no evidence was found for increased cancer incidence in children from exposed fathers [74–76]. Some attributed the increased risk of childhood leukemia near the nuclear plant to population mixing and a yet unidentified infectious agent [69, 77, 78].

Furthermore, additional studies on populations near nuclear plants in other countries did not show significant effects on the young population living in the vicinity of nuclear plants, with some exceptions [79]. Other clusters of childhood cancers were reported near the Krümmel nuclear power plant in Germany [80], the Dounreay nuclear reactor in Scotland [77], and the nuclear fuel reprocessing plant near La Hague, France [81]. Nevertheless, no plausible (genetic) explanations have been proposed to date for these clusters, and because of a lack of knowledge regarding the biological mechanism behind these observations, it was concluded that there were “no indications” for an increased risk of childhood cancer [79].

But, in his critical report [82], Nussbaum rightfully reminded epidemiologists that they should not ignore a fundamental rule earlier espoused by Altman and Bland [83], “The absence of evidence of an effect does not constitute evidence of absence of that effect.”

Although the possibility that paternal exposure to ionizing radiation increases the susceptibility of the offspring to cancer remains controversial, it cannot be excluded that epigenetic effects may play a role in the unexplained excess of cancer incidence observed in children from fathers working in the nuclear industry. Notably, studies in human and animal populations exposed to radiation from the Chernobyl nuclear power plant accident in 1986 showed DNA damage in sperm and an overall increase in generation of reactive oxygen metabolites [84, 85].

Further analyses in the offspring of fathers exposed to low doses of radiation during cleanup of the nuclear plant showed an elevated frequency of chromosome aberrations, which may lead to increased morbidity over their lifetimes [86]. To our knowledge, no studies on Chernobyl victims were performed to verify potential transgenerational epigenetic effects through the paternal germ line. Analyses on epigenetic markers and long-term follow-up studies are needed to help resolve this important question. Animal data provides evidence for transgenerational epigenetic changes from exposure to high and low dose ionizing radiation (see Box 2).

**Does paternal lifestyle, diet, or obesity promote transgenerational epigenetic changes?**

Maternal smoking before and during pregnancy is a well-recognized risk factor for adverse health outcomes in the child; however, paternal preconceptional exposure to PAHs from cigarette smoking is likewise associated with childhood cancer [34]. The Avon Longitudinal Study of Parents and Children (ALSPAC) indicates that the earlier the father starts smoking in life, the higher is his son’s BMI [95]. Paternal cigarette smoking at the time of conception is also linked with DNA damage in cord blood of the offspring, while maternal passive smoke exposure is not a predictor for DNA damage [96]. This indicates that cigarette smoke metabolites may induce transgenerational epigenetic inheritance of vulnerability for DNA integrity defects through the paternal germ line. Interestingly, cigarette smoke induces changes in miRNA profiles of human spermatozoa. The altered miRNAs were suggested to target epigenetic compounds important in DNA methylation and histone modification [97].

Another lifestyle-related factor is obesity. The obesity burden and concomitant health problems are global issues. More alarming is that obese parents tend to give birth to children who will also become obese [98]. Furthermore, these children not only have a higher BMI than children from non-obese parents, there is also a higher risk of congenital abnormalities at birth [3], behavioral problems [6], cardiometabolic dysfunction, and other chronic disorders in later life [5]. As with other parent-child associations, these harmful health conditions are generally attributed to the mother’s lifestyle or diet. However, Figueroa-Colon et al. [99] emphasized for the first time in humans that the father’s body composition can also affect the offspring. They reported a significant association between paternal body fat and long-term changes in the percentage of body fat in their prepubertal children. In contrast, a separate correlation analysis on maternal anthropometric characteristics did not show a significant influence of BMI on the offspring’s body composition.

Long-term cohort studies, such as the Framingham Heart Study, show associations between early-onset paternal (but not maternal) obesity and aberrant levels of circulating alanine transaminase (ALT) in the offspring. Elevated ALT levels are associated with liver dysfunction and obesity, providing evidence for an as yet unknown underlying transgenerational influence on metabolic processes affecting the offspring of obese fathers [100].
Box 3
Animal models: Evidence for transgenerational epigenetic effects from paternal lifestyle and nutrition-related exposures.

It is widely accepted that the intrauterine environment, including maternal nutrition, is important in determining an offspring’s birth weight and risk for chronic disorders in childhood and adult life (reviewed by [2]). Research with the A\textsuperscript{null} mouse model also demonstrated that maternal nutrition during pregnancy can alter the phenotype of the offspring by changing the epigenome [8]. Experiments in rats also show that environmental factors, such as a parental high-fat diet, already affect the offspring if the exposure takes place before in utoe development. A high-fat diet of the parents before and during mating results in offspring with increased body-fat accumulation, increased weight gain, and altered expression of lipoprotein lipase and leptin in adipose tissues [108]. Maternal obesity in rat disturbs postnatal steroid levels and development of male germ cells [109]. Paternal food deprivation in male mice before conception leads to offspring with impaired glucose metabolism [25]. Carone et al. [110] demonstrated that male mice consuming a low-protein diet from weaning to sexual maturity had different RNA content and chromatin packaging of sperm as compared to controls; they also fathered offspring with altered DNA methylation at specific liver CpG islands, including a potential enhancer for the key lipid regulator PPAR\textsubscript{\alpha}; and, expression of hepatic genes involved in lipid and cholesterol biosynthesis were elevated. Ng et al. reported that a paternal high-fat diet results in lower DNA methylation at a putative regulatory region of the interleukin 13 receptor alpha 2 gene, coupled with impaired glucose tolerance and increased body weight in rat offspring [26]. These data strongly indicate that a suboptimal diet during male gametogenesis can influence the metabolic status of the offspring and affect phenotypic outcomes. Although a diet-induced epigenetic effect during spermatogenesis was suggested, a “sperm signature” from paternal diet was only recently reported by the group of Michelle Lane. High-fat paternal diet may increase histone acetylation [111], ROS and sperm DNA damage [112–114]. In addition, male mice consuming a high-fat diet showed altered global methylation and microRNA content in mature sperm, and altered transcriptional profiles in the testes; interestingly, these alterations were linked to metabolic disturbances in the next generations [115].

Genistein is a dietary compound also known to affect DNA methylation patterns. It is a phytoestrogen present in soy or soy-derived products. While its effects on the epigenome have been widely discussed in offspring after relatively short perinatal or in utero exposures [116, 117], and changes in DNA methylation have been demonstrated in organs of exposed animals, including mouse prostate [118], Eustache et al. explored genistein exposures from conception to adulthood in male mice and found deleterious effects on male reproductive development, adult reproductive organs, and fertility. Another important finding of this lifelong genistein exposure was a change in the testis transcriptome, with a general repressive effect on gene expression [54]. Importantly, a major effect was seen at low doses as compared to high doses, and variable results were detected if mixtures with vinclozolin, an anti-androgenic food contaminant, were used. These results underscore the complex interplay of synergistic or antagonist actions of food-born nutrients and/or contaminants, resulting in different molecular or phenotypic outcomes.

Other examples of nutritional compounds or supplements influencing the epigenome are vitamins. Singh et al. [119] suggested that nutritional deficiencies of vitamins or micronutrients, are potential triggers for disturbances in chromatin packaging and DNA integrity during spermatogenesis, and hence the maintenance of the male reproductive health. Folate is a naturally occurring water-soluble vitamin and a key source of the one-carbon group necessary to methylate DNA. Folate, or its synthetic form folic acid, is crucial for normal embryonic development; hence, most studies focus on effects of folate deficiencies during pregnancy (reviewed by [120]). An interesting approach by Mejios et al. [121] showed that both maternal and paternal folate deficiency can influence global DNA methylation in rat offspring. Further studies on animal models are needed to confirm a direct epigenetic effect of folate on sperm DNA and to explore the mechanisms involved to transmit these defects permanently through fertilization and embryonic growth.

Literature showing a direct effect of other lifestyle-related exposures on sperm epigenetics is scarce. To date, two studies demonstrate a correlation between alcohol use and demethylation at gene regulatory sites of IGF2 and H19 in humans [122], and in mice [123].

A Swedish study on historical data of three generations indicates a transgenerational response to variable food availability during the slow growth period (SGP), before the prepubertal growth peak. Longevity of males was reduced if the grandfather [101] or the father [102] was exposed to an excess of food during the SGP. Similar results were found in females if the paternal grandmothers were exposed to an excess of food supply [102]. Furthermore, the risk of death from diabetes in the descendants was four times increased if the paternal grandfather was exposed to a plentiful food supply in his SGP [21]. These remarkable gender-specific associations suggest that during the SGP epigenetic changes in the germ line may underlie these transgenerational effects, but this has not yet been determined.

The first epidemiologic evidence of epigenetic changes in the offspring being triggered by paternal obesity came from analyses of DNA methylation in the Newborn Epigenetics Study...
birth cohort. The father’s BMI was shown to be inversely related to the level of DMR methylation at the imprinted insulin-like growth factor 2 (IGF2) locus [22]; similar results were seen at DMRs of other imprinted genes involved in early growth regulation [103]. Interestingly, these results were independent of maternal obesity. As stated before, the inherited methylation marks for DMRs involved in regulating imprinted gene expression are established during gametogenesis [15], and their deregulation is associated with several chronic or metabolic diseases in the offspring [16–18]. A follow-up analysis of NEST children is needed in order to correlate the obesity-induced findings with metabolic outcomes at later age.

Two study cohorts on maternal nutrition highlight the importance of the timing of exposure to nutritional insults. Periconceptional exposure to food deprivation in the Dutch famine cohort or seasonal dietary circumstances in a Gambian study cohort showed strong associations with poor health outcomes and altered DNA methylation in the offspring [104, 105]; however, neither study addressed the importance of the fathers’ diet. Since the fathers were likely to be exposed to the same famine or nutritional conditions as the mothers, a paternal effect cannot be excluded in these cohorts. As suggested by Lecomte et al. [106], large epidemiological studies are needed where stratified analyses by maternal and paternal influences are carried out, and attempts to dissociate parental obesity from nutritional status should help us understand which phenotype is related to which nutrient deficiency or abundance. Finally, an extensive nutritional study on multiple populations was published in 1939 by Weston Price, an American nutritionist and dentist who investigated multiple tribal diets around the world [107]. Price reported that in several primitive tribes there was a consciousness that the food eaten by both parents before conception has significant influences on birth characteristics, ultimate overall health, and character of the child. He noticed a higher frequency of facial deformities when these “primitive” tribes adopted the Western diet. Furthermore, he commented specifically about the effect of the father’s diet on the offspring’s dental and facial phenotypes. Nevertheless, since Price did not report his original measurements or statistical analyses, these observations need to be interpreted with some caution. Studies on animal males consuming different diets provide evidence for epigenetic effects in the male reproductive system and in the offspring (see Box 3).

**Epigenetic mechanisms:** How and when is the paternal environmental information transmitted to the next generation?

The increasing number of reports on associations between paternal environmental exposures and fertility or risk of disease in the next generation evokes the compelling question of how and when the effects of environmental...
exposures are transferred to the male gametes, and how these effects are sustained through developmental processes. Besides the potential for genetic damage or DNA mutations in sperm cells, animal studies, and epidemiological data indicate that transfer of information through generations may also occur via epigenetic mechanisms. There are a number of potential windows of susceptibility during the lifespan of the father where environmental effects can impact the epigenetic profile of his gametes. We summarized four windows of susceptibility during development of the paternal germ line and zygote in Fig. 1, and discuss the potential roles of DNA methylation, histone modifications, and/or presence of non-coding RNAs during these early developmental processes, in the following paragraphs.

Paternal embryonic development: A first window of susceptibility

During embryonic development, primordial germ cells undergo genome-wide epigenetic erasure as they migrate to the genital ridge. Animal models indicate that this process may appear in waves of active and passive DNA demethylation mechanisms, affecting the bulk of the genome and imprinted genes at different times. However, some portions of the genome have been reported to remain resistant to DNA methylation erasure [124, 125]. These protected genomic regions, currently limited to IAPs, LTR-ERV1 elements, and a few single-copy sequences, open the potential for transgenerational inheritance of DNA methylation profiles over multiple generations. Defects in complete erasure, or in maintenance of the protected regions, could be the first potential effect from internal or external factors during early development (green lightning bolt, Fig. 1).

Paternal prepuberty and spermatogenesis: Second and third windows of susceptibility

Following the epigenetic erasure, DNA methylation is gradually re-established throughout spermatogenesis [9, 10]. The literature suggests that de novo methylation at imprinted gene loci occurs mainly during differentiation from primordial germ cells to spermatogonia, i.e. before puberty in human. Hence, this period of life represents a second, and presumably important, window of susceptibility (blue lightning bolt, Fig. 1). Given that methylation patterns seem to be established by the time germ cells are differentiated to mature spermatocytes [14, 15], the early phase of each reproductive cycle (i.e. development from spermatogonium to spermatocytes) is a third potential vulnerability window (orange lightning bolt, Fig. 1).

De novo methylation and its maintenance are established through DNA (cytosine-5)-methyltransferase (DNMT) enzymes, including DNMT3A and DNMT3B, and DNMT1, respectively. DNMTs are expressed throughout spermatogenesis [15]. Further, the DNA methyltransferase-like protein DNMT3L possesses no DNA methyltransferase activity, but is crucial to the establishment of DNA methylation patterns during spermatogenesis [126, 127]; it interacts and stimulates de novo methylation activity of DNMT3A and DNMT3B [128, 129]. It has been suggested that maintenance of paternal imprints during all stages of spermatogenesis is a dynamic process that might result in a fluctuation in methylation at some CpG dinucleotide sites [15]. This normal fluctuation may be vulnerable to skewing by exposure to environmental factors that influence the transcriptional activity of DNMTs. The autocrine human growth hormone (hGH) has been described to influence DNA methylation through activation of signaling pathways that lead to transcriptional upregulation of the de novo DNA methyltransferase enzymes [130]. Environmental toxins, such as endocrine disruptors, or circulating obesity-related hormones (e.g. estrogen, leptin, and insulin) can accumulate in scrotal fat, potentially affecting sperm DNA methylation through their influence on DNMT activity; thereby contributing to infertility and pregnancy failures [131]. Pathak et al. reported how the estrogen pathway could be involved in genomic imprinting. In rat spermatooza, the estrogen-estrogen receptor β complex interacts with Dnmt1 and binds an estrogen response element at the H19 DMR, catalyzing methylation of the H19 CpG island. DNA methylation at this site may be counteracted by administration of an estrogen receptor modulator, Tamoxifen [132]. These reports strengthen the idea that environmental estrogens may interfere with a normal crosstalk between estrogen signaling and imprinting during spermatogenesis.

Nutritional compounds, such as dietary fatty acids, can directly stimulate transcription of specific genes or transcription factors (such as PPARα [133, 134], potentially also affecting the establishment of epigenetic mechanisms during spermatogenesis; but this is still an unexplored area. Supplementation of methyl donors including folic acid and vitamin B12 are able to increase the flux through a DNA methylation pathway at specific loci, resulting in DNA hypermethylation. This has been studied mainly in maternal or pregnancy models [8, 135], while a recent animal study on males suggests that also the paternal germ line is susceptible to DNA methylation changes through dietary folate intake [121]. Besides a potential environmental epigenetic effect through interaction with hormonal signaling pathways during spermatogenesis, other downstream effects of the surrounding environment on sperm and surrounding cells, e.g. Sertoli cells and leucocytes, include altered ROS concentrations. Various factors such as long-term exposures to chemicals or pesticides [56, 59], heavy metals [61], low dose ionizing radiation [93], chronic conditions such as diabetes [136, 137] and obesity [113, 114], or increasing levels of fatty acids [112] can promote ROS generation. Changes in ROS may modulate sperm DNA methylation and chromatin structure, ultimately influencing regulation of imprinted genes important in growth and development; or other genes, such as those responsible for maintenance of genome stability, altering DNA damage responses and repair mechanisms. Consequently at birth, cord blood or samples of other tissues may reveal DNA strand breaks and/or DNA methylation abnormalities, sustained throughout life and increasing risk for disease.

Periconception and zygote stage: Fourth window of susceptibility

In order to persist throughout embryogenesis, the acquired epigenetic signature needs to withstand reprogramming that occurs after fertilization. Until recently, the role of histones in conveying epigenetic information in mature spermatozoa was doubted because it was believed that all histone proteins are replaced by protamines (i.e. protamines 1 and 2) during late spermatogenesis. This facilitates a highly condensed state of the chromosomes, represses transcriptional activity, and prevents DNA damage [19]. After fertilization, the protamines are removed and replaced with maternal histone proteins, which then undergo the epigenetic modifications required for cellular differentiation. It is now clear, however, that there is selective retention of up to ~10% of the histone proteins in condensed chromatin of the sperm DNA (reviewed by [138]). The modifications present on histone proteins in sperm provide one mechanism by which epigenetic information is carried to the next generation. This retention of histones may relate to the selective establishment of specific DNA methylation patterns, as observed for developmental genes critical to early embryogenesis, including imprinted genes, microRNAs, and homeobox genes [139]. For these genes, histone retention provides a regulatory mechanism in which paternal DNA is poised for immediate activation after fertilization [20]. Lane et al. [126] demonstrated in mouse that intracisternal A-particles (IAPs) are largely resistant to DNA demethylation during preimplantation. Consequently, acquired epigenetic states of IAPs can also lead to persistent heritable changes in transcription of neighboring genes. Histone retention at yet undefined genes or gene promoters cannot be excluded as a potential mechanism for inheritance of environmentally induced epigenetic marks through this window. Importantly, nucleosomal patterns are disrupted in men with infertility [140]. Although the processes described here may explain in part how early environmental messages can be transmitted to the embryo, the complex mechanisms of selective removal, replacement, and retention of epigenetic factors (such as histones or methylation marks) in the fertilized oocyte makes this period of development vulnerable to environmental damage; hence, we define the zygote as a fourth developmental stage where paternal periconceptional influences may play an indirect role (pink lightning bolt, Fig. 1).

Non-coding RNAs as potential messengers of epigenetic information

Besides DNA methylation and histone modifications, the presence of RNA molecules in spermatozoa suggests another type of regulatory mechanism that is implicated in conveying epigenetic information; thereby potentially leading to phenotypic changes in the next generation [141]. An extensive review on the “hidden features” of RNAs in spermatozoa has been authored by Kumar et al. [49]. The types of RNA molecules present include mRNAs and non-coding RNAs. Their retention in spermatozoa begins to occur during early stages of spermatogenesis. Among the non-coding RNAs, several small RNAs have been identified in the sperm, which raises the possibility that these small molecules can carry hereditary information from one generation to the next [142, 143]. RNAs present in the mature spermatozoa are delivered to the oocyte, and from RNA-Seq analysis, many seem to have essential functions during early embryogenesis [144]. The genetic origins of RNAs identified in sperm are highly correlated with regions of the genome that are hypomethylated and enriched in histone proteins, especially those with H3K4me3 (but not H4K27me3) modifications. Spermatozoal RNAs seem to possess the capacity to direct histone modifications and DNA methylation, for instance in response to paternal smoking [97], while the chromatin structure and DNA modifications in turn may affect transcription of RNAs. This “epigenetic crosstalk”, suggested by Rando, may be influenced by the paternal environment [145]. The longevity of such effects is presently unclear, but enzymes such as DNMT2 may protect these small RNA molecules from stress conditions during early embryogenesis [146].

Conclusions and outlook

Environmental variation may lead to transgenerational epigenetic changes resulting in differences in gene expression and ultimately different phenotypes or diseases in the following generations. Besides the generally assumed inherited genomic defects from environmental insults, epigenetic changes may accumulate (i.e. from chronic exposures) or persist and result in heritable modifications of the genome. This may influence male fertility or if the epigenetic modification is subtle it may be transmitted to the zygote through yet unidentified carriers, ultimately affecting health status in the offspring. Aside from the need to better understand the mechanisms by which environmental factors can alter epigenetic reprogramming in sperm, there are a number of unanswered questions. Is there a threshold of tolerance for epigenetic skewing beyond which there is certainty of an effect on the next generation? Environmental insults to epigenetic programming in male gametes may not cause equivalent defects in every cell stage of sperm maturation. Indeed, analysis of individual cloned alleles shows some variability in methylation at sporadic CpG dinucleotide sites during normal spermatogenesis [15]. Paternal age is also likely to be a major contributory factor to the epigenetic integrity of the sperm. Advanced paternal age is a well-established risk factor for child morbidities, and it is plausible that the ability to reprogram the epigenome declines with advancing years, as do other cellular processes [147]. We hypothesize that the male gametes are at higher risk for epigenetic damage during the epigenetic reprogramming periods, and that environmental factors can alter the fidelity of this process. Such an “environmental message” may be carried to the next generation through epigenetic modifications in the form of incomplete or unstable methylation at certain regions of the genome, changes in the levels of DNMTs, histone modifications, and defects in the transmission of non-coding RNAs during the process of
fertilization. Research on human sperm, and long-term epidemiologic investigations of multiple generations are necessary in order to obtain better insights into the epigenetic mechanisms underlying transgenerational environmental effects through the paternal lineage. This will lead to a better understanding of the etiology of certain (childhood) disorders, and may ultimately have implications for public health recommendations. We conclude that a healthy occupational environment and lifestyle for future fathers may be more important than ever realized before.

Acknowledgments
We thank Prof. Dr. Dirk Vanderschueren, Prof. Dr. Carl Spiessens, and Dr. Goedele Paternot for their thoughtful comments. This work was supported by the University of Leuven (KU Leuven), Duke University School of Medicine, Duke Nicholas School of the Environment, National Institutes of Health (P01ES022831, RO1ES016772, RO1DK085173), the U.S. Environmental Protection Agency (RD-83543701), and the U.S. Department of Energy (DE-FG02-10ER64931). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH, U.S. EPA, or U.S. DOE.

Conflict of interest
The authors declare that they have no competing interests.

References
The following references are relevant to the topic:


SESSION 3
I. INTRODUCTION

A. The Problem: Obesity, Diabetes, and Their Interactions

Obesity is defined as having an excess of body fat (267). However, for practical clinical and research purposes, obesity and overweight are most commonly defined by body mass index (BMI), the ratio of weight in kilograms divided by height in meters squared. Overweight is defined as a BMI of 25–29.9 kg/m² and obesity as a BMI of >30 kg/m². The category of obesity is further divided into subcategories of class I (BMI 30.0–34.9 kg/m²), class II (BMI 35.0–39.9 kg/m²), and class III (BMI ≥40 kg/m²) (355a). However, BMI does not provide a good measure of carcass adiposity, which is the true definition of obesity. For that reason, other measures such as waist circumference, waist-hip ratio, as well as percent body fat using DEXA, CT, and MRI have also been used (153). Such additional measures may be important because so-called normal weight obesity, the combination of normal BMI and high body fat content, is associated with a high prevalence of cardiometabolic dysregulation, metabolic syndrome, and cardiovascular risk and/or mortality factors (446). However, these other measures are not as widely used for a variety of reasons, including the expense of imaging equipment.

Diabetes is a chronic disease that is defined by hyperglycemia and is a heterogeneous condition that can be subdivided into a number of classes. Type 1 diabetes mellitus (T1DM) accounts for ~5% of all cases of diabetes. It usually occurs in childhood or adolescence and is generally considered to be an autoimmune disease which attacks the pancreatic β-cells leading to profound hypoinsulinemia (222, 459). Type 2 diabetes mellitus (T2DM) is the most common form, accounting for almost 90% of cases. It usually occurs later in life, often, but not always, in association with obesity and almost always associated with early-onset peripheral insulin resistance and later β-cell dysfunction and insulin deficiency (110, 430). Its etiology is less well characterized and, along with T1DM, may well have an underlying genetic predisposition (22, 122, 126, 525). Specific diagnostic criteria have been defined by a number of organizations, including the World Health Organization and the American Diabetes Association. These are either a
hemoglobin A1C of $\geq 6.5\%$, or fasting plasma glucose of $\geq 126$ mg/dl, or a plasma glucose concentration of $\geq 200$ mg/dl 2 h after a 75 g oral glucose tolerance test or a random plasma glucose measurement $\geq 200$ mg/dl (www.diabetes.org). There are currently estimated to be around 382 million individuals worldwide that have diabetes. In the United States (US) alone, 25.8 million individuals (8.3% of the population) have diabetes. It was estimated that diabetes caused at least $548$ billion dollars in health expenditure in 2013, and this figure is set to continue growing (International Diabetes Federation). Understanding the factors driving this increase is therefore of great economic and social importance.

B. Prevalence and Associated Morbidity and Mortality of Obesity

The prevalence of obesity and overweight in the United States is high. In 2007–2008, 32% of US men and 36% of US women were obese, and an additional 40% of men and 28% of women were overweight (149). In 2010, more than one-third of US children and adolescents were overweight or obese (368). About 5% of Americans have a class III obesity, i.e., a BMI of $>40$ kg/m$^2$ (149). The prevalence of obesity and overweight has increased by 134 and 48%, respectively, since 1976–1980 (492). While overweight and obesity trends among women have remained stable, rates in men have continued to rise (149) with a 50 and 25% long-term risk of developing these conditions, respectively, in the Framingham study (531). These figures vary widely among sex, ethnic, and racial groups (149), as does the relationship between BMI and disease risk such that obesity prevalence is not a definite predictor of the degree of disease risk.

In general, obesity reduces life expectancy by 6–20 yr depending on age and race (152, 397), particularly among adults below the age of 65 (4, 114, 151, 152, 422). Cardiovascular disease, T2DM, cancer, and respiratory diseases are the leading causes of death in obese individuals (422). It is less clear whether being overweight carries the same increased mortality risk (4, 151, 286, 397, 422). The association between overweight/obesity and mortality risk, however, varies by sex, ethnicity, and age, which may be why data are mixed (71, 188, 229, 320, 497, 519). Being overweight or obese is associated with an increased risk of coronary heart disease (52, 91, 555). T2DM is strongly associated with obesity or overweight in both men and women (191), and a BMI of $>25$ kg/m$^2$ was associated with a 2.2-fold greater risk of death from diabetes, a greater association than with any other cause of death (422). However, as with other diseases, the relationship between BMI and T2DM risk also varies by ethnicity (314, 499). Other diseases associated with obesity include various types of cancer (70, 112, 201, 433), ischemic stroke (358, 501, 579), heart failure (245), dementia (202), venous thrombosis (7), gallstones (489), gastroesophageal reflux disease (386), renal disease (145), sleep apnea (570), and osteoarthritis (83). Particularly pertinent to this review, maternal obesity is associated with gestational complications and adverse fetal and neonatal health outcomes (348, 513). However, there remains a controversy as to the higher rate of mortality among the overweight and obese, particularly using self-reported BMI (244). Some report the so-called obesity paradox whereby the overall mortality was lower among those with T2DM and cardiovascular comorbidity and weight loss but not weight gain was associated with increased mortality and morbidity (124, 125).

C. Genes × Environment Interactions: Imprinting (Epigenetics) as a Concept

Although a number of common genetic susceptibility loci for obesity and T2DM have been identified over the last decade, the rapid rise in prevalence of these conditions in the last two decades, a time frame which is not compatible with a change in our genetic make-up, suggests that the environment in which we live is an important determinant of obesity risk. Environmental factors that have been attributed to this rapidly increasing prevalence of obesity include increased consumption of highly processed foods that are high in saturated fat and refined carbohydrates as well as reduced physical activity (421). However, the wide variation in BMI among individuals living in the same “obesogenic” environment has led to the opinion that obesity risk is determined by a complex interaction between our genes and the environment in which we live. How these interactions could occur at the molecular level through epigenetic mechanisms and how there may be critical time periods during development when this is more likely to occur will be discussed in more detail below.

D. Historical Background

1. Early concepts of energy homeostasis regulation

In 1940, Hetherington and Ranson (209, 210) first demonstrated that lesions of the ventromedial hypothalamus caused rats to massively overeat and become obese. As later became apparent, to produce the massive obesity associated with the “classic” VMH lesion, damage usually extended to a quite large area including both the ventromedial (VMN) and arcuate (ARC) nuclei (127, 249, 462). However, it was not until several years after this fact became evident that the importance of the ARC and its resident proopiomelanocortin (POMC) and neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons in the regulation of energy and glucose homeostasis were recognized (38, 42, 43, 189, 467). Later, it was shown that large lesions of the lateral hypothalamic area (LHA) produce profound anorexia and weight loss (15), which led Stellar (493) to put forward the dual center hypothesis whereby the VMH was the “satiety center” and
the LHA was the “feeding center.” This concept held sway for many years and led to the largely hypothalmo-centric view of energy homeostasis control that still dominates the thinking and research of many investigators. However, we now recognize that such control resides within a distributed network of sites within the brain (183, 184) and that lesions in one part of this network can alter the defended level of body weight and adiposity (242). The observation that the level of defended body weight can be altered by lesions of areas such as the VMH and LHA led to the idea of a set-point whose level is set depending on the neural substrates as well as internal and external environments (242).

However, it was obvious that the brain required some means of monitoring the metabolic status of the periphery to enable it to control overall energy homeostasis. Kennedy (247) was among the first to suggest that body fat storage might be the source of such feedback. He suggested that adipose tissue produces a signal, in proportion to its mass, that is sensed by the brain to regulate changes in intake or expenditure, and this keeps body fat within a predefined set-point. This negative-feedback system has been termed the “lipostatic” hypothesis (247). In fact, the lipostatic factor postulated by Kennedy was eventually shown to be leptin, a hormone produced by adipose tissue in proportion to its overall mass (577). However, the basic concept of a set-point remains highly controversial, and extensive tomes have been written in defense (243) and rebuttal of this concept (396, 488, 558). What does seem clear is that in most humans, and some rodent strains that become obese, the defended body weight can be moved upward fairly easily while long-term attempts to move them below their higher body weight by caloric restriction is met with failure in upwards of 90% of individuals (288, 292, 302). The underlying reason for this observation remains unknown, but its existence serves as the main focus for most research which attempts to find treatments for obesity.

2. The discovery of leptin and how it changed things

In 1949, investigators at the Jackson laboratory in Bar Harbor reported a colony of mice showing severe obesity (223). These mice were first distinguishable from littermates at 4 wk of age but became four times heavier than wild-type littermates as adults. Offspring of heterozygous matings demonstrated the 3:1 ratio characteristic of a recessive gene, which was subsequently designated ob (now Lep) (223). In 1966, a second mouse strain with severe obesity syndrome was identified by Coleman and colleagues (220). Mice homozygous for the mutation were designated diabetes (db) and displayed early-onset obesity, hyperphagia, and diabetes. These fortuitous observations represented a major breakthrough in the field of the genetics of obesity, although the nature of the defective gene(s) remained to be discovered. Prior to the era of sophisticated transgenic approaches, Coleman and colleagues went on to perform heroic parabiosis experiments. They surgically connected the circulatory system of either wild-type or obese ob mice with diabetic db mice and found that it produced weight loss and hypophagia in wild-type and ob mice without affecting db mice. Based on these observations, Coleman and colleagues (220) proposed that ob mice lacked a circulating satiety factor and that db mice overproduced that circulating factor but could not respond to it. In 1994, Friedman and collaborators (577) cloned the defective gene of the ob mouse. Using positional cloning, they found that the ob gene encodes a 4.5-kb RNA secreted by adipose tissue in proportion to its mass (577). As predicted, administration of the recombinant OB peptide reduced body weight and food intake of obese mice (73, 197, 399). Based on these physiological effects, Friedman named the peptide “leptin” from the Greek root leptos for “thin.” However, db mice were insensitive to the weight loss-inducing effect of leptin, suggesting that the db locus encodes the leptin receptor, which was subsequently cloned in 1996 (82, 283). Leptin appears to act primarily on the brain to mediate its effects on feeding and metabolism because central administration of leptin has a marked effect on feeding (73), and the strongest expression of leptin receptor occurs in the hypothalamus (283, 527). In fact, leptin fulfills all of the predicted “lipostatic” properties proposed by Kennedy in 1953 (247). Moreover, the observation that leptin is one of the first major metabolic hormones to appear during embryogenesis (215) suggests a role for leptin in perinatal development.

3. Early studies implicating the perinatal environment in the pathogenesis of obesity and diabetes

Some of the earliest evidence in support of the importance of the early life environment in determining long-term health came from studies in the United Kingdom and Sweden in the 1930s demonstrating that, within any one age group, death rates were most affected by the date of birth and not the year of death (248). Further support for the importance of the neonatal environment on long-term health emerged almost 50 years later in studies in Norway by Forsdahl (155) demonstrating that geographical variations in atherosclerotic disease were not associated with current mortality rates but correlated strongly with past infant mortality rates. The earliest evidence that nutrition during neonatal life could influence long-term metabolic health came from the study of individuals who were born during the Dutch Hunger Winter that occurred in the western part of the Netherlands at the end of World War II. These data suggested that low nutrient intake during early postnatal life actually reduced the risk of obesity at age 19 (428). These observations were supported by pioneering studies in rats by Kennedy (246) where he altered the plane of nutrition during the suckling period through manipulation of litter size. Rats reared in small litters where there is little competition for the mother’s milk gain more weight during lactation and remain fatter and heavier throughout life even when fed a standard laboratory chow diet. In contrast, rats reared in large litters receive less milk and conse-
Focus on the potential importance of the fetal environment arose from studies by Barker and colleagues (198) demonstrating a strong association between birth weight and subsequent risk of development of T2DM and other features of the metabolic syndrome. These studies demonstrated that individuals with the lowest birth weight were around six times more likely to have T2DM or impaired glucose tolerance at age 64 compared with those individuals with the highest birth weight. These findings have now been reproduced in over 50 studies worldwide. The relationship between birth weight and T2DM holds true in monozygotic (identical) twins (51, 417), suggesting that the fetal environment plays a critical role in mediating the relationship between birth weight and long-term metabolic health. While nutrient supply is one important determinant of fetal growth, assessing the importance of fetal nutrition in mediating these relationships is difficult in humans. However, evidence from studies of individuals who were in utero during periods of famine have provided direct evidence that alterations in maternal nutrition during pregnancy can influence long-term risk of T2DM. Prior to the “Dutch Hunger Winter,” the western part of the Netherlands was a well-nourished population. The abrupt onset of the famine and its short duration (5 mo) provided a unique opportunity to retrospectively study the effects of maternal nutrient restriction on offspring glucose tolerance. At age 50, those individuals who were in utero during the famine had worse glucose tolerance compared with those individuals born either the year before or the year after the famine (427). Those exposed during late gestation were most affected, suggesting that the third trimester represents a particularly vulnerable developmental period in terms of long-term regulation of glucose homeostasis. In contrast, risk of cardiovascular disease and obesity was more pronounced in those individuals exposed to famine during early gestation (428). This highlights the different critical periods of development for different organ systems. A subsequent, larger, study of a population exposed to the Chinese Famine (1959–1961) showed a similar association between exposure to suboptimal nutrition in utero and increased risk of T2DM in later life (309). In both studies, it was demonstrated that exposure to a nutritionally rich environment in later life exacerbated the detrimental effects of undernutrition in utero. The causative relationship between poor nutrition in utero and long-term health has been further substantiated by studies in animal models (see below).

II. CENTRAL REGULATION OF ENERGY AND GLUCOSE HOMEOSTASIS

A. The Central-Peripheral Conversation in the Control of Energy and Glucose Homeostasis

Energy homeostasis is defined as the balance between energy intake on the one hand and output as thermogenesis (heat production) on the other. When intake exceeds output, energy is stored primarily as fat in adipose depots. When food supplies are limited and intake is restricted, those adipose stores are called upon as the major energy source over long periods of time. While it is generally agreed that the brain is the controller of energy and glucose homeostasis, it is able to carry out this function only because it receives vital information about the metabolic and physiological status of the body from enteroceptive inputs from the various organs via metabolic signals and neural afferents. Afferents from the majority of viscera are carried primarily within the vagus (Xth) cranial nerve that has its cell bodies in the nodose ganglion. Their central axons terminate within the caudal part of the nucleus of the solitary tract (NTS) in the medulla (96, 442, 443, 466). Other small unmyelinated nerves from the viscera, which travel with somatic efferents, have their cell bodies in the dorsal root ganglia of the spinal cord. Their central processes also terminate in the caudal NTS. Thus the NTS represents the first important neural link between the viscera and the brain. These neural inputs carry sensations of stretch, pain in the viscera, as well as from chemical sensors within the portal vein, carotid body, and small intestines (96, 442, 443, 466). Importantly, the brain also monitors the metabolic status of the body by the transport of hormones such as leptin, insulin, and ghrelin and substrates such as glucose, free fatty acids, lactate, ketone bodies, and cytokines across the blood-brain barrier (BBB) (28, 29, 31, 362). The BBB excludes many toxins and molecules that do not have dedicated transporters from entering the brain by virtue of tight junctions between the vascular endothelial cells and apposition of astrocyte foot processes on cerebral microvessels. However, tight junctions in some vessels in areas such as the ARC may vary in permeability depending on the nutritional state of the individual (273). Finally, these neural, hormonal, and substrate signals from the body are integrated within a distributed network of brain sites that contain specialized metabolic sensing neurons (see below) which gather these signals from the body, together with indirect neural inputs from the primary senses of taste, smell, sight, hearing, and sensation, to alter their membrane potential, neural activity, neuro-transmitter and -peptide release, as well as gene transcription (303).
B. Metabolic Sensing Neurons: the Basic Integrators and Regulators of Glucose and Energy Homeostasis

In the 1950s Jean Mayer (322) first postulated that there were neurons in the hypothalamus that sensed changes in glucose oxidation as a means of regulating feeding. It was not until 1964 that Oomura et al. (372) and Anand et al. (16) identified such glucosensing neurons. The majority of neurons utilize glucose as their primary fuel to produce ATP when their activity increases. When neuronal activity increases, neuronal glucose transporters 3 (Glut3) increase the uptake of glucose proportionally (530). Most neurons can also utilize lactate, long-chain fatty acids, and ketone bodies as alternate fuels in some instances (47, 131, 312, 445). However, whereas metabolic sensing neurons also utilize glucose as a primary fuel, ambient extracellular levels of glucose and other metabolic substrates are “sensed” by these neurons using a variety of signaling and metabolic pathways as a means of regulating their activity. Thus, while most neurons utilize such substrates to fuel their ongoing activity, metabolic sensing neurons do as well, but also use these same substrates to regulate their activity (50, 280, 301, 303, 338).

These neurons either increase (glucose excited) or decrease (glucose inhibited) their activity as ambient glucose levels rise and are conversely inhibited and excited as glucose levels fall (16, 20, 304, 373). Thus, after a meal, glucose-excited neurons are generally activated, while glucose-inhibited neurons are inactivated. During fasting or insulin-induced hypoglycemia, glucose inhibited neurons are powerfully activated (450, 452, 484). Within the ventromedial portion of the hypothalamus (VMH), which is composed of the ARC and VMN, ~10–15% of neurons are either glucose excited or inhibited (305). Of those, 40–65% utilize the pancreatic form of glucokinase as a gatekeeper for the regulation of glucose-induced changes in their activity (236). Formation of ATP within glucose-excited neurons leads to inactivation of an ATP-sensitive K\(^+\) (K\(_{ATP}\)) channel leading to membrane depolarization, entry of calcium via a voltage-dependent calcium channel, increases in activity, propagation of an action potential, and release of neurotransmitters and peptides from their axon terminals (20, 305). Glucose-inhibited neurons form nitric oxide and, via activation of AMP-activated kinase and soluble guanylyl cyclase, increase neuronal firing when glucose levels fall by an action on the cystic fibrosis transmembrane receptor (148). Catabolic ARC POMC neurons are predominantly glucose excited (221), while anabolic ARC NPY/AgRP (351) and LHA orexin/hypocretin neurons (350) are mostly glucose inhibited in type. However, other glucosensing neurons have been identified which utilize several other ion channels and transporter mechanisms to regulate their activity (239, 365, 375, 390).

There remains a controversy as to whether physiological changes in blood and/or brain glucose are actually involved in the regulation of feeding as Mayer originally proposed (129, 172, 305). To summarize this controversy, studies using very high or low levels of glucose or glucose availability, especially in the brain, can inhibit or stimulate feeding, respectively (186, 474, 479, 529). Some investigators have shown a relationship between spontaneous, small dips in blood glucose preceding meals in rats and humans (72, 74, 313). However, others have failed to confirm such a relationship between blood or VMH glucose levels and meal onset (129). Also, manipulation of VMH neuronal glucosensing by altering glucokinase activity fails to affect either short- or long-term feeding (129), while it does markedly alter the counterregulatory responses to insulin-induced hypoglycemia (290). Such results suggest that hypothalamic glucosensing neurons are not critical regulators of normal feeding but are important for the defense against hypoglycemia.

Many of these same VMH glucosensing neurons are also fatty acid sensors which respond to long-chain fatty acids by altering their activity (230, 278, 280, 281, 337, 374). While early work suggested that this fatty acid sensing was mediated by intracellular metabolism of long-chain fatty acids (230), it now appears that much of this sensing is mediated by fatty acid translocator/CD36 (which appears to act as a receptor and may also be a transporter of fatty acids) in many VMH neurons and that this regulatory step is independent of neuronal fatty acid oxidation (278, 280, 281). Furthermore, although impairment of VMH glucosensing has no effect on energy homeostasis, altering fatty acid sensing by depletion of VMH neuronal CD36 inhibits linear growth as well as causes redistribution of fat stores from visceral to subcutaneous adipose depots and marked insulin resistance (278). Thus, while the glucosensing properties of VMH metabolic sensing neurons do not appear to be critical for the regulation of energy homeostasis, their ability to sense and respond to long-chain fatty acids is critical for some aspects of both energy and glucose homeostasis. Importantly for this review, the interaction among an obesity-prone genotype, diet, and the presence of maternal obesity has a major effect on both the glucose- and fatty acid-sensing properties of these VMH metabolic sensing neurons (281).

In addition to their responses to glucose and long-chain fatty acids, the activity of many of these same neurons is also altered by ambient levels of lactate (485) and ketone bodies (279, 510), both of which are produced locally by astrocytes (48, 49, 131). They also respond to hormones produced in the periphery such as leptin (225, 486), insulin (487, 541), and ghrelin (99) which are transported across the BBB. Thus the term metabolic (or nutrient) sensor is an apt term for these neurons. Importantly, while a great deal of the research on such neurons has focused on ARC and
VMN neurons, glucosensing neurons have been identified in the lateral hypothalamus (16, 350), hypothalamic paraventricular nucleus (PVN) (128), amygdala (578), basal ganglia (285), NTS (343), and several other brain areas known to be involved in the regulation of both energy and glucose homeostasis (289, 305). Most of these neurons make critical connections with brain areas that provide efferent output to a variety of neuroendocrine, autonomic, and behavioral centers required for such homeostatic processes. The network of brain areas containing these metabolic sensors forms a distributed network that functions as an integrated system. Thus the early observations that destruction of the VMH or LHA leads to marked disturbances in energy and glucose homeostasis (209, 210, 240, 241, 341, 354) do not mean that these are satiety and feeding centers; simply means that destroying one node of this distributed network can lead to dysfunction of its integrated function. While there is a great deal of redundancy in this distributed network, many of its component parts can undergo plasticity, particularly during early pre- and postnatal development through alterations in neural connections and expression of neuropeptides and peptides (58, 59, 62, 98, 391–393, 490).

C. Homeostatic and Reward-Based Systems

To ensure adequate nutrition, it is necessary for the brain to have intrinsic neural circuits that sense and regulate the levels of various nutrients in the blood and body stores. As mentioned above, a primary importance has been given to the hypothalamus, in part because this brain region can integrate hormonal, autonomic, and somatomotor control mechanisms and, in turn, induce a variety of neuroendocrine homeostatic responses (FIGURE 1). However, we now know that the central systems regulating energy homeostasis involve a distributed and interconnected neural network (181, 182, 301). For example, the ARC, that was originally thought to be exclusively “anorexigenic,” contains two chemically identified neuronal types that play opposite roles in energy balance regulation: the POMC neurons that are anorexigenic but also the NPY/AgRP neurons that are orexigenic (94, 483). Moreover, POMC neuronal activity can be modulated indirectly via transsynaptic GABAergic inputs arising from NPY neurons, showing the anatomical intricacy of these neural networks (17, 100, 516). Arcuate POMC and NPY neurons project to multiple hypothalamic and extrahypothalamic sites to regulate feeding (65, 94). Of particular importance are projections to the PVN because it is the most thoroughly characterized pathway involved in feeding and energy balance regulation, and the PVN is anatomically connected to endocrine, autonomic, and somatomotor systems (461, 506, 544). For example, the paraventricular part of the PVN contains corticotropin-releasing hormone and vasopressin neurons that regulate adrenocorticotrophic hormone secretion and thyroid-stimulating hormone neurons that influence thyroid-stimulating hormone production in the pituitary. In addition to neuroendocrine neurons, the PVN also contains neurons that send direct projections to preautonomic sites, such as the brain stem and spinal cord (438, 506). In addition to forebrain structures, the caudal brain stem, and particularly the dorsal vagal complex, plays an essential role in the regulation of energy homeostasis. The dorsal vagal complex comprises the dorsal motor nucleus of the vagus nerve, NTS, and area postrema. Although the hypothalamus predominantly integrates long-term adiposity signals, dorsal vagal complex neurons appear to be more involved in the short-term control of feeding in response to satiety signals (see Refs. 46, 182 for reviews).

If feeding were controlled solely by homeostatic systems, most individuals would likely maintain a stable, relatively lean body weight. However, virtually any mammal will eat beyond its homeostatic needs when exposed to highly palatable foods such as a high-fat/high-sucrose diet. Such observations support the contention that the hedonic (“reward”) system plays an important role in regulating feeding behavior (FIGURE 1). The hedonic system deals with the rewarding value of stimuli (e.g., food) and has neural circuits which encode wanting (incentive motivation) and liking (experienced pleasure) of those stimuli. A key neurobiological substrate involved in incentive motivation to eat is the mesolimbic dopaminergic pathway. This pathway is composed of dopamine neurons in the ventral tegmental area (VTA) of the midbrain that connects to limbic centers such as the nucleus accumbens, the amygdala, hippocampus, and medial prefrontal cortex (45). The observation that rodents with defective dopamine signaling in this mesolimbic system become aphagic and adipsic and can even die of starvation supports the idea that the mesolimbic dopaminergic system plays an incentive role in feeding regulation (507, 526). In addition to being activated by a variety of addictive substances, including cocaine and alcohol, VTA dopamine neurons are also directly modulated by metabolic hormones such as leptin and ghrelin. Leptin exerts a direct inhibitory influence on VTA dopamine neurons, and hyperphagia of leptin-deficient mice is blunted in the absence of dopamine (146, 163, 217, 507). In contrast, ghrelin increases the activity of VTA dopaminergic neurons and direct injection of ghrelin into the VTA promotes feeding (3, 354). These studies show that metabolic hormones are not only involved in the short- and long-term control of energy homeostasis, but also modulate motivated behaviors and both our need and desire to eat.

D. Central Roles for Leptin, Insulin, and Ghrelin

1. Leptin

The discovery of leptin reinforced the concepts originally proposed by Woods and Porte for insulin (561) that our
subconscious motivation to eat can be powerfully and dynamically regulated by hormonal signals from the periphery. Although this 16-kDa protein is primarily produced by white adipose tissue, it rapidly became clear that leptin acts primarily in the brain to mediate its effects on feeding and metabolism. Leptin injection blunts food intake and decreases body weight, and this effect is more robust when the hormone is injected intracerebroventricularly compared with peripherally (73, 196, 197, 399). The long (signaling) form of the leptin receptor (LepRb) is expressed at high levels in the brain (76, 135, 470), and neuron-specific deletion of LepRb results in a phenotype that is a virtual carbon copy of whole-body leptin receptor-deficient db/db mouse. Reactivation of leptin receptors in the brain of db/db mice rescues their obese and diabetic phenotype, further demonstrating the importance of the brain in mediating leptin’s effects (90, 117).

Soon after the cloning of leptin and its receptors, considerable research focused on neurons located in the ARC, in part because of the proximity of this nucleus to the median eminence, a region outside of the BBB (87). Also the ARC contains the highest density of leptin receptors of any brain region (76, 135, 283, 334, 470) and lesions of VMH (that includes the ARC) prevent leptin’s actions in the brain (263, 460). Within the ARC, leptin stimulates the activity of neurons that contain POMC-derived peptides and inhibits the activity of neurons that co-produce NPY and AgRP (333). Gain- and loss-of-function mutations of leptin receptors selectively in POMC neurons produce phenotypes that suggest a prominent role for POMC neurons in mediating leptin’s effects on energy ex-
penditure and glucose homeostasis with a more moderate effect on body weight regulation (27, 44).

Prior to 2005, a widely held view was that most, if not all, of leptin’s effects are mediated by neurons located in the ARC. However, peripheral leptin administration also acts on neurons in other brain regions such as the VMN, LHA, VTA, and NTS (76, 134, 194, 195, 468). Such observations slowly moved the attention of the field away from the arcuate-centric notion of leptin action. Thus mice lacking LepRb in SF1-expressing neurons of the VMN develop mild obesity when fed a chow diet and are markedly sensitive to high-fat diet-induced obesity, supporting a role for VMN neurons in leptin’s regulatory actions (121). In addition, targeted deletion of LepRb in LHA neurotensin neurons causes early-onset obesity due to hyperphagia and locomotor inactivity (284). Notably, neurotensin neurons appear anatomically well-poised to relay leptin’s actions on the mesolimbic dopaminergic system, suggesting that neurotensin neurons may be a crucial point of convergence for homeostatic and hedonic interactions that regulate ingestive behavior. Supporting a role for leptin on brain reward circuits, leptin receptors are expressed and functional on dopaminergic neurons in the midbrain and direct manipulation of LepRb in VTA dopamine neurons influences feeding behavior (146, 163, 217). Another site of particular interest outside the hypothalamus is the NTS, a hindbrain nucleus involved in the processing of meal-related satiety signals where LepRb mRNA was shown to be expressed (335). But it was another 12 yr before the functional relevance of these NTS LepRbs was demonstrated. Downregulation of LepRb in the medial NTS led to increased body weight and adiposity and caused chronic hyperphagia, likely due to a reduction in leptin’s potentiation of gastrointestinal satiation signaling such as cholecystokinin (CCK) (204). The NTS also receives neural inputs from the hypothalamus, and recent studies have demonstrated that leptin’s modulation of energy expenditure and brown adipose thermogenesis is via a GABAAergic ARC-PVN-hindbrain pathway (258). In summary, the effects of leptin on the central control of energy homeostasis are anatomically distributed and appear to involve a complex, distributed, and interconnected neuronal network involving neurons located in throughout the brain.

2. Insulin

Despite its sole production by the β-cells in the pancreas, plasma insulin, like leptin levels, generally parallel overall levels of carcass adiposity (23, 416). In addition, plasma insulin levels also vary over a wide range during ingestion and absorption of nutrients. While peripheral insulin’s main actions are on glucose homeostasis, several lines of evidence suggest that insulin can act centrally to affect many brain functions. First, there are abundant levels of insulin receptors in several brain areas including the olfactory bulb, hippocampus, and hypothalamus (147, 226, 238, 573). There is still a debate about whether insulin is actually produced within the brain (376, 463), but it does appear that, despite its large size, it is transported across the BBB (30). During brain development, insulin acts on its brain receptors (sometimes in association with insulin-like growth factor I) as a trophic factor for facets of neural development (206, 423, 432) including neurite outgrowth (206, 464) and neuronal differentiation (355) and survival (359). However, when injected into the hypothalamus of rat neonates, insulin alters neuronal density in the VMN in association with increased body weight gain as adults (410). While controversial (159), some studies suggest that insulin might cross the placenta to enter the fetal circulation in humans (332). For example, in rats, insulin injections in third trimester dams predispose to adult obesity in offspring (232). However, maternal hyperinsulinemia might increase transplacental glucose transport to the fetus (378). Maternal hyperinsulinemia and hyperglycemia could thus cause fetal hyperglycemia with attendant hyperinsulinemia (235) and later increases in fetal weight in offspring of mothers with gestational diabetes (511). On the other hand, insulin clearly does cross the gut wall in the early postnatal development in rodents (213, 349) such that elevations in maternal milk insulin levels can be absorbed by the offspring as potential mediators of obesity development in later life (176).

In addition to these developmental effects, insulin has important glucose-dependent actions on the activity of hypothalamic metabolic sensing neurons (451, 487) as one way in which a signal relating to adiposity can be “sensed” by the brain. There is a large amount of literature on the effects of centrally injected insulin on food intake, energy, and glucose homeostasis. Both chronic and acute intracerebroventricular infusions of insulin reduce food intake (9, 360, 562) and reducing periventricular insulin receptors causes increased food intake, adiposity, and peripheral insulin resistance (367). However, reducing insulin receptors focally in the VMH causes glucose intolerance without altering body weight (388). In mice with selective neuronal knock-out of insulin receptors, females have increased food intake, and both males and females develop diet-induced obesity, mild insulin resistance, and hypertriglycerideremia (68). However, such mice reportedly had no abnormalities of brain development or neuronal survival. Direct injections of insulin into the hypothalamus (415) or via the carotid arteries (426) alter hepatic glucose production (415), although the physiological significance of these studies has been questioned because of the large doses or nonphysiological conditions used to assess these central actions of insulin (306). Thus there is a great deal of conflicting information about the physiological role of insulin on brain development and the regulation of energy and glucose homeostasis. On balance, it seems likely that insulin is transported across the BBB and does have effects on all of these parameters.
3. Ghrelin

Ghrelin was originally discovered as an endogenous ligand for the growth hormone secretagogue receptor (GHSR) (254). In adults, ghrelin is mainly synthesized within oxyntic mucosa cells of the stomach, whereas the primary source of ghrelin production during neonatal life appears to be the pancreas (254, 454). In part because of its discovery from its linkage to GHSR, ghrelin was originally reported to stimulate growth hormone (GH) secretion (254). But it rapidly became evident that it also exerts an important role on feeding behavior. When injected peripherally or centrally, ghrelin promotes feeding, suppresses energy expenditure, and causes weight gain (276, 352, 563). Remarkably, ghrelin-induced hyperphagia occurs within 5 min and persists for 24 h after injection. The observations in both human and other animals of a preprandial rise and a postprandial decline in plasma ghrelin levels suggested that ghrelin plays a specific role in hunger and meal initiation (105, 106, 515). Based on these physiological effects, it is not surprising that GHSRs are abundantly expressed in various brain regions involved in somatic growth, food intake, and body weight regulation such as the hypothalamus, hindbrain, and midbrain (342, 580). Empirical studies employing direct intra-ARC injections of ghrelin and selective lesions of the ARC demonstrated the primary importance of ARC neurons, specifically in mediating ghrelin’s action on feeding (509, 563). Within the ARC, the highest proportion of neurons activated by systemic ghrelin injection coexpress NPY and AgRP (100, 540, 554). Consistent with these findings, pharmacological blockade of NPY or its receptors blunts the effects of ghrelin on food intake (276, 352). Ghrelin can also regulate the activity of POMC neurons in the ARC, but this effect appears indirect and likely involves trans-synaptic GABAergic inputs arising from NPY neurons (17, 100, 516).

Leptin and ghrelin therefore appear as two complementary, yet antagonistic, regulators of energy balance. Notably, the distribution pattern of GHSR resembles that of LepRb (401), suggesting that leptin and ghrelin might reciprocally regulate many of the same neurons. However, whether there is a direct interaction between leptin and ghrelin signaling at the cellular level remains unclear. For example, although ARC neurons coexpress GHSR and LepRb, GHSR knockout mice display unaltered leptin sensitivity (401). Nevertheless, similar to leptin, the regulatory actions of ghrelin on feeding likely involve a complex and distributed neural network. In addition to its actions on hypothalamic neurons, ghrelin also regulates mesolimbic dopaminergic neurons in the midbrain to modulate more complex aspects of feeding such as food-reward behavior (3, 85, 354, 400, 478). More recent genetic evidence demonstrated that reactivation of GHSR signaling selectively in hindbrain neurons does not ameliorate ghrelin-induced food intake but rescues hypoglycemia of GHSR null mice, suggesting that hindbrain neurons relay ghrelin’s effects on glucose homeostasis (471).

E. Neuronal Plasticity

The mammalian brain ensures adaptive behavior through its large capacity for cellular and circuit plasticity. One unique property of the hypothalamus, compared with other brain structures such as the cortex and hippocampus, is that its regulation is to a large degree activity-independent, but instead is controlled by physiological signals that reflect environmental conditions. The biological processes involved in neuronal plasticity fall into two major categories: the birth of new neurons (neurogenesis) and the reshaping of existing neural circuits (synaptic remodeling). Low rates of neurogenesis are observed in the mature hypothalamus under basal conditions (255, 256), and median eminence tanyocytes appear to be a possible source of these newborn neurons (282). This constitutive hypothalamic neurogenesis can be enhanced by hormonal factors. For example, central injections of ciliary neurotrophic factor (CNTF) induced marked neurogenesis in the hypothalamus that appears to participate in the weight loss effects of CNTF in ob/ob and DIO mice (256). Moreover, microimplantation of neural progenitors that express lep receptors into the hypothalamus of newborn db/db mice allows differentiation of the donor cells into neurons that integrate into functional neural circuits that lead to reduced hyperphagia and obesity (107). Nonneurotropic factors, such as aging and neurodegeneration, can also promote hypothalamic neurogenesis (405). Hypothalamic neurogenesis can also be downregulated. For example, high-fat feeding alters cellular remodeling as demonstrated by a reduction in the number of newly generated cells and the maintenance of old neurons in the mature hypothalamus (327). Together, these findings demonstrate that neurogenesis might represent an important adaptive cellular mechanism in response to environmental insults.

Neuronal plasticity of hypothalamic feeding circuits also occurs through rearrangement of synapses. The excitatory and inhibitory synaptic inputs to the POMC and NPY neurons are markedly altered in adult ob/ob mice; leptin deficiency increases excitatory inputs on NPY/AgRP neurons while it decreases excitatory synaptic inputs to POMC neurons (406). Acute leptin injection in adult ob/ob mice rapidly (within hours) reverses these effects, both at the electrophysiological and ultrastructural levels. Other hormones, such as ghrelin and corticosterone, also have organizational effects on hypothalamic neural circuits by modulating the synaptic inputs of ARC POMC and NPY neurons in adult mice (193, 406). Moreover, a significant remodeling of synapses has been reported in obesity-prone (DIO) rats, with an increase in inhibitory inputs to POMC neurons in the ARC of DIO rats compared with diet-resistant (DR) rats (218). The capacity of nutritional challenges
to cause structural changes also appears to differ between DIO and DR rats. High-fat feeding causes a loss of synapses onto POMC neurons in DIO rats, but a gain in synaptic coverage in obesity-resistant DR rats (218). Together, these observations indicate that remodeling of brain circuits involved in energy balance regulation occurs throughout the entire lifespan and is influenced by both metabolic and physiological cues and pathological insults. This neuronal plasticity allows the elaboration of adaptive behavioral and physiological responses that are essential for optimal regulation of energy balance.

F. Gut-Brain Interactions

1. Neurohumoral inputs

The brain receives a wide variety of signals from the gastrointestinal (GI) tract, via either sensory afferents or hormonal signals. The vagus nerve is indisputably the most important neural link between the gut and the brain. It is the longest of the cranial nerves and innervates the entire alimentary tract. It comprises fibers carrying afferent sensory information from the periphery to the brain, but also fibers carrying efferent motor information from the brain to the viscera (420). Afferent signals carried by the vagus nerve include information about gastric stretch, enteroendocrine signals from hormones released within the GI tract, and blood glucose and fatty acid levels. The caudal brain stem, and particularly the NTS via its vagal afferents and efferents, acts as a nodal point in the gut-brain axis. Vagal afferents from the GI tract synapse within subregions of the NTS, and the activation of these afferents regulates postprandial function by inhibiting food intake (465). In turn, the NTS sends reciprocal projections to other regions of the brain involved in feeding regulation such as the hypothalamus, amygdala, and nucleus accumbens. The NTS therefore represents a major portal through which visceral afferent information for homeostatic reflexes enters the brain.

Vagal afferent fibers are also sensitive to a variety of peripheral factors, including CCK, an endogenous peptide released by duodenal enteroendocrine cells (310). CCK is released after a meal and inhibits food intake [i.e., reduces meal size and induces meal termination (480)] in part by increasing the firing rate of vagal afferents projecting to the NTS (170, 347). The regulatory action of CCK on vagal-NTS projections appears to be mediated via the CCK-A receptor subtype (64, 259, 277, 395).

In addition to CCK, the gut secretes a number of other hormones that signal to the brain to regulate feeding. These hormonal effectors include ghrelin, peptide YY (PYY), and glucagon-like peptide-1 (GLP-1). Ghrelin is produced mainly by the gastric mucosa and is the only known peripheral hormone that promotes feeding. That secretion of ghrelin is increased in response to starvation, increased before a meal, and suppressed by meals, supports the hypothesis that ghrelin is primarily involved in meal initiation (105, 106, 515). The hypothalamus is a primary site of ghrelin’s orexigenic effects. The highest density of ghrelin receptors and ghrelin-responsive neurons is found in the hypothalamus, particularly in the ARC, VMN, and PVN (211, 352, 342, 580). The observations that blockade of the gastric vagal afferent abolishes the feeding response to intravenous ghrelin and that GHsRs are expressed in vagal terminal suggest that ghrelin also induces some of its regulatory effects through the vagus nerve (115). For example, ghrelin does not stimulate feeding in human patients with surgical procedures involving vagotomy (115). However, data to the contrary exist regarding an essential role for the vagus in transmitting peripheral ghrelin’s effects on feeding (19).

PYY is produced by L-type enteroendocrine cells, mainly in the ileum and colon, in response to the caloric content of the meal (5). The bioactive peptide, PYY3–36, is stimulated in proportion to the energy content of food and peaks 1–2 h postprandially. Peripheral administration of PYY3–36 inhibits food intake in rodents and humans (34, 35). PYY3–36 has a high affinity for the NPY Y2 receptors, which are widely distributed throughout the periphery and CNS, including in vagal endings (253). Consistent with these findings, gastric vagotomy blocks the anorectic effects of PYY3–36 (1, 253). In addition, PYY3–36 acts on hypothalamic neurons to reduce feeding and ARC injection of PYY3–36 inhibits food intake and inhibits the electrical activity of NPY nerve terminals causing a reduction of the inhibition of POMC neurons (35).

GLP-1, GLP-2, and oxyntomodulin are produced by the posttranslational processing of the preproglucagon gene in the gut and the brain stem (24). The GLPs are produced by intestinal L-cells in response to fatty acids or carbohydrates. GLP-1 is released into the circulation after a meal to inhibit gastric secretion and emptying and induce postprandial secretion of insulin (24, 268). Direct oxyntomodulin injection into the ARC causes a sustained reduction in refeeding after a fast, indicating the importance of the hypothalamus and particularly the ARC in mediating oxyntomodulin’s anorectic action (113). However, intra-ARC administration of the GLP-1 receptor antagonist exendin9–39 does not block the anorectic action of GLP-1, indicating that oxyntomodulin and GLP-1 use different neural pathways to mediate their feeding effects (113). Sites of action of GLP-1 include neurons in autonomic control sites such as brain stem catecholamine neurons (363, 366).

2. Gut microbiota

Gut microflora and their interactions with obesity have become a subject of great interest in recent years. Leptin-deficient ob/ob mice have significant reductions in Bacteroides and increases in Firmicutes, two major gut bacterial phyla (307). Similarly, some obese humans demonstrate an
increase in Firmicutes in their stools (308), and prolonged ingestion of a high-fat diet is associated with decreased bacterial abundance and increased Firmicutes content (520). Importantly, bacterial transplants from lean and obese mice into otherwise high-fat obesity-resistant, germ-free mice cause them to develop the weight gain phenotype of the donors, suggesting a causal role of gut microbiota in the development of obesity (521, 522). Also, increased body and fat mass in human twin pairs discordant for obesity could be transmitted to germ-free mice by transplantation of the fecal microbiota of those humans (438). The mechanism by which alterations in microbial gut flora might determine the propensity of an individual to become obese has not been established. However, one hypothesis is that these microflora might alter nutrient absorption by changing the absorptive surface of the gut in association with inflammatory changes induced by some diets (429, 520, 521). Such changes in gut permeability might become more important as the individual matures since large molecules such as antibodies, leptin, and insulin cross the neonatal intestinal barrier and enter the circulation (287, 349). Regardless of the specific mechanism, early postnatal nutrition and milk content might alter gut microbiota as an explanation for the increased obesity of diet-resistant pups cross-fostered to obese DIO dams (75, 176, 272, 315).

G. Peripheral Organs and Glucose Homeostasis

1. Pancreas

The pancreatic β-cells within the islets of Langerhans are the only cells that have the capability to secrete insulin. They are therefore central to the appropriate regulation of glucose homeostasis. The islets of Langerhans were first identified in 1869 by the German anatomist Paul Langerhans and, despite the fact that they constitute <5% of pancreatic mass, they are critical for maintenance of glucose homeostasis. They contain five major cell types: α-cells (that produce glucagon), δ-cells (that produce somatostatin), PP cells (that produce pancreatic polypeptide), ε-cells (that produce ghrelin), and β-cells (that produce insulin and amylin). Pancreatic β-cells produce insulin primarily in response to elevated levels of glucose. However, production can also be increased in response to other factors such as certain amino acids, free fatty acids, and the sulfonylurea class of antidiabetic drug. The stimulation of insulin secretion involves changes in β-cell electrical activity and ultimately exocytosis of insulin (reviewed in Rorsman and Braun, 447). T2DM is thought to arise in general when pancreatic β-cells malfunction such that they cannot further increase insulin secretion to compensate for progressive peripheral tissue insulin resistance. This may arise because of an inherent or progressive reduction in β-cells mass (reviewed in Weir and Bonner-Weir, 545), genetic defects that reduce β-cell function (reviewed in Bonnefond et al., 54), program-

2. Liver

The liver is the major site of glucose production under fasting conditions, and thus resistance to the action of insulin to inhibit hepatic glucose production can contribute to hyperglycemia (66). There are a number of mechanisms by which hepatic insulin resistance can occur. Nonalcoholic fatty liver disease (NAFLD), which is thought to affect up to 30% of the population in the Western world, is thought to be a major contributing factor (571). Under physiological conditions fatty acids enter hepatocytes and are either oxidized by mitochondria or stored in the form of triglycerides. However, under conditions where there is an imbalance between influx and oxidation excessive storage occurs. This can occur, for example, when lipid storage capacity of adipose tissue becomes exceeded, leading to increased flux of fatty acids into the liver and consequently increased deposition of triglycerides and other lipid intermediates such as phosphatidic acid and diacylglycerol (21). These can result in activation of various kinases (e.g., inhibitor of kappa B kinase and Jun NH2-terminal kinase) that inhibit insulin signaling through serine phosphorylation of IRS-1 and consequently cause hepatic insulin resistance. In addition, there is evidence to suggest that under conditions of hyperinsulinemia, as a consequence of resistance to the action of insulin in relation to inhibition of hepatic glucose production, insulin’s ability to promote de novo lipogenesis can remain intact. This will further promote hepatic triglyceride accumulation (66). There is good evidence to suggest that fatty liver and hepatic insulin resistance can develop as a result of both early environmental (86) and genetic factors (168).

3. Skeletal muscle

Skeletal muscle is the major site of glucose disposal postprandially and thus insulin resistance at this site is a substantial contributor to the development of T2DM. Skeletal muscle takes up glucose in an insulin-dependent manner as a result of the stimulation of translocation of the glucose transporter GLUT4 to the plasma membrane via stimulation of the phosphoinositol 3-kinase-protein kinase B (Akt) pathway. In addition to this insulin-stimulated pathway, there is an alternative pathway that potentiates glucose uptake into skeletal muscle that is activated by exercise and caloric restriction (453). This is mediated by AMP kinase, which has therefore become a focus of potential therapeutic strategies for insulin resistance and associated syndromes. As with liver, skeletal muscle is a major site of triglyceride accumulation in situations where the adipocyte lipid storage capacity has been exceeded. There is a strong positive...
correlation between muscle triglyceride content and insulin resistance (385). The mechanism(s) by which increased lipid accumulation induces insulin resistance in skeletal muscle remains a subject of debate (reviewed in 55). However, it has been suggested that such lipotoxicity results in increased levels of bioactive lipid metabolites such as ceramides that are known to inhibit activation of protein kinase B. Paradoxically intramyocellular triglycerides are also increased in highly insulin-sensitive trained athletes (reviewed in 89). This suggests that it is not the presence of the triglycerides per se that is causing the insulin resistance and that perhaps if their turnover is increased, for example, by regular exercise, generation of lipotoxic intermediates is reduced.

4. White adipose tissue

In recent years, the contribution of adipose tissue to whole body glucose homeostasis and regulation of energy balance has been increasingly recognized, and it is therefore no longer considered merely a site of lipid storage. It can both directly and indirectly influence glucose homeostasis. Adipose tissue takes up glucose in an insulin-dependent manner. Although it was initially considered to account for only ~5% of postprandial glucose uptake, studies with transgenic animals have suggested that loss of insulin-dependent glucose uptake to adipose tissue leads to substantial loss of glucose tolerance (2). In addition to directly taking up glucose, adipose tissue can indirectly affect whole body glucose homeostasis through release of factors including free fatty acids, adipokines (e.g., resistin and adiponectin), and inflammatory mediators (e.g., TNF-α) that influence glucose uptake and/or insulin action in other tissues, especially skeletal muscle (reviewed in 165). It is well established that obesity-associated insulin resistance is associated with inflammation of adipose tissue and consequently increased production of inflammatory markers and cytokines (including TNF-α, IL-6, and IL-1β) that inhibit insulin signaling (reviewed in 144). Adipose tissue is also the major site of leptin production, a major regulator of energy balance across the life course (discussed in detail elsewhere in this review).

III. PERINATAL BRAIN DEVELOPMENT

The hypothalamus develops from the rostral diencephalon after induction by the underlying prechordal plate. Classical birth dating studies using [3H]thymidine or the thymidine analog BrdU revealed that the majority of neurons composing the hypothalamus are born between embryonic day (E) 11 and E14 in mice and E12 and E17 in rats (14, 101, 227, 317, 383). Hypothalamic neurons acquire their terminal peptidergic phenotype soon after they are generated. For example, melanin concentrating hormone neu-rons in the LHA are born between E12 and E13 in rats, and its mRNA is detected in the LHA as early as E13 (63). More recent genetic cell lineage experiments also indicated that hypothalamic progenitor cells can give rise to neurons that express antagonistic neuropeptides in adult life. For example, embryonic Pomc-expressing precursors can subsequently adopt either a POMC or an NPY phenotype (383).

Although hypothalamic neuronal proliferation and differentiation occurs primarily during the second half of gestation in rodents, the rodent hypothalamus remains relatively immature at birth and continues to grow during the first 2–3 wk of postnatal life. Axonal tract tracing experiments in mice showed that hypothalamic axonal connections are not formed at birth. For example, ARC axons reach their target nuclei between postnatal day (P) 6 and P16 (60). Axon terminals containing NPY/AgRP are found in a pattern that coincides with the innervation of axons from the ARC (25, 187, 361). Effector efferent projections from the VMN and dorsomedial nucleus (DMN) appear to develop prior to those from the ARC and are fully established by P6 and P10, respectively (60). Synapses are another key component of neuronal connectivity. We still know relatively little about the exact time point (if any) at which synapse assembly is fully established in the hypothalamus, but a few reports indicate that synapses mature gradually in the hypotalahmus from birth to adulthood (319, 328).

Brain stem projections develop relatively early in rodents. Brain stem catecholaminergic inputs to the PVN are present as early as P1 in rats (440). However, different neurotransmitter systems show different developmental patterns. For example, the density of noradrenergic projections to the PVN is relatively low at birth and gradually increases to reach adult levels at weaning. In contrast, adrenergic projections are relatively high in the PVN of newborn rats but gradually decrease until weaning (440). Reciprocal descending projections from the hypothalamus to the caudal brain stem also develop early in life. Retrograde tracing experiments showed that hypothalamic neurons, such as those in the DMN, PVN, and LHA, send axonal projection to dorsal vagal complex neurons at birth and continue to develop to achieve adult-like patterns at weaning (439, 441). In summary, projections to and from the hypothalamus and brain stem develop primarily after birth and appear chemically and structurally immature until weaning.

The considerable importance of postnatal hypothalamic development in rodents differs from that in humans and non-human primates where the hypothalamus develops almost entirely during fetal life. For example, in Japanese macaques NPY/AgRP fibers innervate the PVN as early as gestational day 100 (i.e., late second trimester) and a mature pattern of NPY/AgRP projections is not apparent until gestational day 170 (180). These findings emphasize the importance of recognizing species differences in terms of timeline of developmental events. Although the regional development of the rodent hypothalamus proceeds on a
timelines of days, the same developmental process takes weeks to months in human and non-human primates. Similar to non-human primates, the human hypothalamus also develops primarily prenatally with NPY-containing axons detected in the ARC and PVN as early as at 21 weeks of gestation (262).

IV. GENETIC BASIS OF OBESITY

A. Single Gene Mutations

Although single gene mutations that cause obesity are rare, their identification has helped greatly in our understanding of energy homeostasis regulation. One very successful approach to identify monogenic forms of obesity has been to focus on children who were extremely obese from an early age and to use a combination of biochemical and genetic approaches to identify the affected locus (reviewed in 366). O’Rahilly and colleagues (343) used this approach to identify a pair of cousins who were severely obese as a result of having undetectable levels of leptin. They were established as having a homozygous frame shift mutation in the leptin gene (345). Treatment of these and other leptin-deficient individuals with daily injections of recombinant leptin normalized their body weight, thus proving causality between the single gene mutation and the obese phenotype (143). To date, there are still only 24 confirmed instances of individuals with this mutation (S. Farooqi, personal communication). Furthermore, these studies demonstrated that human food intake regulation, as in the leptin-deficient ob/ob mouse, was dependent on a functional leptin-signaling pathway. Since these initial studies, it has been demonstrated that human obesity can result from defects in various components of the leptin signaling pathway including the leptin receptor (88), POMC (270), and the melanocortin-4 receptor (MC4R) (569). The latter is now thought to be the most common monogenic form of obesity, with some studies demonstrating that ~1 in 200 obese people have disease-causing mutations in the MC4R (12, 274). There are now over 20 single gene disorders that have been shown to cause severe obesity. In addition to direct components of the leptin signaling pathway, they include genes such as prohormone convertase 1 (which is required for the processing of pro-peptides into active peptides such as POMC) (228), SIM 1 (a transcription factor required for hypothalamic development) (425) and SH2B1 (an adaptor protein that modulates signaling through tyrosine kinase and JAK-associated cytokine receptors) (123). It is notable that these single gene mutations generally influence central sensing and control of energy homeostasis rather than through peripheral systems. Further analyses of these individuals demonstrate that the defects influence appetite and satiety resulting in increased food intake. In contrast, little or no effect is observed on energy expenditure, with MC4R mutation patients being the exception and showing a small but significant reduction in metabolic rate (264).

B. Obesity as a Polygenic Disorder

As above, although there are several single gene mutations that have been identified which cause obesity and diabetes in humans (142), approximately two-thirds of obesity is inherited in what is probably a polygenic fashion (57, 502). Genome-wide association studies (GWAS) were greatly facilitated by the International HapMap (www.hapmap.org) defining common single-nucleotide polymorphisms (SNPs) and existing linkage disequilibrium that provided near-genomic coverage of common genetic variations. We are now in the fourth wave of GWAS studies of obesity that has used a variety of variables such as BMI as a continuous trait or extremes of obesity in large populations of children or adults. FTO was one of the first genes identified, originally as having a high association with T2DM but later showing that this was through its association with obesity (158). Similarly, although homozygous inheritance of mutations of the MC4R leads to severe obesity (142), variants near the MC4R gene have a relatively strong association with obesity (269, 581). Other variants with obesity associations are BDNF, TMEM18, SH2B1, NEGR1, MTCH2, FAIM2, and GNPDA2 (36, 203, 216, 219, 434). It is important to point out that, as opposed to being causal for obesity, the way that direct mutations of the MC4R gene are (142), these GWAS genes are merely associations. Many are in noncoding areas of the genome and might be markers rather than playing any contributory role in obesity causation (456). However, several of the genes such as BDNF, MC4R, SH2B1, NRXN3, TMEM18, and NEGR1 are known to be involved in the regulation of energy homeostasis, reward, and/or neural development (142, 158, 179, 205, 321). Importantly, FTO has been shown to play a critical role in leptin receptor trafficking (500). There are also likely to be many other genes that singly or in combination contribute to the genetic propensity to become obese which have yet to be identified by such studies. In addition, epigenetic modifications of some of these known or yet to be identified genes are likely to play a critical role in determining their expression under conditions of varying environmental conditions.

V. PERINATAL ENVIRONMENT AND THE DEVELOPMENT OF OBESITY AND T2DM

A. Prenatal Influences

1. Parental undernutrition

Addressing the consequences of parental undernutrition is technically challenging in a human context. The best evidence for a direct effect of undernutrition during pregnancy on long-term metabolic health of the offspring has come from the study of individuals who were in utero during
periods of famine such as the Dutch Hunger Winter (427) and the Chinese Famine (309) (see above). These have demonstrated effects of severe undernutrition during pregnancy and risk of T2DM in the offspring. Few human studies have established an effect of more physiological differences in diet during pregnancy on long-term health of the offspring. Studies of a large Danish cohort revealed that dairy protein consumption during pregnancy was positively associated with birth weight (370). However, data are not yet available regarding the possibility that increased dairy protein consumption is also associated with reduced risk of T2DM. The importance of appropriate micronutrient intake during pregnancy for the health of the offspring at age six has been suggested by a study of an Indian cohort (564). This revealed that low maternal vitamin B12 at 18 wk of pregnancy and high maternal erythrocyte folate concentrations at 28 wk of pregnancy were associated with increased insulin resistance in the offspring. Similar observations were made in a Nepalese cohort with maternal vitamin B12 deficiency being associated with insulin resistance in the offspring at age 6–8 yr (498).

In contrast to the paucity of evidence from humans, there is extensive evidence from animal models to suggest that maternal undernutrition during pregnancy is associated with increased risk of glucose intolerance, insulin resistance, and obesity in the offspring (FIGURE 2). This includes detrimental consequences of total caloric restriction, macronutrient, as well as micronutrient deficiency. Varying degrees of total caloric restriction have been demonstrated to result in metabolic dysfunction in the offspring. Reducing caloric intake by 50% during the last week of pregnancy and throughout lactation in rats led to loss of glucose tolerance in the offspring as they aged (169). Similar effects were observed in a sheep model where nutrient restriction was initiated in late pregnancy (167), perhaps suggesting that the third trimester is a critical time period of exposure for risk of T2DM (as also indicated from studies of the Dutch Hunger Winter Cohort). More severe caloric restriction (to 30% ad libitum) in the rat has also been shown to be associated with insulin resistance and obesity in the offspring (532).

The most extensively studied rodent model of macronutrient deficiency is that of isocaloric protein restriction. Offspring of dams fed diets containing 5–8% protein demonstrate impaired insulin secretion in adulthood (336) that is exaggerated if the offspring are fed a high fat diet, 556 and insulin resistance (382). These defects in insulin action and secretion are associated with an age-dependent loss of glucose tolerance and development of a T2DM phenotype in later life (402). If followed by rapid postnatal catch-up growth, maternal protein restriction during pregnancy is associated with increased adiposity in the offspring (381). The propensity to the development of obesity is further exaggerated if the offspring are weaned onto a highly palatable diet (381). Although most studies of parental macronutrient deficiency have focused on nutrient restriction in the mother, there are now emerging studies to suggest that there can be detrimental consequences of paternal nutrient deficiency; offspring of males fed a low-protein diet displayed increased expression of genes involved in lipid and cholesterol biosynthesis in the liver (77).

![FIGURE 2](image-url). Developmental origins of metabolic disease. The developmental programming of key regulatory systems by the perinatal environment and/or genetic background represents a possible mechanism by which alterations in maternal and/or early postnatal nutrition predispose the offspring to obesity and type 2 diabetes. This figure was created in part using illustrations from “Servier Medical Art” with permission.
A number of studies in animal models have also investigated the effects of micronutrient deficiency in the offspring. Maternal zinc deficiency in rats has been associated with increased leptin levels, insulin resistance, and impaired glucose tolerance in the offspring (233). Similar observations were observed in a rat model of maternal dietary chromium restriction (384). Maternal anemia in the rat leads to increased blood pressure in the offspring but as yet has not been associated with changes in offspring adiposity or glucose intolerance (166).

2. Parental obesity

Initial programming studies focused primarily on the detrimental consequences of parental undernutrition on the long-term metabolic health of the offspring (see above). However, following the growing epidemic of obesity during the last decade, a rapidly increasing number of studies have focused on the consequences of parental overnutrition and/or obesity during the periconceptual period on the risk of obesity and T2DM in the offspring. The potential detrimental consequences of maternal obesity during pregnancy on offspring risk of obesity in humans was first inferred from a number of observational studies which demonstrated that children born to obese mothers were more at risk of obesity/increased adiposity than those born to obese fathers (79, 214, 275, 369, 424, 550). This was further supported by evidence that maternal BMI is independently associated with offspring BMI (261, 551), adiposity (164), and insulin resistance (53, 339) and that there are strong associations between maternal weight gain during pregnancy and offspring adiposity (103, 436). Some evidence for these associations being causal has come from studies of siblings born before and after the mother had bariatric surgery to reduce her weight. These studies demonstrated that offspring born after surgery had a reduced risk of obesity and insulin resistance compared with those born prior to surgery (265, 481). It has recently been shown that these differences in risk were also associated with changes in DNA methylation (i.e., an epigenetic change) of a large number of loci, including genes involved in glucose homeostasis (190). These studies show a disproportionate risk of disease in offspring born to the same mother under different in utero conditions thereby providing evidence that an obesogenic environment experienced during this critical period of development directly influences long-term risk of obesity.

Studies in animal models support the findings from the human studies described above, supporting a causal relationship between maternal overnutrition/obesity during pregnancy and offspring adiposity and insulin resistance (reviewed in Alfaradhi and Ozanne, 10) (FIGURE 2). The earliest studies addressed the detrimental consequences of maternal high-fat feeding where rat dams were fed a saturated-fat-rich diet during pregnancy and lactation leading to obesity, insulin resistance, and dysregulated glucose homeostasis in the offspring (192, 512). These effects were not only a consequence of maternal diets rich in saturated fats. Maternal diets rich in n-6 polyunsaturated fatty acids have also been demonstrated to have detrimental effects on insulin sensitivity and adiposity in rat offspring (69). Similar detrimental effects of maternal high-fat feeding have been observed in non-human primate models. These include increased adiposity, as well as increased hepatic triglyceride deposition in the offspring at 6 mo of age (325). Such abnormalities in mouse models have been shown to persist into adulthood (67). Encouragingly, it has been demonstrated that dietary intervention prior to pregnancy in rats that had been fed a high-fat diet for 90 days, at least in part ameliorated the detrimental effects of the high fat diet on offspring adiposity and insulin resistance (574).

Although the studies above show proof of principle that maternal high-fat feeding has detrimental consequences for the metabolic health of the offspring, the experimental diets utilized are not representative of the obesogenic diets currently consumed by women (and men) living in the western world. In addition, in general, the rodent models of maternal high fat feeding are not associated with maternal obesity, unless they are fed for long periods of time, as rodents are good at regulating their caloric intake in response to high-fat foods which they do not find that palatable. A more recent approach has therefore been to employ highly palatable/cafeteria style diets rich in simple sugars that override the natural satiety signals of rodents and are more representative of a typical human Western diet. Feeding of such diets to pregnant rodents leads to the development of insulin resistance, increased adiposity, and impaired glucose tolerance in the offspring (84, 455, 469). These changes are associated with altered structure and function of central appetite regulatory circuits (84, 251, 535). The effects of maternal diet-induced obesity on offspring body weight and adiposity are most apparent when the offspring themselves are fed an obesogenic diet highlighting an interaction between fetal and early postnatal nutrition (37, 473). This provides one potential explanation for the variation in obesity observed in individuals all living in the same obesogenic environment. Detrimental effects of a maternal obesogenic diet have also been observed in larger animal models such as sheep. Feeding pregnant ewes an obesogenic diet that was 150% of their energy requirement led to increased intramuscular triglyceride and skeletal muscle insulin resistance (567). There is also evidence to suggest that the effect of maternal high-fat feeding/obesity can be transmitted through at least two generations, through both the male and female lineage (130).

Finally, in animal models there is now also emerging evidence to suggest that paternal obesity at the time of conception is associated with metabolic dysfunction in the offspring. Chronic high-fat feeding in male rats led to increased adiposity, glucose intolerance, and insulin resistance in female offspring (357). Most recently it has been shown that these effects of paternal diet-induced obesity on offspring obesity and insulin resistance persist to at least
two generations, at least in mice (162). This is accompanied by changes in gene expression in testes and sperm and global DNA methylation in the sperm.

3. Prenatal stress and offspring obesity and diabetes

Psychosocial stress is a common factor in human existence. Such stress increases the likelihood of becoming obese and diabetic in humans (316, 449) and rodents (140, 431, 503). Not surprisingly, severe stress during pregnancy can have major adverse effects on offspring. Many of these effects are likely due to the fact that cortisol, which is released in stressful situations, can cross the placenta and alter the development of the brain and other organs (137, 237, 364). In addition to a range of abnormalities in behavior and cognitive function (364), there is evidence that severe maternal psychosocial stress is associated with higher BMI, percent body fat, insulin resistance, and abnormal lipid profiles (137, 139) and hypothalamic-pituitary-adrenal dysregulation in young adult offspring (138). Much of our knowledge of the mechanisms underlying these abnormalities comes from rodent studies in which dams are subjected to different types of stress and/or corticosteroids during various stages of gestation. As a broad generalization, depending on the stage of pregnancy, prenatal stress or exogenous glucocorticoids can have a major adverse impact on the development of the brain, including neurotransmitter systems and brain areas involved in the regulation of energy and glucose homeostasis (161, 237, 437) and pathways regulating motivated and reward behaviors (208, 323). Depending on the timing of stress and the sex of the offspring, adverse offspring outcomes of prenatal stress include permanent dysfunction of the neuroendocrine axis (237) and stress responsiveness (160, 208), delayed learning (160) and abnormal glucose tolerance, hyperphagia, as well as increased body weight and adiposity (111, 363, 387, 508, 546). Importantly, prenatal stress results in less maternal grooming and attention in offspring (81, 418), which can have important effects on offspring behavior and metabolic phenotype (80, 81, 95). In keeping with these fetal/neonate-maternal interactions, at least some of the abnormalities in offspring stress responsivity can be reversed by blocking the mother’s stress-induced corticosterone response (32), by fostering their offspring to nonstressed dams (32) or by postnatal handling (482). Intriguingly, in addition to an effect of maternal stress on developing offspring, paternal stress prior to mating significantly reduced the stress responsivity of resultant offspring with global changes in transcriptional regulation suggestive of epigenetic programming (444). Unfortunately, no data were presented with regard to either alteration in adiposity or glucose tolerance in this study. Nevertheless, such studies, if they can be translated to the human condition, suggest that much of the damage done by prenatal stress can be undone by either ameliorating the mother’s stress response or by postnatal manipulations that control the offspring-mother interactions.

4. Gestational diabetes

Initial epidemiological studies highlighted the association between low birth weight and increased metabolic disease risk in later life, observations that have been reproduced in over 40 populations worldwide (356). However, in some of these studies, such as those of native North American population, increased risk of T2DM and metabolic syndrome was also observed at the high birth weight end of the spectrum (324). These populations have a high prevalence of T2DM, obesity, and consequently gestational diabetes (>10% of all pregnancies) (157). Therefore, the increased risk of metabolic disease in individuals with high birth weight was proposed to reflect an increased risk of diabetes in the macrosomic offspring of women with gestational diabetes (108, 318, 403, 536). This hypothesis is supported by sib pair studies that have demonstrated a greater prevalence of T2DM and high BMI in siblings born after the mother was diagnosed with T2DM compared with those born prior to the development of T2DM (109). Further evidence for the association between maternal gestational diabetes and increased offspring weight being causative has come from a retrospective study that demonstrated that intensive treatment by diet and/or insulin of gestational diabetic mothers attenuated this association (212).

Studies in animal models have also provided strong evidence that gestational diabetes can cause increased risk of diabetes in the offspring (FIGURE 2). In most rodent studies, the effects of maternal diabetes have generally been assessed using models where diabetes is induced in the mother by chemical destruction of the maternal β-cells using streptozotocin (reviewed by Van Assche et al., 528). The phenotype of the offspring is determined by the severity of the glucose intolerance induced in the mother. The offspring of mildly diabetic mothers are large at birth and in neonatal life demonstrate an apparent enhanced development of their endocrine pancreas. However, in adulthood they have a deficit in their insulin secreting capacity (199) and develop impaired glucose tolerance (6, 472). The offspring are also hyperphagic, leptin resistant, and obese (491). This is associated with hypothalamic defects (409) including a reduction in neuronal connections between the ARC and the PVN (491). If the maternal diabetes is severe, the offspring are born small for gestational age. As a result of overstimulation by the high glucose levels, the offspring β-cells are almost completely degranulated with lower insulin content and the offspring become insulin resistant as adults (6). In light of the growing epidemic of obesity, a growing number of animal models of maternal diet-induced obesity are being established (see above and below). In some of these it has been demonstrated (unsurprisingly) that the dams develop impaired glucose tolerance during pregnancy. Although gestational diabetes is not the only altered metabolic parameter in these models, it is conceivable that at least some of the detrimental consequences of maternal obesity in the offspring are caused by accompanying gestational diabetes.
B. Postnatal Influences on Offspring
Metabolic Outcomes

1. Maternal-infant interactions

Early infancy exposure to a variety of experiences and metabolic milieu can have an important impact on the ways in which the infant learns to cope with their environment. The content of breast milk is influenced by the physiological and metabolic state of the mother and can have important effects on the metabolic state and feeding preferences of their infants. Hormones such as leptin and insulin are secreted into the milk and, during early infancy, can be absorbed directly into the bloodstream of suckling infants (78, 176, 213, 234, 349). In addition, the milk content of nutrients such as essential fatty acids which are required for neural development (524) are heavily influenced by the genetic and metabolic status of the mother (176). While many studies support a protective effect of breast versus formula feeding during infancy against later obesity and glucose intolerance (104, 154, 266, 379), some suggest that factors such as maternal diabetes might have an adverse effect on the metabolic development of their offspring (408). In rodents, cross-fostering of genetically obesity-resistant (DR) pups to obese dams with a genetic propensity to become obese on high-fat diets (DIO) causes them to become obese and insulin resistant when subsequently exposed to a high-fat diet as adults (176). Much of this effect may be attributed to abnormalities in milk content of nutrients such as poly- and monounsaturated fatty acids and hormones such as insulin and leptin which are essential for normal brain development (176). Similarly, dietary choices of the breast-feeding mother or early exposure to specific tastes and orders in infant formulas can have marked effects on dietary and taste preferences of the developing infant (154, 329–331, 518). In both humans and experimental animals, the major issue left unanswered is what basic mechanisms underlie these persistent changes in behavior as well as metabolic and physiological function. Some are associated with changes in the anatomical development of pathways critical to these functions (62), while others may be due to epigenetic changes in gene expression, or both.

2. Catch-up growth in intrauterine growth retardation and accelerated postnatal growth

Accelerated early neonatal growth and/or obesity has been shown to amplify the detrimental consequences of being born small for gestational age on metabolic health outcomes. The original Hertfordshire studies by Hales et al. (198) demonstrated that the men with the worst glucose tolerance at age 64 were those that were in the lowest quartile of birth weight but who were obese as adults. Likewise, in the Dutch Hunger Winter studies, the worst glucose tolerance was observed in individuals who were exposed to famine in utero but became obese as adults (427). The particular detrimental effects of rapid growth during childhood following fetal growth restriction emerged from a study of primary school children in South Africa. Those with a low birth weight who gained weight rapidly during early childhood had the worst glucose tolerance at age 7 (102). Studies in Finland also demonstrated that men and women who develop T2DM are those born small for gestational age and then cross BMI centiles between the ages of 2 and 11 (141). These detrimental effects of catch-up growth may be related to the observation that during periods of such accelerated growth there is preferential accumulation of fat mass rather than lean tissue (344). Studies in animal models reinforce this concept that rapid postnatal growth following in utero growth restriction is detrimental to long-term metabolic health, including increased risk of obesity. Rodent models of maternal protein restriction, caloric restriction, and intrauterine artery ligation, which all demonstrate low birth weight, develop increased adiposity when suckled by normally fed dams during the lactation period and therefore undergo postnatal catch up growth (381, 475, 532).

There is now also growing evidence to suggest that accelerated postnatal growth not only exaggerates the effects of suboptimal growth in utero but can also have detrimental effects on later health regardless of an individual’s birth weight. This is particularly prominent in relation to risk of increased adiposity and obesity. At least three systematic reviews demonstrate in humans that accelerated postnatal growth increases risk of subsequent obesity (26, 346, 371). These studies show associations, but do not provide information regarding the causes of the accelerated growth. However, in humans, both observational and randomized feeding trials suggest that nutritionally induced rapid weight gain in the first half of infancy predicts later obesity and cardiovascular risk factors such as higher blood pressure (173, 523, 547). Studies comparing breast-fed infants to formula-fed infants revealed that the former were at reduced risk of obesity (18, 200). These observational studies do not provide causal evidence that nutrition per se mediates these relationships. However, it is well known that formula-fed infants gain more weight over the first year of life than breast-fed infants (120). Causal relationships between nutrition during infancy and subsequent metabolic health have emerged from randomized intervention studies and control trials. In these studies low levels of nutrient intake during the neonatal period are protective against risk of obesity and cardiovascular disease (257, 476, 477). The precise duration of this early neonatal critical time window for determination of obesity risk is not clear. However, it has been suggested that it could be as little as the first postnatal week of life (495). Animal models have again confirmed these studies in humans. Use of a range of animal models has repeatedly confirmed the fact that early overnutrition in the neonatal period predisposes to later obesity (FIGURE 2). Raising rodent pups in small litters increases their intake and markedly increases their propensity to be-
come obese as adults (231, 246). Similarly, overfeeding neonatal rats for the first 18 days of life by intragastric tubes markedly increases their body weight gain (549). On the other hand, raising rodent pups in large litters restricts their access to food and can protect even genetically obesity-prone animals from becoming obese (231, 392).

VI. GENE-ENVIRONMENT INTERACTIONS

A. Epigenetics

The term *epigenetics* (literally meaning “above the genetics”) was first defined by the developmental biologist Conrad Waddington as the “interactions of genes with their environment which bring the phenotype into being” (539). The epigenetic changes that mediate this interaction include alterations in DNA methylation, covalent modifications of histone tails (e.g., acetylation, methylation, phosphorylation, and ubiquitination), and expression of noncoding RNAs (e.g., miRNAs). The phenomenon of epigenetics therefore explains how one genotype can give rise to multiple different phenotypes through alterations in the epigenotype. It also provides a molecular framework through which the environment can interact with the genome to alter gene expression and thereby influence phenotype. As gene-environment interactions are key to the concept of developmental programming, much attention has been directed towards the potential role of epigenetic mechanisms in mediating the effects of a suboptimal exposure of a fetus in utero to permanent changes in its long-term metabolic health including risk of T2DM and obesity. Epigenetics provides an attractive mechanism to underlie the cellular memory by which a suboptimally exposed cell during a critical period of development stably affects gene expression following multiple rounds of cell division.

The potential for diet during pregnancy to permanently alter the epigenotype and therefore adult phenotype and disease susceptibility was first demonstrated 15 years ago using the Agouti viable yellow (Avy) mouse (559). The Avy allele is epigenetically sensitive as a result of a retrotransposon insertion upstream of the Agouti gene. When the retrotransposon is hypermethylated and thus silenced, the agouti gene is expressed only in skin and produces a lean mouse with an agouti-colored coat (termed pseudo-agouti). In contrast, hypomethylation of the retrotransposon generates a ubiquitously expressed transcript that causes yellow coat color and obesity. It has been demonstrated that when Avy pregnant dams are fed a diet supplemented in methyl donors and cofactors (e.g., choline, folate acid, vitamin B12) they tend to have offspring that are pseudo-agouti and lean rather than being yellow and obese as seen when the Agouti gene is ubiquitously active (543, 559). This effect of maternal diet on offspring coat color occurred by increasing the level of methylation at the Avy allele.

Rodent models have also demonstrated that physiological changes in maternal diet in wild-type animals can alter the epigenotype of the offspring (reviewed in 380). Transcription factors have emerged as common targets of epigenetic programming by changes in the early environment. They are also conceptually attractive targets of programming. Through epigenetic programming of such factors, a coordinated network of genes can be modified since transcription factors themselves regulate expression of gene networks. Examples of transcription factors that are epigenetically programmed through changes in DNA methylation and histone modifications include the pancreatic β-cell developmental transcription factors and MODY genes PDX-1 (as a result of placental insufficiency) (389) and HNF-α (as a result of maternal protein restriction) (457). Both of these studies demonstrated that the programmed changes were dynamic and changed as the animals aged, perhaps explaining the importance of the ageing process in development of the diabetic phenotype in both of these animal models of programming. Peroxisome proliferator-activated receptor-α in the liver is another example of a transcription factor that is epigenetically programmed by maternal protein restriction in rats (311). Studies in ovine models have also demonstrated that maternal undernutrition can lead to epigenetic alterations in the offspring. For example, periconceptual undernutrition in sheep led to changes in the methylation of the POMC locus in the fetus later on in gestation (496). Altered DNA methylation of a gene involved in gluconeogenesis (PEPCK1) has also been reported in a non-human primate model of maternal undernutrition during pregnancy (360).

In addition to programmed epigenetic changes in response to fetal/maternal undernutrition, animal models have also highlighted the potential for maternal overnutrition to influence epigenetic modifications in the offspring. Using a mouse model of maternal high-fat feeding, Vuetic et al. (357) demonstrated that components of central reward pathways such as the µ-opioid receptor were susceptible to epigenetic programming by maternal overnutrition. Maternal diet-induced obesity in rats has also been shown to lead to programmed changes in DNA methylation of pro-adipogenic genes (including C/EBP-β) in adipose tissue in the offspring (56). Growing evidence suggesting that epigenetic programming by overnutrition not only occurs through the maternal line but can also be transmitted through the paternal line is emerging. Chronic high-fat feeding of male rats led to pancreatic β-cell dysfunction in female offspring that was associated with changes in methylation of the Il13ra2 locus (357). Furthermore, consistent with epidemiological evidence, overnutrition during the neonatal period leads to permanent changes in epigenetic modifications. Using a model of neonatal overfeeding through litter size manipulation, Plagemann and colleagues (407, 414) demonstrated programmed changes in the POMC and insulin receptor loci in the hypothalamus.
Data from humans in relation to evidence for epigenetic modifications contributing to the developmental origins of T2DM and obesity are much more limited and are often hindered by the lack of availability of metabolically relevant tissues from living humans. The majority of studies have therefore focused on clinically accessible tissues such as white blood cells or umbilical cord. However, a major goal has been to identify epigenetic changes in these tissues that are reflective of epigenetic changes in tissues such as adipose tissue, the brain, and the endocrine pancreas. Genome-wide methylation analysis of cord blood cells demonstrated that intrauterine growth restriction in humans was associated with altered methylation of the HNF-α locus, again highlighting the potential importance of programming of transcription factors (132). Human studies have also demonstrated association between patterns of early postnatal growth and epigenetic modifications. Groom et al. (185) reported a link between rapid postnatal growth and differential methylation of the TACSTD2 locus, a gene associated with childhood adiposity. Evidence for the effects of diet during pregnancy and epigenetic changes in the offspring in humans is sparse, and most has come from studies of individuals who were in utero during the Dutch Hunger Winter. Initial studies of this cohort identified differential methylation of the Igf2 locus six decades after exposure to the famine in utero (207), and a further five vulnerable loci were identified in a subsequent study (514). Other human studies have demonstrated the potential use of epigenetic modifications as markers of future risk of metabolic disease. In two separate cohorts, Godfrey et al. (175) demonstrated that methylation of the retinoid X receptor in umbilical cord tissue correlated strongly with percent fat mass later on in childhood and explained ~25% of the variation in adiposity.

In addition to studies showing associations between changes in early patterns of growth and nutrition, there are also a limited number of studies showing epigenetic variation in candidate genes associated with T2DM and obesity. Small but significant differences in methylation of FTO (39), insulin (568), and KCNQ1 (517) loci have all been shown to correlate with disease risk. Furthermore, there is evidence that lifestyle factors associated with changes in obesity risk can alter promoter methylation of key genes in skeletal muscle including PGC-1α, PDK4, and PPAR-δ (33).

### B. Hormonal Influences

As discussed above, a plethora of data from rodent and human studies have suggested that changes in nutrition during perinatal life have a significant impact on the development of obesity and related diseases in later life. Hormones, such as leptin, insulin, and ghrelin, are dynamically regulated by nutritional and metabolic status and are therefore major signals to the developing fetus and neonate of nutrient availability (FIGURE 2). In addition, hormones produce a multitude of effects on functions in the developing fetus and neonate that are well outside the functions they serve in later life. Thus the biological actions of several metabolic hormones are different during neonatal versus adult epochs. For example, in sharp contrast to the potent effects of leptin and ghrelin on feeding in adults, peripheral leptin or ghrelin injections have no significant effects on milk intake or body weight during the first 2–3 wk of postnatal life in rats and mice (340, 404, 490). These observations suggest that leptin and ghrelin might exert different functions during neonatal life such as altering neural development. Early observations by Bereiter and Jeanrenaud (40, 41) reported structural defects in the obese ob/ob mice, including a reduction in soma size of cells in the VMN and dorsal motor vagal nucleus neurons, as well as alterations in the dendritic orientation of VMN and LHA neurons. Twenty years later, Ahima and Flier (8) showed that the same mutant mice display an immature pattern of expression of synaptic and glial proteins. This pioneer work paved the way for subsequent research on leptin in brain development and plasticity.

The availability of ob/ob mice and more modern neuroanatomical tools to study neural circuits allowed more detailed studies on the role of leptin on hypothalamic development. Axonal tracing of ARC neurons demonstrated that the leptin deficiency permanently disrupts the development of projections from the ARC to each of its major targets, including the PVN (61). Remarkably, peripheral leptin injection in ob/ob neonates restores the density of ARC axons to a density that was comparable to that of wild-type littersmates, but the treatment of adult ob/ob mice with leptin is largely ineffective (61). Also, leptin restores normal brain weight in ob/ob mice but only when the hormone is injected during early life (494). These observations suggest that leptin acts primarily during a restricted critical neonatal period to exert its neurotrophic effects. Notably, obeseogenic environments, such as maternal obesity, diabetes, and postnatal overnutrition, can cause hyperleptinemia throughout postnatal life and impair central leptin sensitivity during critical periods of hypothalamic development (62, 174, 250, 491). Notably, this early leptin resistance is associated with a disrupted development of ARC neural projections to the PVN (62, 174, 250, 491). In contrast, maternal undernutrition during pregnancy and lactation or the postnatal period blunts the naturally occurring postnatal leptin surge and also causes abnormal development of ARC projections (97, 118, 572), and daily leptin treatment during early postnatal life in pups born to undernourished dams normalizes their metabolic abnormalities (533). These findings show the importance of neonatal leptin in life-long metabolic regulation and raise the importance of early endocrine intervention in metabolic (mal)programming.

More recent studies have also implicated ghrelin in the development of metabolic systems. Ghrelin is one of the first...
major metabolic hormones to appear during development. It is expressed in embryos as early as the morula stage and continues to be expressed in the developing fetus and neonate. During perinatal development, ghrelin is transiently expressed in the pancreatic α-cells where it colocalizes with glucagon (116). But ghrelin is also produced by the pancreatic β-cells (419). This transient expression of ghrelin appears to play a role in pancreas development. Newborn rats exposed to ghrelin for 7 or 14 days had reduced pancreatic weights, attenuated pancreatic DNA synthesis, and reduced DNA content (119). The morphological effects of neonatal ghrelin appear widespread because chronic neonatal ghrelin injections also reduce growth of the stomach, as evidenced by a decrease in gastric weight, DNA synthesis, and DNA content. On the other hand, ghrelin injections in adult animals increase pancreatic and gastric weight, DNA synthesis, and DNA content (119, 542), indicating that ghrelin can induce biphasic effects on gastric growth depending on the age of exposure.

Ghrelin also exerts developmental effects on the brain. In vitro incubation of hypothalamic and brain stem cells with ghrelin induces proliferation with many of the resultant newborn cells acquiring a neuronal and/or glial phenotype (224, 575, 576). Insulin has also long been associated with brain development. Consistent with a trophic role of insulin in the developing hypothalamus, offspring of insulin-deficient mothers display a reduced number of ARC neurons, and this reduction of neuronal cell number is preventable by the normalization of glycemia using pancreatic islet transplantation (156). Moreover, hypoinsulinemic pups born to protein-restricted dams display a reduction in the number of astrocytes (411), while the offspring of gestationally diabetic mothers, which have increased insulin levels, have increased numbers of astrocytes (409, 412). In addition to influencing hypothalamic cell numbers, insulin can also influence hypothalamic neuronal connectivity. Pups born to insulin-deficient dams display abnormally organized POMC and NPY/AgRP neural projections that could result from the attenuated responsiveness of hypothalamic neurons to the neurotrophic actions of leptin during neonatal development (491). Notably, intrahypothalamic insulin injections during early postnatal life cause life-long metabolic dysregulation, raising the importance of neonatal insulin in the developing brain on life-long metabolic regulation (410, 412).

C. Rodent Models of Gene-Environment Interactions

1. Mouse models

Although transgenic and knockout experiments are typically conducted in mice, a significant variability in adiposity, DIO, and obesity-related diabetes exists among the mouse strains commonly used in laboratory research (see 548 for a review). The inbred C57BL/6J (B6) strain is probably the most widely used strain to conduct transgenic and knockout experiments, in part because of its susceptibility to develop obesity on high-fat diets. C57BL/6J mice are not obese on a standard chow, but when fed a high-fat diet they develop hyperglycemia, hyperinsulinemia, and hyperleptinemia (133, 505, 548). In contrast, some strains, such as 129/Sv and A/J mice, are almost totally resistant to obesity and diabetes when fed a high-fat diet (503). Remarkably, both 129/Sv and C57BL/6J mice eat an equal number of calories when fed a high-fat diet (13), suggesting that C57BL/6J mice have a higher feeding efficiency and gain greater weight per calorie consumed. Even within the C57 mouse strain there are significant differences among sub-strains in response to the high-fat diet. Thus C57BL/6J mice fed a high-fat diet exhibit a marked metabolic phenotype, whereas C57BL/6KsJ mice only display a weak phenotype (93). Furthermore, in some laboratories it has been noted that C57BL/6J mice within the same colony exhibit a bimodal response to high-fat diet; half develop DIO, and half are obesity-resistant (136). Given the fact that they all share the identical genotype, this marked difference in metabolic phenotypes when offered a high-fat diet suggests the presence of an as yet to be determined epigenetic influence. Background genes also appear to play an important role in determining the metabolic phenotype of mice with normally occurring mutations or mice that have been genetically altered by introduction of transgenes. For example, ob/ob and db/db mice on the C57BL/KsJ background are obese and develop severe diabetes and a marked hyperglycemia, whereas ob/ob mice on the C57BL/6J background are obese but only exhibit mild diabetes and hyperglycemia (92). Similarly, mice with a double-heterozygous deletion of the insulin receptor and insulin receptor substrate-1 become insulin resistant and severely hyperinsulinemic on the C57BL/6J background, but on the 129/Sv background these double mutant mice only exhibit a mild hyperinsulinemia (271). Together, these observations indicate that background genes in mice greatly influence the development of obesity and obesity-related diseases, such as T2DM, in response to either an obesogenic environment or genetic defects.

2. Rat models

The selectively bred DIO and DR strains of rats have proven to be a valuable model for studying the interactions of genes with environment. These strains were derived from the outbred Charles River Sprague-Dawley rat. Sprague-Dawley rats from this breeder have the fairly unique characteristic of showing a wide variation in body weight and adipose gain when placed on a relatively high-fat (31%), high-sucrose (25%) diet, designated as a “high energy” (HE) diet (296). Approximately half the rats placed on such a diet overeat for 4–6 wk and become obese (296). The remaining rats overeat for only a few days and gain no more weight than controls fed a low-fat chow diet (299). Importantly, these outbred rats have been selectively bred to produce
DIO and DR strains which have maintained their distinctive phenotypes for more than 50 generations. The obesity of the DIO rat appears to have a genetic origin since breeding DIO males with another obesity-resistant strain of rats passes on this phenotype to the offspring of these crosses in an apparently polygenic manner of transmission similar to most human obesity (57, 298, 502). This model is an excellent one for the study of human obesity since, like most obese humans, it maintains its higher body weight and adipose set-points even when switched to a low-fat diet or after being calorically restricted for many weeks (291, 302). This defense of a higher body weight set-point is what occurs in obese humans and is likely the reason for the high recidivism rate in the medical treatment of obesity and the extreme measures many previously obese individuals must undertake to keep off lost weight (326, 448, 538, 557).

The DIO/DR model is extremely useful for the study of gene-environment interactions associated with maternal obesity and insulin resistance since dams can be fed the same high-fat diet but only the DIO dams become obese and insulin resistant during gestation and lactation (176, 177, 294, 300). This obesity of DIO dams is not accompanied by an increase in offspring body weight unless such offspring are also fed HE diet from weaning. As opposed to DIO offspring, offspring of DR dams, whether the dams were made obese with a highly palatable diet or stayed lean on HE diet during gestation and lactation, gained no more weight or adiposity than controls regardless of their postweaning diets. However, maternal obesity, regardless of genotype, was associated with enlargement of the VMN and DMN and differentially affected the density of norepinephrine and serotonin transporters in the PVN (294). On the other hand, offspring of DIO dams, regardless of whether their dams were lean or obese during gestation and lactation, showed defective development of the α-melanocyte stimulating hormone (α-MSH, a catabolic peptide derived from POMC) and AgRP pathways projections from the ARC POMC and NPY/AgRP neurons to the PVN. These defective projections appeared to be due to the inherent leptin resistance of the DIO rat (176, 178, 295, 297, 299, 392), since leptin is required for normal development of this pathway (62).

Although it is uncertain whether DIO pups are born with inherent leptin resistance, it does appear in the first few days of life (62), making this early postnatal period an important focus of potential interventions that might alter later life development of obesity. In fact, cross-fostering DR pups from lean DR dams to obese, but not lean, DIO dams fed HE diet causes them to become obese and insulin resistant when they are fed HE diet as adults (176). This is associated with an “anabolic” shift in the expression of hypothalamic genes including increased ARC AgRP and decreased VMN leptin, insulin, and MC3R expression (176). As mentioned above, the major difference between obese DIO dams and all others was the high insulin and leptin and low mono- and polyunsaturated fatty acid contents of their milk, suggesting an early influence on both brain development and obesity-proneness in this otherwise highly obesity-resistant strain (176). However, cross-fostering DIO pups from either lean or obese DIO dams to lean DR dams had no effect on their propensity to become obese or insulin resistant when fed HE diet as adults. While fostering DIO pups with lean DR dams failed to alter their obesity-prone phenotype, raising them in large litters, which severely restricts intake (246), prevents them from becoming obese as adults. This obesity resistance was associated with normalization of their ARC leptin receptor binding and leptin sensitivity with a presumptively resultant normalization of their ARC-PVN α-MSH and AgRP pathway outgrowth (392). Thus early dietary interventions can have a major effect on later life development of obesity that is highly dependent on the interventions and the genetic makeup of the individual.

While it is clear that interventions during gestation and early postnatal development can have major impacts on the long-term development of systems involved in the regulation of energy and glucose homeostasis, it appears that the critical period for altering some of these systems may extend well into adolescence and early adulthood in both humans and other mammals. An excellent example of this is the effect of early-onset exercise on altering the development of obesity in selectively bred DIO rats. These rats are intrinsically less active than DR rats both in their home cages (260) and in running wheels. Yet, when running wheels are made available, only DIO rats lose weight despite running only half as much as DR rats (293). When early postweaning DIO rats are fed a high-fat diet and simultaneously provided with a running wheel for 3 wk or more, they have a persistent protection from becoming obese which is associated with increased leptin signaling in the ARC and VMN (394). Because ARC-PVN α-MSH and NPY/AgRP pathways have already completed their development by the time the wheels are introduced (60), the increased leptin signaling and obesity protection are not associated with changes in the density of these fiber pathways (391). However, this does not exclude the possibility that other brain areas that have not fully developed might be altered by such early-onset exercise. In addition, it is possible that the increased leptin signaling might be associated with epigenetic changes, although this possibility has not been explored.

VII. HOW CAN WE USE THIS INFORMATION TO PREVENT AND TREAT OBESITY AND DIABETES?

This review presents data that clearly demonstrate the major importance of the perinatal environment and genetic predisposition in determining the development of neural pathways and organs involved in the regulation of energy and glucose homeostasis. While we do understand many of
the predisposing factors, it remains challenging to identify those individuals who are most at risk and the predisposing factors that push them into a vicious cycle of obesity and insulin resistance from which few can recover. Because organs, particularly the brain, undergo the majority of their development during the perinatal period, there is a premium on identifying at risk individuals and risk factors during this critical period. Importantly, while most organs undergo continuing change of structure and function throughout life, the brain is much less plastic with regard to changing the connections of critical neuronal pathways established during critical periods of early development. The problem is that, even if we could reliably identify such individuals and risk factors, we are a long way from knowing how to alter the perinatal environment to prevent offspring from being set on the path to near-permanent predisposition to obesity and diabetes.

Also, we understand even less about the factors that make obesity, once it develops, a near-constant condition in so many individuals. Given our current state of knowledge, there are some possible guidelines, although some of these are based on animal research that might not apply to humans. First, several factors increase the probability of offspring obesity and/or diabetes. These include obesity in one or both parents, gestational diabetes, intake of a high-fat, calorically dense diet during pregnancy and lactation, gestational undernutrition with postnatal overfeeding (“catch up growth”), genetic mutations known to cause obesity in affected individuals, and possibly some gene variants which have a high association with obesity such as FTO. However, it is important to recognize that these latter gene variants are only associations, and we are a long way from understanding the combinations of genes and the epigenetic modifications of these and other genes that promote obesity. Similarly, while research in animal models has identified several factors that appear to adversely alter the development of neural pathways involved in the regulation of energy and glucose homeostasis, it is unclear if these same factors apply to humans and, if they do, the stage of gestational and postnatal development which is most at risk. Finally, even if we could identify at risk individuals and obesogenic factors, changing the perinatal environment is a socioeconomic and cultural challenge for which we have so far failed to find a practical solution in the vast majority of at risk individuals. The hope would be that continued research into the factors that predispose individuals to become obese might identify those that lend themselves to relatively simple, straightforward interventions.

ACKNOWLEDGMENTS

Present addresses: S. Bouret, The Saban Research Institute, Neuroscience Program, Childrens Hospital Los Angeles, Univ. of Southern California, Los Angeles, CA 90027 (e-mail: sbouret@chla.usc.edu); and S. E. Ozanne, Univ. of Cambridge Institute of Metabolic Science and MRC Metabolic Diseases Unit, Cambridge CB2 2QR, UK (e-mail: seo10@mole.bio.cam.ac.uk).

Address for reprint requests and other correspondence: B. E. Levin, Neurology Service (127C), VA Medical Center, 385 Tremont Ave., East Orange, NJ 07018 (e-mail: levin@njms.rutgers.edu).

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01 30066 and 53181 and the Research Service of the Veterans Administration (to B. E. Levin). S. E. Ozanne is supported by the MRC Metabolic Diseases Unit (MRC_MC_UU_12012/4). S. G. Bouret is supported by the National Institutes of Health Grants R01DK84142 and P01ES022845, United States Environment Protection Agency Grant RD83544101, the Foundation for Prader-Willi Research, and the EU FP7 integrated project (grant agreement no. 266408, “Full4Health”).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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GENE-ENVIRONMENT CAUSES OF OBESITY


Strategies for reversing the effects of metabolic disorders induced as a consequence of developmental programming

M. H. Vickers1* and D. M. Sloboda1,2,3,4

1 National Research Centre for Growth and Development, Liggins Institute, University of Auckland, Auckland, New Zealand
2 Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada
3 Department of Obstetrics and Gynecology, McMaster University, Hamilton, ON, Canada
4 Department of Pediatrics, McMaster University, Hamilton, ON, Canada

Edited by:
Catalina Pico, University of the Balearic Islands, Spain

Reviewed by:
Nina Eikelis, Baker IDI Heart and Diabetes Institute, Australia
Marianne Tare, Monash University, Australia

*Correspondence:
M. H. Vickers, National Research Centre for Growth and Development, Liggins Institute, University of Auckland, 2-6 Park Avenue, Grafton, Auckland 1142, New Zealand.
email: m.vickers@auckland.ac.nz

INTRODUCTION

Obesity and the metabolic syndrome have reached epidemic proportions worldwide with far-reaching health care and economic implications. The rapid increase in the prevalence of these disorders suggests that environmental and behavioral influences, rather than genetic causes, are fueling the epidemic. The developmental origins of health and disease hypothesis has highlighted the link between the periconceptual, fetal, and early infant phases of life and the subsequent development of metabolic disorders in later life. In particular, the impact of poor maternal nutrition on susceptibility to later life metabolic disease in offspring is now well documented. Several studies have now shown, at least in experimental animal models, that some components of the metabolic syndrome, induced as a consequence of developmental programming, are potentially reversible by nutritional or targeted therapeutic interventions during windows of developmental plasticity. This review will focus on critical windows of development and possible therapeutic avenues that may reduce metabolic and obesogenic risk following an adverse early life environment.

Keywords: developmental programming, obesity, metabolic syndrome, maternal nutrition, leptin, insulin resistance, animal models

Abbreviations: ARH, arcuate nucleus of the hypothalamus; BBB, blood brain barrier; DEX, dexamethasone; Fas, fatty acid synthase; Fk-1, vascular endothelial growth factor receptor 2; GH, growth hormone; GLP-1, glucagon-like peptide 1; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IUGR, intrauterine growth restriction; MLP, maternal low protein; PARs, predictive adaptive responses; PDX1, pancreatic and duodenal homeobox 1; PGC-1, peroxisome-proliferator-activated receptor-gamma co-activator-1; PPAR, peroxisome proliferator-activated receptor; SGA, small for gestational age; SL, small litter; SOCS-3, suppressor of cytokine signaling 3; STAT-3, signal transducer and activator of transcription 3; UN, undernutrition; VAT, visceral adipose tissue; VEGF, vascular endothelial growth factor; WAT, white adipose tissue.

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Abbreviations: ARH, arcuate nucleus of the hypothalamus; BBB, blood brain barrier; DEX, dexamethasone; Fas, fatty acid synthase; Fk-1, vascular endothelial growth factor receptor 2; GH, growth hormone; GLP-1, glucagon-like peptide 1; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IUGR, intrauterine growth restriction; MLP, maternal low protein; PARs, predictive adaptive responses; PDX1, pancreatic and duodenal homeobox 1; PGC-1, peroxisome-proliferator-activated receptor-gamma co-activator-1; PPAR, peroxisome proliferator-activated receptor; SGA, small for gestational age; SL, small litter; SOCS-3, suppressor of cytokine signaling 3; STAT-3, signal transducer and activator of transcription 3; UN, undernutrition; VAT, visceral adipose tissue; VEGF, vascular endothelial growth factor; WAT, white adipose tissue.

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resistance, and dyslipidemia in later life (Barker et al., 1989, 1990; Osmond et al., 1990). From these initial observations, the importance of maternal nutrition and, in particular the effect of poor maternal nutrition on birth weight and adult disease risk was addressed in human studies of famine exposure. The most widely reported of these being the Dutch Hunger Winter of 1944–1945 (Ravelli et al., 1976, 1999; Roseboom et al., 1999, 2001); demonstrating that the timing of the exposure was a major determinant in phenotypic outcomes. Whereas famine exposure during early gestation was associated with adult hypertension (Ravelli et al., 1976), reduced maternal famine during late gestation was associated with an increased adult adiposity and glucose intolerance (Law et al., 1992; Ravelli et al., 1999). Famine exposure late in pregnancy led to a greater impairment of glucose tolerance than during early or mid-gestation. The rate of obesity was higher in men exposed in the first half of gestation and lower in men exposed in the last trimester of gestation as compared to non-exposed men. These relationships however do not speak to causation and other reports of prenatal famine exposure have yielded contradictory results. Retrospective studies investigating offspring exposed to famine during the siege of Leningrad did not show any relationship between birthweight and adult metabolic sequelae (Stanner and Yudkin, 2001). The disparity between the Dutch and the Leningrad studies may be explained using the PARs framework. Following the short period of the Dutch Winter Hunger, food supply was returned to normal levels and maternal nutrition restored; thus in many pregnancies fetuses would have been acutely “starved” in utero and subsequently well nourished in postnatal life. This may represent a circumstance that the PARs hypothesis may work: describe as a nutritional mismatch between the intrauterine and postnatal – the actual and the predicted – environments. Conversely, in the Leningrad cohort, maternal nutritional status was poor both before and after pregnancy and thus one can speculate that any fetal adaptations may have been appropriate for the predicted postnatal environment.

In historically undernourished, recently urbanized populations such as India, where low birth weight individuals are exposed to a high-fat Western diet, the incidence of obesity and type 2 diabetes is reaching epidemic proportions (Yajnik, 2000). Work by Yajnik and colleagues have shown that although Indian babies are born low birth weight, they exhibit increased visceral adiposity (Yajnik, 2000). This is consistent with other studies, that have demonstrated in small babies a disproportionate abdominal fat mass during adult life, despite a lower body mass index (BMI; McMillen et al., 2005). Although there is considerable debate regarding whether accelerated postnatal growth after fetal growth restriction (catch-up growth) in early postnatal life is beneficial or not, most studies suggested that postnatal “catch-up” growth is associated with adverse outcomes in later life (Eriksson et al., 1999; Ong et al., 2000). Interestingly, work by Parsons et al. (2001) found that men with a lower birth weight, who then exhibited catch-up growth to achieve a greater proportion of their adult height by age 7, had a risk of obesity comparable to that of men with higher birth weights. Work by Eriksson et al. (2003) has demonstrated that ponderal index at birth was a reliable predictor of later obesity and also found that an early adiposity rebound in babies born of low birth weight was associated with obesity in adult life.

DEVELOPMENTAL PROGRAMMING OF OBESITY – EVIDENCE FROM ANIMAL STUDIES

Animal models have been extensively used to provide empirical data to support the developmental origins of health and disease (DOHaD) hypothesis and essential to the search for the mechanistic links between early life influences and disease risk in later life. Although epidemiological data suggest that developmental programming occurs within the normal range of birth size (Barker, 2007a,b), most experimental models tend to significantly restrict fetal growth; in the past there was an assumption that those insults that impair fetal growth are likely to be those that trigger developmental programming. Several approaches have been developed to induce early growth restriction in animals in an attempt to elucidate the relationship between growth restriction and adult disease risk, potentially providing a framework for investigating underlying mechanisms. However, intrauterine growth restriction (IUGR) is not essential to developmental programming, but is merely a surrogate for evidence that fetal development may have been affected. Fetal growth restriction is either present or absent in experimental studies depending on the insult used and the insult; impaired adult metabolic function is consistent regardless of the presence or absence of growth restriction. In the rat, obesity and metabolic disorders have been induced in offspring by maternal global undernutrition (Woodall et al., 1996a; Vickers et al., 2000, 2001, 2002; McArdle et al., 2006), maternal protein restriction (Langley-Evans et al., 1999), maternal uterine artery ligation (Rajakumar et al., 1998; Simmons et al., 2001), maternal synthetic glucocorticoid treatment (Nyirenda et al., 1998), maternal anemia (Lewis et al., 2001), or prenatal cytokine exposure (Dahlgren et al., 2001).

MATERNAL UNDERNUTRITION

The early work of Barker and colleagues highlighted fetal nutrition as the primary factor driving the developmental origins of adult disease. Within the laboratory, fetal undernutrition is most commonly achieved through maternal dietary restriction during pregnancy. At present, rodent models investigating the mechanistic links between maternal undernutrition and adult disease risk generally utilize one of two dietary protocols: global undernutrition or iso-caloric low protein diets. The maternal low protein (MLP) diet during pregnancy and lactation is one of the most extensively utilized models of nutritional programming (Snoeck et al., 1990; Langley and Jackson, 1994; Desai et al., 1996; Ozzanne et al., 1999; Petry et al., 2001). In this model, pregnant rats are fed ad libitum a low protein diet containing 5–8% (w/w) protein (casein), generally a little under half the protein content but equivalent in energy of a control diet containing 18–20% (w/w) protein (Snoeck et al., 1990; Langley-Evans, 2000). Offspring from protein-restricted mothers are 15–20% lighter than controls at birth (Desai et al., 1996). Maintenance of a MLP diet during the period of lactation increases this weight difference and permanently limits later growth. If MLP offspring are cross-fostered at birth to lactating mothers fed a control diet, they exhibit rapid catch-up growth (Desai et al., 1996). This catch-up growth appears to have a detrimental effect on life span, which results in premature death that is associated with accelerated loss of kidney telomeric DNA (Jennings et al., 1999).

The experimental observations made in the MLP diet model point toward a number of potential mechanisms that may underlie...
the pathogenesis of obesity and diabetes in these offspring; including both structural and functional changes to a number of organ and endocrine systems. Gene ontogeny analysis of visceral adipose tissue (VAT) demonstrated a global up-regulation of genes involved in carbohydrate, lipid, and protein metabolism in offspring of MLP animals; indicative of dynamic changes in the transcriptional profile of key metabolic genes. These observations are limited by the fact that a MLP diet is isocaloric compared to the normal protein control diet and therefore by default contains an increased fat and carbohydrate content because of passive over-consumption on the MLP diet to reach protein targets (Sorensen et al., 2008).

Global maternal undernutrition models have been developed with a number of different levels of undernutrition during different periods of pregnancy. In the rat, moderate nutritional restriction (70% of normal intake) in the first 18 days of pregnancy resulted in offspring with significant IUGR, but offspring body weight catches up to that of controls by postnatal day 20 (Ozaki et al., 2000). These offspring displayed characteristics of insulin and leptin resistance. These abnormalities increase with age and are most pronounced in male offspring (Ozaki et al., 2000) and the postnatal phenotype is markedly exacerbated when the offspring are fed a high-fat diet post-weaning. Consistent with the MLP model, transcriptional profiling in the UN model demonstrated that maternal undernutrition induced metabolic programming, favoring fat as an energy source and resulted in mitochondrial dysfunction affecting postnatal hepatic function and subsequently, via the resultant metabolic changes in other organs, leads to the evolution of a phenotype similar to that of the metabolic syndrome (Morris et al., 2009).

In addition to caloric and protein restriction models, specific micronutrient deficiencies have also been investigated. The effect of maternal iron deficiency results in several features of the metabolic syndrome in offspring (Lewis et al., 2001, 2002). Work by Gambling et al. (2003) has highlighted that the timing of iron supplementation is critical in reversing the effects of maternal anemia on the developing fetus and postnatal sequelae in offspring. These data correlate well with human studies showing that iron supplementation during pregnancy leads to a higher mean birth weight and reduced incidence of low birth weight infants (Cogswell et al., 2003). Maternal chromium restriction significantly increased body weight and fat percentage, especially central adiposity, in both male and female rat offspring (Padmavathi et al., 2010a, b). Maternal magnesium restriction can predispose rat pups to insulin resistance and glucose intolerance (Venu et al., 2005). Maternal total vitamin restriction increases body fat content but not insulin resistance in rat offspring up to 6 months of age (Venu et al., 2004). Conversely, high multivitamin intake during pregnancy has been shown to program the male offspring who go on to demonstrate characteristics of the metabolic syndrome in adulthood, possibly through its effects on central mechanisms of food intake control (Szeto et al., 2008).

MATERNAL NUTRITION EXCESS

Epidemiological studies have demonstrated that fetal growth restriction correlates with adult disease, implying that fetal nutritional deprivation is a strong stimulus for programming (Armitage et al., 2005). As such, experimental animal models were developed using controlled maternal caloric intake or protein or macronutrient deficiency. However, in many developed societies, maternal and postnatal caloric intake is either insufficient or excessive. A number of studies have shown that the relationship between birth weight and obesity risk is not a simple inverse linear association but is a U-shaped curve; a higher prevalence of adult obesity occurs in individuals in low or high birth weight categories and this has often been attributed to both low and high planes of maternal nutrition (Armitage et al., 2005; McMillen et al., 2005; Samuelson et al., 2008; Howie et al., 2009; Morris and Chen, 2009). Indeed in pregnancies which have been complicated by maternal diabetes, gestational diabetes, or impaired glucose tolerance, offspring have been shown to be at greater risk of developing obesity (Holemans et al., 2004). There is a similarity in offspring phenotypes derived from both ends of the maternal nutritional spectrum; offspring of both under- and over-nourished mothers display common metabolic derangements including obesity and insulin resistance. The components of the maternal diabetic and/or obeseogenic environment that mediate the detrimental effects on hypothalamic appetite control and consequently offspring health are not clearly established. A common phenotypic outcome across a range of programming models is resistance to leptin in adulthood resulting in a loss of inhibition of food intake (Ozanne, 2011). However, there has been less focus on whether leptin resistance develops during the very early stages of life, at a time when leptin itself has no effect on food intake, but may play a mechanistic role in metabolic programming (Mistry et al., 1999; Glavas et al., 2010).

Maternal obesity in the rat has been shown to reprogram hypothalamic appetite signaling pathways and leptin signaling at birth (Morris and Chen, 2009). These hypothalamic changes, together with lower leptin levels in offspring of obese mothers may contribute to the lower expression of key appetite regulators at birth, suggestive of altered fetal neuronal development in
response to maternal obesity. These alterations may contribute to eating disorders later in life. Activation of STAT-3 signifies leptin sensitivity and recent work in female rats by Shin et al. (2012) has shown that at birth, despite IUGR offspring being hypo leptinemic, hypothalamic leptin signaling was activated as seen by enhanced STAT-3. Further, it has been shown that offspring of high-fat fed (HF) dams exhibit an alteration in hypothalamic leptin-dependent STAT-3 phosphorylation, independent of the level of post-weaning nutrition (Ferezou-Viala et al., 2007).

It is notable that evidence for the programming of obesity and several other features of the metabolic syndrome from both nutrient restriction (caloric, protein, iron) and fat-feeding studies, suggest a possible commonality of mechanism (Armitage et al., 2004).

CRITICAL WINDOWS FOR INTERVENTION

Until recently, developmental programming was seen to be an irreversible change in developmental trajectory, the consequences of which had to be managed, e.g., obesity and Type 2 diabetes. Few studies have addressed the possibility of reversibility or prevention of the postnatal programmed phenotype.

The adipokine leptin has received significant interest as a potential programming factor; alterations in the profile of leptin in early life are associated with altered susceptibility to obesity and metabolic disorders in adulthood. Maintenance of a critical leptin concentration during early development facilitates the normal maturation of tissues and signaling pathways involved in metabolic homeostasis. A period of relative hypo- or hyperleptinemia during this window of development will induce some of the metabolic adaptations which underlie the developmental programming of appetite control and metabolic function.

It has now been shown in a range of animal models and a number of species that perturbations in the profile of leptin in early life are associated with altered susceptibility to obesity and metabolic disorders in adulthood. It has been proposed that deficiencies in leptin during critical windows of development could lead to a hardwiring of obesity (Horvath and Bruning, 2006). Manipulation of early life leptin concentrations in animal models using exogenous leptin and/or leptin antagonists have clearly shown a role for leptin in determining metabolic fate in later life (Vickers, 2007; Attig et al., 2008a). The source of leptin during this critical developmental window may be varied and likely includes maternal transplacental transfer of leptin, endogenous fetal leptin but may also include milk leptin during lactation. Leptin in maternal milk is likely an important factor in the maturation of organ systems and feeding pathways in the neonate. Breast milk leptin correlates positively with maternal plasma leptin concentrations although the concentration in breast milk is significantly lower than that found in maternal plasma (Ucarr et al., 2000). Breast fed infants have higher serum leptin levels compared to formula fed infants; and formula fed infants may be at an increased risk of developing obesity (Savino et al., 2009). Experimentally, oral intake of physiological doses of leptin during lactation in rats has been shown to prevent obesity in later life (Pico et al., 2007) supporting the hypothesis that milk leptin plays a favorable role in developmental programming.

In adult mammals, leptin acts on the brain to reduce food intake by regulating the activity of neurons in the arcuate nucleus (ARH). Bouret et al. have shown that neural projection pathways from the ARH are permanently disrupted in leptin-deficient ob/ob mice (Bouret and Simerly, 2004; Bouret et al., 2004a). Treatment of ob/ob neonates with exogenous leptin rescues the development of ARH projections, and leptin promotes neurite outgrowth from ARH neurons in vitro. It is well established that small for gestational age (SGA) neonates have diminished cord blood leptin concentrations and as children are hypoleptinemic (Iniguez et al., 2004). These children go on to develop obesity and leptin resistance in adult life; which can be mimicked experimentally in the rat (Vickers et al., 2000). Thus, perinatal nutritional perturbations that alter leptin levels may have enduring consequences for the formation and function of neuronal circuits that regulate food intake and body weight (Elmqquist et al., 1998; Bouret and Simerly, 2004; Bouret et al., 2004a,b). Leptin treatment to neonatal female rats born to undernourished mothers prevented the development of metabolic compromise in adulthood (Vickers et al., 2005). The complete normalization of the programmed metabolic phenotype by neonatal leptin treatment implied that leptin can reverse prenatal adaptations resulting from fetal undernutrition. Moreover, the effects were specific to low birth weight animals, whilst leptin had no effect in animals born to control mothers. Whether this leptin effect acts centrally or peripherally is unclear – one possibility is that the window of developmental plasticity is still open and the high leptin levels reverse the c McCluskey effects of prenatal undernutrition (Gluckman et al., 2007). Consistent with this, neonatal leptin treatment to IUGR piglets partially reversed the growth restricted phenotype by correcting growth rate, body composition, and development of several organs involved in metabolic regulation (Attig et al., 2008b). It has recently been reported that intranasal leptin reduces appetite and induces weight loss in rats with diet-induced obesity (Schulz et al., 2011) but this approach has yet to be utilized in the context of developmental programming. It must be noted however that in rodents the projections of neurons in the ARH to the paraventricular nucleus, dorsomedial nuclei, and lateral hypothalamic area, areas that also influence appetite regulation, develop postnatally and correspond to a period of late gestation in the sheep and human (Muhlhauser et al., 2008).

The use of a leptin antagonist in early life mimics the effects of maternal caloric restriction. Attig et al. (2008a) administered a specific ObRb antagonist that blocked leptin’s action in the neonatal rat, later predisposing offspring to leptin resistance and increased body weight gain when fed a high energy diet postnatally. Further work using leptin antagonists has shown that postnatal leptin is necessary for maturation of numerous organs in the newborn rat (Attig et al., 2011) with leptin antagonism resulting in aberrant pancreas, kidney, and ovarian development. We have shown that administration of pegylated leptin antagonist (increased half life compared to standard leptin antagonist) to rat neonates can also modify their responsiveness to diet-induced obesity in adult life but this is dependent upon prior maternal nutrition and post-weaning diet (Beltrand et al., 2012).

Pregnancy is characterized by a state of leptin resistance comprising an adaptive response that facilitates maternal energy
storage in preparation for the high metabolic demands of pregnancy and subsequent lactation (Ladyman et al., 2010). However, despite resistance to the central anorectic effects of leptin, it has also been reported that maternal leptin treatment to dams fed a low protein diet can prevent the adverse metabolic programming induced as a consequence of protein malnutrition (Stocker et al., 2004). Increased exposure of the fetus to maternally derived glucocorticoids has been established as a mechanism regulating metabolic demise in offspring; indeed, leptin administration in models of low maternal protein intake resulted in the normalization of placental 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2) levels (Stocker et al., 2004), resulting in a potential reduction in fetal exposure to maternal glucocorticoids. Of note, leptin administration to normally nourished dams also conferred protection against diet-induced obesity in offspring (Stocker et al., 2007) suggesting that effects may be independent of maternal dietary intake.

Maternal supplementation with methyl donors has also been reported to modify some deleterious effects of programming on offspring. Maternal folic acid supplementation in the rat following protein restriction prevents epigenetic modification of hepatic gene expression in the offspring including PPAR-α and -γ (Lillycrop et al., 2005). Whether the reversal of hypomethylation in this setting improves metabolic outcome in offspring is not known. A recent study using a similar model reported that maternal protein and folic acid intake during gestation does not program leptin transcription or circulating leptin concentrations in rat progeny (Chmurzynska et al., 2011). Maternal glycine (Jackson et al., 2002) and choline (Bai et al., 2012) supplementation can also prevent MLP-induced hypertension in offspring in adulthood although the mechanisms are not yet fully understood.

The role of catch-up growth on development of the adult obese phenotype is still under debate. Work by Plagemann (2006) argued that it may not be fetal undernutrition and low birth weight per se that predisposes to adult-onset obesity; rather it is the overfeeding of underweight newborns that may substantially contribute to their long-term disease risk. When IUGR offspring are permitted rapid catch-up growth by nutrient availability, these offspring will demonstrate evidence of increased body weight and body fat, and leptin resistance as adults. Conversely, if catch-up growth is delayed by postnatal nutrient restriction, these offspring exhibit normal body weight, body fat, and plasma leptin levels as adults (Desai et al., 2005; Howie et al., 2011). However, in contrast to the observations by Lopez et al. (2007) have reported that perinatal overfeeding (using small litters, SL) does not induce alterations in either the anorectic response to central leptin administration or expression of leptin receptors and neuropeptides in adult rats. The leptin resistance to peripheral leptin in adult SL rats may be related to impaired leptin transport across the BBB. This transport mechanism could be triglyceride-mediated as reported by Banks et al. (2004). SL offspring have significantly elevated plasma triglycerides as adults (Plagemann et al., 1999). As triglycerides inhibit the transport of leptin across the BBB they could be a key factor in the onset of the peripheral leptin resistance, which is a hallmark of obesity.

Although leptin treatment has beneficial effects in ameliorating metabolic disorders that result from developmental programming, leptin treatment to offspring of normal pregnancies may have adverse long-term metabolic effects which may relate to alterations in the amplification and timing of the neonatal leptin surge (Itoh et al., 2011). Itoh et al. (2011) showed that giving neonatal leptin treatment to control mice lead to impaired glucose tolerance in adult offspring. In the rat, the effects of exogenous leptin in male offspring are directionally dependent upon maternal nutritional status (Vickers et al., 2008). A study by Yura et al. (2005) showed that treatment of male control offspring with leptin in the neonatal period resulted in a modest increase in the risk for obesity as compared to saline treated controls. This concurs with a previous study of leptin given to normal neonatal rats which also showed programmed hyperleptinemia and hyperinsulinemia in adulthood, and lead to leptin resistance by reducing the expression of the hypothalamic leptin receptor (Toste et al., 2006). However, work by Pico et al. (2011) has shown that oral administration of leptin at physiological doses to rat neonates had later positive metabolic effects that prevented development of overweight and obesity. Thus, studies in rodents suggest that early leptin treatment may program either a later lean or obese phenotype. These differences in metabolic outcomes across studies may relate in part to the dosage used, source of leptin, gender, and method of administration (Pico et al., 2011).

Interventional studies in humans demonstrate that leptin administration in subjects with congenital complete leptin deficiency or subjects with partial leptin deficiency (subjects with lipodystrophy, congenital or related to HIV infection, and women with hypothalamic amenorrhea) reverses the energy homeostasis and neuroendocrine and metabolic abnormalities associated with these conditions (Chan et al., 2011; Mantzoros et al., 2011). In contrast, leptin’s effects are largely absent in the obese hyperleptinemic state, primarily as a result of leptin resistance or tolerance (Mantzoros et al., 2011). However, failure of leptin in the clinic as an intervention in obesity, and withdrawal of numerous anti-obesity drugs from clinical use, e.g., sibutramine, has stimulated new approaches in the development of anti-obesity drugs. These efforts are focused on utilizing leptin-related synthetic peptides as leptin receptor antagonists or leptin-related synthetic peptide analogs or mimetics (Grasso, 2011).

The current animal data fit with the PAR hypothesis proposed by Gluckman and Hanson (2004a). Following the PAR hypothesis, in response to a given in utero or early postnatal nutritional plane (either high or low), cellular processes are invoked to cope with the predicted environment. This hypothesis suggests that disease only manifests when the actual nutritional environment diverges from that which was predicted. Since the development of critical pathways involved in energy homeostasis in rodents continue well into the postnatal period, it can be modified by both pre- and postnatal environmental manipulation (e.g., prevention of catch-up growth) and thus obesity can be potentiated, reversed, or attenuated postnatally. It is likely that similar principles hold true for humans although the timing of pathway development occurs earlier than in rodents.

Work in the rodent has shown that treatment with both growth hormone (GH) and insulin-like growth factor (IGF)-1 can resolve several aspects of the metabolic phenotype in developmentally programmed offspring. In a model of maternal undernutrition to induce fetal growth restriction, offspring fed either a control
or high-fat diet postnatally demonstrated hypertension, obesity, hyperphagia, hyperinsulinemia, and hyperleptinemia; the effects of which were markedly amplified in the presence of a postnatal high-fat diet (Vickers et al., 2000). Adult treatment with GH normalized systolic blood pressure and reduced fat mass but simultaneously exacerbated the hyperinsulinemia as a result of the diabetogenic actions of GH (Vickers et al., 2002). IGF-I infusion in adult females led to a complete normalization of adiposity, appetite, fasting plasma insulin, and leptin concentrations in developmentally programmed offspring (Vickers et al., 2001). These studies highlight the role of the somatotropic axis in metabolic disturbances although the longer term efficacy of such treatment regimes is not known. Trials with GH in small for gestation age children have shown a normalization in systolic blood pressure which was maintained for the 6 year duration of treatment (Holemans et al., 2004).

Epidemiological and experimental studies have shown that early life adversity leads to glucose intolerance and an enhanced risk for type 2 diabetes in offspring. Work by Simmons et al. has shown that treatment of neonatal rats with the glucagon-like peptide (GLP)-1 analog Exendin-4 (EX-4) reversed the adverse consequences of being born growth restricted and prevented the development of diabetes in adulthood (Stoffers et al., 2003; Park et al., 2008; Raab et al., 2009). Neonatal EX-4 prevented the progressive reduction in insulin-producing β-cell mass that was observed in IUGR rats over time and restored to normal levels the expression of pancreatic duodenal homeobox (PDX), a critical regulator of pancreas development and islet differentiation. It has recently also been shown that EX-4 increases histone acetylation activity and reverses epigenetic modifications that silence PDX1 in the intrauterine growth retarded rat (Pinney et al., 2011). Although adiposity was not examined in this study, GLPs are known to modify food intake, increase satiety, delay gastric emptying, and suppress glucagon release and therefore further studies are needed to explore these signaling pathways.

The role of possible direct nutritional interventions has been highlighted in a study by Wyrvoll et al. (2006). Pregnant rats were treated with synthetic glucocorticoids (Dexamethasone; DEX) from embryonic day 13 to term; offspring were cross-fostered to mothers on either a standard diet or a diet high in omega-3 fatty acids, pups remained on these diets post-weaning. Maternal DEX reduced birthweight and delayed the onset of puberty in offspring and elicited hyperleptinemia and increased fat mass in offspring by 6 months of age. These effects were ameliorated by a high omega-3 diet, demonstrating that direct manipulation of postnatal diet, other than that associated with postnatal caloric restriction, can limit adverse outcomes of maternal glucocorticoid administration.

The use of taurine supplementation either during pregnancy and/or lactation has long been known to be efficacious in preventing pancreatic β-cell dysregulation and restoring normal proliferation and apoptosis of rat pancreatic islets following a MLP diet (Boujendar et al., 2002). The mechanism by which taurine regulates the apoptotic rate of endocrine cells involving IGF-II and Fas signaling pathways (Boujendar et al., 2002) are not completely understood. Maternal LP diet leads to up-regulation of VEGF and Flk-1, associated with the lower fetal islet vascularization; maternal supplementation with taurine prevented such damage and may have a potential role in islet vasculogenesis (Boujendar et al., 2003). Recent findings suggest that a maternal LP diet causes long-lasting mitochondrial changes, which may contribute to the development of type 2 diabetes later in life, and that a lack of taurine may be a key causative factor for these dysfunctional mitochondrial changes (Lee et al., 2011); it may be through this route that taurine serves also to regulate DNA synthesis.

**Physical activity and exercise as an intervention**

Most of the investigations of developmental programming have focused on metabolic and cardiovascular disorders and little attention has been paid to indices such as physical activity and effects of exercise. The studies that have examined activity have primarily focused on stress and anxiety behaviors, not direct physical activity per se. Clinical and epidemiological studies of changes in physical activity resulting from a poor fetal environment are limited and it appears that two main factors have contributed to this limitation; first, lifestyle influences obscure linkages between metabolic predisposition and maturity onset behavioral patterns; and second, the need to use subject diaries, that describe perceived activity levels in clinical cohorts have inherent errors in the precision of reporting. Epidemiological studies of survivors of the Dutch famine of 1944–1945 have shown that prenatal exposure to famine resulted in not only altered food preferences toward unhealthy diets but also showed trends toward reduced physical activity in adulthood (Lussana et al., 2008).

A number of recent reports in animal models suggest that several aspects of physical activity are determined by factors operating in early life. Studies in rats, using a variety of maternal manipulations, have shown that voluntary locomotor behavior is significantly reduced in offspring in postnatal life. Maternal undernutrition in the rat resulted in reduced physical activity before the development of an obese phenotype with programming-induced alterations in physical activity levels being reported as early as postnatal day 35 (pubertal age; Vickers et al., 2003). Similarly, a maternal LP diet has been shown to result in a significant reduction in voluntary locomotor activity in offspring (Bellinger et al., 2006; Langley-Evans, 2007). The window of LP exposure is critical in determining effects on locomotor activity in offspring and sometimes manifests in a gender-specific manner. In the mouse, preconceptional LP feeding during one female ovulatory cycle prior to natural mating led to a reduction in locomotor activity in offspring (Watkins et al., 2008). In the mouse, maternal diet-induced obesity leads to a reduction in physical activity in offspring (Samuelsson et al., 2008).

Despite growing evidence for the developmental programming of sedentary behavior, little is known about the effect of exercise as a maternal intervention. Any programming-induced compromise in the control of resting metabolism, either through deficits in oxidative fiber number or intramuscular energy sensing could act as the initial trigger for increased susceptibility to developing the metabolic syndrome (Nassis et al., 2005; Gardner and Rhodes, 2009). It has been suggested that as little as regular low-moderate intensity exercise, independent of any reduction in bodyweight, is needed to prevent the development of metabolic disorders in offspring (e.g., via activation of muscle-specific PGC-1 for example; Arany et al., 2007; Gardner and Rhodes, 2009). Using a rat
model, Miles et al. (2009a) showed prenatally undernourished rats increased their preference for wheel running versus lever pressing for food in a choice task. Further, despite a predisposition to develop obesity under sedentary conditions, obesity development was prevented in IUGR offspring when exercise was available (Miles et al., 2009b).

ROLE OF EPIGENETICS

Epigenetics is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence (Henikoff and Matzke, 1997). Because of their inherent malleability, epigenetic mechanisms are susceptible to environmental influences and this environmental susceptibility is expected to be enhanced during early development. As such, this process is emerging as an important regulator of the changes in gene expression undergone by adipose tissue during obesity. Importantly, epigenetic marks may be reprogrammed in response to both stochastic and environmental stimuli, such as changes in diet and the in utero environment (Jaenisch and Bird, 2003).

Within tissues and organs that control metabolic homeostasis, a range of phenotypes can be induced by sustained changes in maternal diet via modulation of genes that control DNA methylation and by histone acetylation, which suggests epigenetic programming (Sebert et al., 2011). Leptin’s 3-kb promoter region is embedded within a CpG island and contains many putative binding sites for known transcription factors, such as Sp-1 sites, cAMP response element, glucocorticoid response element, and a functional CCAAT/enhancer binding protein (C/EBP-α) site which contains a CG dinucleotide and is sufficient for tissue-specific gene expression (Gong et al., 1996; Stoger, 2006). It has been shown that leptin’s promoter is subject to epigenetic programming and leptin’s expression can be modulated by DNA methylation.

Such potential mechanisms underlying epigenetic modification of tissue function resulting in a predisposition to altered leptin and insulin signaling are discussed by Holness and Sugden (2006). For example, activation of the leptin receptor induced expression of suppressor of cytokine signaling-3 (SOCS-3). This protein inhibits leptin signal transduction and also potently inhibits insulin receptor signaling. Altered SOCS-3 methylation may therefore have lasting effects on the leptin–insulin feedback loop; the adiposinsular axis (Kieffer and Habener, 2000). Impaired glucose tolerance during pregnancy is associated with altered leptin gene DNA methylation with potential functional impacts (Bouchard et al., 2010). In patients analyzed before and after bariatric surgery-induced weight loss, a decrease in WAT leptin expression (about 50%) did not correspond to changes in promoter methylation density. Thus, methylation density in the leptin promoter may constitute one control level for cell-type specific leptin expression, whereas weight loss induced changes in leptin expression does not appear to be methylation-dependent (Marchi et al., 2011).

Fixed genomic variation explains only a small proportion of the risk of adiposity. In animal models, maternal diet alters offspring body composition, accompanied by epigenetic changes in metabolic control genes. Little is known about whether such processes operate in humans (Godfrey et al., 2011). Recent work has shown the utility for perinatal epigenetic analysis in identifying individual vulnerability to later obesity and metabolic disease (Godfrey et al., 2011). In this study, epigenetic gene promoter methylation at birth was associated with adiposity in children. Identification of in vivo methylation of the leptin promoter provides a molecular entry point to study the timing, factors, and conditions that lead to tissue-specific methylation patterns of gene promoters (Stoger, 2006). Overall therefore, the application of epigenomic approaches and the determination of targets (e.g., imprinted or non-imprinted genes and methylation sites) for early life effects on epigenetic gene regulation are exciting and important new areas of investigation (McMillen and Robinson, 2005).

DISCUSSION

Numerous epidemiological studies have described a relationship between an adverse prenatal environment and the development of metabolic disease and obesity in later life. Both clinical/epidemiological studies and experimental research have clearly shown that the propensity to develop increased adiposity in later life is increased when early life development has been adversely affected. The pathogenesis is not based on genetic defects but on altered genetic expression as a consequence of an adaptation to environmental changes during early life development. However, little is known about the interaction between the pre-and postnatal nutritional environment on either amplification or resolution of the programming phenotype depending on the degree of nutritional match/mismatch. Thus, experiments to examine the PARs hypothesis are required in conjunction with transgenerational work to further the DOHaD paradigm.

The molecular mechanisms underlying developmental programming have only recently begun to be investigated. Epigenetics has now become a mechanism that is fundamental to research into DOHaD. The two most studied epigenetic mechanisms identified in the adaptive developmental programming of metabolic disorders are DNA methylation and histone modifications. Availability of dietary methyl donors and cofactors during a critical window of fetal development may influence DNA methylation patterns. Thus, early methyl donor malnutrition (i.e., excess nutrition or undernutrition) could effectively lead to premature epigenetic aging (changes in age-associated DNA methylation patterns) and thereby confer an enhanced susceptibility to adult disease in later life (Waterland and Jirtle, 2004).

Developmental programming research offers a novel approach to investigate the mechanistic basis of obesity and related metabolic disorders which in human populations predominantly arises from environmental factors and lifestyle choices. It is notable that the variety of different insults in early life (caloric, protein, iron, fat-fed) produce the same detrimental consequences in adult life, suggestive of a common mechanism underlying the developmental early life programming of adult disease. An increasing number of studies are now investigating avenues to reverse or ameliorate the detrimental metabolic effects associated with developmental programming. Of note, intervention studies with leptin in the early period of developmental plasticity have shown promise in reversing the programmed metabolic phenotype; however translation from small animal models to the human setting is difficult as the critical windows observed in the rodent represent a late period...
of in utero development in larger species. However, the emerging focus on studies aimed at reversing the programmed phenotype offers an exciting potential for new advances in our understanding of critical determinants and mechanisms for human obesity and metabolic disorders.

ACKNOWLEDGMENTS
The authors acknowledge funding support from the Health Research Council of New Zealand, the National Research Centre for Growth and Development, the Kelliher Charitable Trust, and the Ministry of Science and Innovation.

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Vickers and Sloboda

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.


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Obesity and Metabolic Syndrome and Functional and Structural Brain Impairments in Adolescence

WHAT’S KNOWN ON THIS SUBJECT: Despite the dramatic rise in prevalence of metabolic syndrome (MetS) among children and adolescents, and that MetS is associated with cognitive and brain impairments among adults, no data on the impact of MetS on the brain exist in children.

WHAT THIS STUDY ADDS: It provides the first data on the impact of MetS on brain in adolescence. We show reductions in cognitive function and brain structural integrity in nondiabetic adolescents with MetS, thus suggesting that even pre-clinical metabolic illness may give rise to brain complications.

abstract

BACKGROUND: The prevalence of metabolic syndrome (MetS) parallels the rise in childhood obesity. MetS is associated with neurocognitive impairments in adults, but this is thought to be a long-term effect of poor metabolism. It would be important to ascertain whether these brain complications are also present among adolescents with MetS, a group without clinically manifest vascular disease and relatively short duration of poor metabolism.

METHODS: Forty-nine adolescents with and 62 without MetS, matched on age, socioeconomic status, school grade, gender, and ethnicity, received endocrine, MRI, and neuropsychological evaluations.

RESULTS: Adolescents with MetS showed significantly lower arithmetic, spelling, attention, and mental flexibility and a trend for lower overall intelligence. They also had, in a MetS-dose-related fashion, smaller hippocampal volumes, increased brain cerebrospinal fluid, and reductions of microstructural integrity in major white matter tracts.

CONCLUSIONS: We document lower cognitive performance and reductions in brain structural integrity among adolescents with MetS, thus suggesting that even relatively short-term impairments in metabolism, in the absence of clinically manifest vascular disease, may give rise to brain complications. In view of these alarming results, it is plausible that obesity-associated metabolic disease, short of type 2 diabetes mellitus, may be mechanistically linked to lower the academic and professional potential of adolescents. Although obesity may not be enough to stir clinicians or even parents into action, these results in adolescents strongly argue for an early and comprehensive intervention. We propose that brain function be introduced among the parameters that need to be evaluated when considering early treatment of childhood obesity. Pediatrics 2012;130:e856–e864

AUTHORS: Po Lai Yau, PhD,a Mary Grace Castro, BS,a Adrian Tağani,a Wai Hon Tsui, MS,a and Antonio Convit, MD,b,c

Departments of aPsychiatry and aMedicine, New York University School of Medicine, New York, New York; and cNathan Kline Institute for Psychiatric Research, Orangeburg, New York

KEY WORDS metabolic syndrome, adolescence, obesity, diffusion tensor imaging, brain abnormalities, cognitive performance, hippocampal volumes, fractional anisotropy

ABBREVIATIONS BP—blood pressure
CRP—C-reactive protein
CSF—cerebrospinal fluid
DLPFR—dorsolateral prefrontal region
DVT—Digit Vigilance Test
FA—fractional anisotropy
HDL—high-density lipoprotein
ICV—intracranial vault
IR—insulin resistance
MetS—metabolic syndrome
MPRAGE—magnetization-prepared rapid acquisition gradient echo
QUICKI—quantitative insulin sensitivity check index
T2DM—type 2 diabetes mellitus
VANOVA—voxelwise analysis of covariance
WM—white matter
WRAML—Wide Range Assessment of Memory and Learning
WRAFT—Wide Range Achievement Test

Each author made substantial contributions to this article. Dr Convit designed, performed, and supervised the study; Drs Yau and Convit, W. H. Taui, M. G. Castro, and A. Tağani acquired and analyzed the data; Drs Yau and Convit wrote the article; all authors have seen and approved the final version of the manuscript.

www.pediatrics.org/cgi/doi/10.1542/peds.2012-0324
doi:10.1542/peds.2012-0324

Accepted for publication May 31, 2012

Address correspondence to Antonio Convit, MD, Brain, Obesity, and Diabetes Laboratory (BODyLab), New York University School of Medicine, 145 East 32nd St, 8th Floor; New York, NY 10016.

E-mail: antonio.convit@med.nyu.edu

PEDIATRICS (ISSN Numbers: Print, 0031-4005; Online, 1098-4275).

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FINANCIAL DISCLOSURE: The authors have indicated they have no financial relationships relevant to this article to disclose.

FUNDING: Funded by the National Institutes of Health DK 083537 and, in part, by grant 1UL1RR029893 from the National Center for Research Resources. Funded by the National Institutes of Health (NIH).
As a result of the childhood obesity epidemic, in 2006, the prevalence of metabolic syndrome (MetS) was already 8.6% among all US children and adolescents. MetS in childhood predicts MetS and type 2 diabetes mellitus (T2DM) in adulthood. The MetS is composed of 5 obesity-associated components, namely elevations in fasting glucose levels or insulin resistance (IR) dependent on the definition used, lower high-density lipoprotein (HDL), hypertriglyceridemia, and hypertension in addition to abdominal obesity. MetS among middle-aged and older adults has been associated with cognitive dysfunction. However, to date, no brain data exist in adolescents. MetS in childhood presents a unique opportunity to evaluate whether brain structure and function are affected by metabolic dysregulation of relatively short duration and before the development of hyperglycemia or clinically manifest cardiovascular disease.

We aim to ascertain whether obesity and MetS, in the absence of T2DM, are associated with impairments in brain health. In addition to cognitive performance and measurements of hippocampal, dorsolateral prefrontal region (DLPFR), and overall CSF volumes, we also ascertained WM microstructural integrity by using sensitive diffusion tensor imaging methods.

**METHODS**

**MetS Classification**

There is currently no general consensus to define pediatric MetS. The prevalence of impaired fasting glucose levels is very low in nondiabetic youth, and, therefore, measures of IR may offer higher sensitivity to detect metabolic abnormalities in this age group. The quantitative insulin sensitivity check index (QUICKI) has been validated as a measure of IR in nondiabetic children and adolescents. We chose a QUICKI value ≤0.3500 to indicate IR. We used the ATP III diagnostic criteria for abdominal obesity and hypertension in children, as well as the NHANES adolescent triglycerides cutoff. However, for HDL we used the more stringent value (waist circumference >88 cm [females] and >102 cm [males]) was used if it was lower than the children's cutoff value; (2) reduced HDL, serum HDL levels <50 mg/dL (females) and <40 mg/dL (males); (3) hypertriglyceridemia, serum triglyceride levels >110 mg/dL; (4) hypertension, for those <18 years of age, blood pressure (BP) ≥90th percentile for age, gender, and height; for those of ≥18 years of age, we used adult criteria, BP ≥130 mm Hg, diastolic BP ≥85 mm Hg; or use of antihypertensive medication; and (5) IR, a QUICKI value ≤0.350. An individual has MetS when he/she meets criteria for at least 3/5 of the MetS components.

**Study Participants**

A total of 129 nondiabetic adolescents (14–20 years of age) were screened for participation in a study to examine the brain consequences of MetS. This study was approved by the NYU School of Medicine institutional review board. All of the participants (and if <18 years of age, one of their parents) signed informed consent. Exclusion criteria were a diagnosis of T2DM or other significant medical conditions (other than IR, polycystic ovary disease, dyslipidemia, and hypertension), Tanner stage <4, use of psychoactive medications, a diagnosis of depression, a history of significant learning disability, or pregnancy. Of the 129 adolescents screened, 18 were excluded (9 did not meet entry criteria, 3 had clinical MRI abnormalities, and 6 did not complete the evaluation), resulting in 111 adolescents (49 with and 62 without MetS) included. One adolescent in the MetS group had a fasting glucose of 110 mg/dL (with a hemoglobin A1c level of 5.8%); all others had fasting glucose levels <100 mg/dL. Given our fasting glucose levels and normal hemoglobin A1c levels, it is highly unlikely we included any adolescents with undiagnosed T2DM.

**Cognitive Evaluations**

Cognitive testing was conducted blind to group membership in a standardized fashion ~1 hour from the last meal over two 1.5-hour sessions. We used standard tests, described in detail elsewhere. For overall intellectual functioning we used the Wechsler Abbreviated Scale of Intelligence (WASI), and for academic achievement we used the Wide Range Achievement Test (WRAT). Memory skills were assessed with the Wide Range Assessment of Memory and Learning (WRAML), and executive function was assessed with the Wisconsin Card Sorting Test, Tower of London Test, Controlled Oral Word Association Test, and Trails B Test. Attention was measured with the Digit Vigilance Test (DVT), WRAML Attention-Concentration Index, and Trails A Test, and psychomotor efficiency was tested with the Digit Symbol Substitution Test. The Wechsler Abbreviated Scale of Intelligence, WRAT, and WRAML are adjusted for age; all others test scores are raw scores.

Obstructive sleep apnea is associated with obesity and can affect the brain and cognition. Sleep apnea was
assessed with a 20-item questionnaire.\textsuperscript{17} A diagnosis of depression was exclusionary, but, to adjust for potential subclinical depressive symptoms on cognition, we administered the Beck Depression Inventory (BDI).\textsuperscript{18}

**MR Image Acquisition**

Standardized MR scans were acquired by using identical parameters on the same 1.5 T Siemens Avanto System over 45 minutes. Please refer to Yau et al\textsuperscript{24} for details on the MR sequences.

**Brain Volumetric Assessment**

All brain volumes were determined blind to participants’ identity and diagnosis. We measured the intracranial vault (ICV) on the magnetization-prepared rapid acquisition gradient echo (MPRAGE) image by following the dural and tentorial margins. Overall, CSF volume was determined by using an intensity threshold to identify CSF voxels within the ICV. The volumes of right and left hippocampus were measured and then averaged by using a highly reliable\textsuperscript{19} and postmortem-validated method.\textsuperscript{20} The DLPFR region was outlined also by using a reliable method.\textsuperscript{21} To account for intersubject variability in brain size, measured brain volumes were adjusted (residualized) to the ICV volume by using linear regression.

**Diffusion Tensor Imaging–based WM Microstructural Assessment**

We used fractional anisotropy (FA) to assess WM microstructural integrity. To prepare the FA maps for voxelwise comparisons in Talarach space, we used Automatic Registration Toolbox software,\textsuperscript{22} which is highly rated for image registration quality and accuracy.\textsuperscript{23} First, the skull-stripped structural native MPRAGE image was normalized to the standard Montreal Neurological Institute brain template by using a three-dimensional nonlinear warping algorithm. Second, a rigid-body linear transformation optimized the registration between T2 and MPRAGE by iteratively correcting for subject motion. Third, with a nonlinear two-dimensional warping algorithm, the non–diffusion-weighted $b_0$ image was iteratively warped to correct for spatial distortions inherent in echo planar acquisitions by using the skull-stripped T2 image as a guide. Finally, to reduce interpolation errors, we combined transformation parameters from steps 1 to 3 and applied them to spatially correct and normalize the native FA maps to Talarach space. Please refer to Yau et al\textsuperscript{24} for a more detailed description of these procedures.

**Statistical Analyses**

Before data analysis, we evaluated normality of continuous variables by using the Kolmogorov-Smirnov (for $n \geq 50$) or Shapiro-Wilk (for $n < 50$) test. Normally distributed variables were evaluated by using 2-tailed independent samples $t$ test (effect size expressed by Cohen $d$). For those that were nonnormally distributed, the Mann-Whitney $U$ test (effect size expressed by $r$) was used. C-reactive protein (CRP) levels $>10$ mg/dL may indicate acute inflammation and were excluded casewise from analyses involving CRP. We used a WM mask created from the average of the MPRAGE images of all participants in Talarach space to restrict group FA comparisons to WM. Two-tailed voxelwise analysis of covariance (VancoVA) analysis examined the group differences in WM FA, with age as a covariate. Linear regression models were used to assess the differences in cognitive performance among adolescents who had 0, 1, 2, 3, 4+ (4 or 5) MetS components. Furthermore, stepwise regression analyses were used to understand whether any one or group of MetS components predicted the brain variables that were statistically different between MetS and non-MetS adolescents, after controlling for age and gender. For these analyses, we used the mean arterial BP (1/3 systolic BP $+ 2/3$ × diastolic BP) as a continuous measure of BP rather than the dichotomous classification used for MetS assignment. Extreme scores, $>3$ SDs from the respective group means, were excluded. Descriptive statistics are presented as counts and percentages for categorical variables and as means and SDs (M $\pm$ SD) for continuous variables. For results of the regression analyses, the unstandardized $\beta$-values ($\beta$), proportion of variance explained by the independent variable ($r^2$), and $F$-ratio are presented in parentheses in the text; the $\delta$ ($\Delta$) represents changes in the statistical values for the current step after accounting for covariates in the previous steps.

**RESULTS**

**Demographic and Endocrine Data**

Groups did not differ significantly on age, socioeconomic status, school grade, gender, or ethnicity (Table 1). As expected, adolescents with MetS had significantly larger waist circumference and BMI, higher degree of IR, worse lipid profile, and poorer BP control (only 1 participant was receiving an antihypertensive medication). Adolescents with MetS also had significant elevations in plasma acute-phase reactant markers of inflammation (CRP and fibrinogen). The groups did not differ significantly on self-reported ratings of obstructive sleep apnea, or subclinical scores of depressive symptoms.

**Cognitive Performance Results**

Adolescents with MetS had lower academic achievement (spelling and arithmetic) and tended to have a lower IQ (Table 2). They also scored lower on measures of attention and mental
TABLE 1 Demographic and Endocrine Data

<table>
<thead>
<tr>
<th>Measures</th>
<th>MetS (n = 49)</th>
<th>Non-MetS (n = 62)</th>
<th>Effect Size</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>17.77 ± 1.42</td>
<td>17.48 ± 1.65</td>
<td>0.19</td>
<td>.33</td>
</tr>
<tr>
<td>Gendera</td>
<td>M 31, F 18</td>
<td>M 34, F 28</td>
<td>.37</td>
<td></td>
</tr>
<tr>
<td>Socioeconomic statusb</td>
<td>2.07 ± 1.03</td>
<td>2.40 ± 1.38</td>
<td>0.09</td>
<td>.36</td>
</tr>
<tr>
<td>School grade</td>
<td>11.79 ± 1.76</td>
<td>11.81 ± 1.91</td>
<td>0.01</td>
<td>.95</td>
</tr>
<tr>
<td>Obesity, %</td>
<td>100 ± 37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meets waist criterion, %</td>
<td>96 ± 27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meets HDL criterion, %</td>
<td>77 ± 26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meets triglyceride criterion, %</td>
<td>42 ± 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meets hypertension criterion, %</td>
<td>20 ± 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMIb</td>
<td>38.43 ± 7.17</td>
<td>27.09 ± 8.59</td>
<td>0.64</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Waist measurement, cm²</td>
<td>115.97 ± 17.70</td>
<td>88.52 ± 21.28</td>
<td>0.64</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>QUICKI scorec</td>
<td>0.31 ± 0.02</td>
<td>0.36 ± 0.04</td>
<td>1.65</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)b</td>
<td>78.96 ± 8.85</td>
<td>75.53 ± 7.38</td>
<td>0.20</td>
<td>.04</td>
</tr>
<tr>
<td>Fasting insulin (μIU/mL)b</td>
<td>23.91 ± 12.73</td>
<td>10.22 ± 9.05</td>
<td>0.67</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.44 ± 0.33</td>
<td>5.25 ± 0.32</td>
<td>0.28</td>
<td>.005</td>
</tr>
<tr>
<td>HDL (mg/dL)c</td>
<td>41.25 ± 6.47</td>
<td>51.37 ± 11.47</td>
<td>1.05</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)b</td>
<td>105.29 ± 42.08</td>
<td>69.76 ± 25.40</td>
<td>0.44</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)b</td>
<td>115.86 ± 12.72</td>
<td>104.94 ± 10.70</td>
<td>0.43</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)b</td>
<td>71.51 ± 10.13</td>
<td>63.42 ± 7.09</td>
<td>0.42</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CRP (mg/L)b</td>
<td>3.11 ± 1.91</td>
<td>1.72 ± 2.59</td>
<td>0.48</td>
<td>.001</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)b</td>
<td>371.98 ± 101.79</td>
<td>288.77 ± 61.55</td>
<td>0.45</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>BDI scoreb</td>
<td>0.66 ± 0.07</td>
<td>0.08 ± 0.08</td>
<td>0.15</td>
<td>.14</td>
</tr>
<tr>
<td>Self-rating of sleep apneab</td>
<td>0.23 ± 0.16</td>
<td>0.18 ± 0.13</td>
<td>0.12</td>
<td>.22</td>
</tr>
</tbody>
</table>

Normally distributed continuous variables were evaluated with the t test (effect size Cohen d) unless indicated otherwise.

BDI, Beck Depression Inventory; F, female; HbA1c, hemoglobin A1c; M, male.

a The χ² test was used for categorical variables.
b Mann-Whitney U test was used (effect size: 0.1, small; 0.3, medium; 0.5, large). Effect sizes are expressed as absolute values.
c Adjusted for unequal variances.

No participant had clinically relevant WM hyperintensities. VANOVA analyses assessing WM microstructural integrity and utilizing conservative statistics with a cluster size of 100 contiguous voxels and a false discovery rate of 1% identified a total of 14 clusters (overall, 4.90 mL in volume), all showing reduction in FA, an indication of diminished fiber organization, among adolescents with MetS (P < .001). The significant clusters were located in major fiber tracts such as the corpus callosum, optic radiations, and medial longitudinal fasciculi. See Fig 1 where we present the 10 largest clusters. Given that 20% of participants with and 6% without MetS were hypertensive and that hypertension is associated with WM disease,26 we confirmed that the group differences remained when also controlling for mean arterial BP (data not shown).

Our non-MetS (control) group also had varying degrees of metabolic dysregulation (see Table 1); thus, we also contrasted our adolescents with MetS (n = 49) with those without any positive MetS components or “completely healthy” (n = 21); this more restricted control group also had similar demographic characteristics to the MetS group. We found the hippocampal volume reductions and increased CSF volumes remained significant and that the cognitive group differences were more dramatic, with 10 of the 17 (up from 7/17) cognitive measures now showing at least a statistical trend, all with larger effect sizes (data not shown). In addition, the VANOVA analysis (P < .005, also at false discovery rate = 1%), revealed a total of 16 clusters (overall, 4.47 mL in volume) all demonstrating FA reductions in the adolescents with MetS.

Impart of Higher MetS Burden on the Cognitive and Brain Findings

Of the 111 participants included, 18 did not have MRI data (most of them could not be accommodated by the scanner because of their large body size), which resulted in 93 adolescents with MR scans (34 MetS and 59 non-MetS adolescents). We found no significant differences in ICV volume (MetS, 1178.65 ± 102.76 mL; non-MetS, 1202.74 ± 116.17 mL; t[91] = −1.00, P = .32, d = 0.22). Adolescents with MetS had significantly smaller ICV-adjusted hippocampal volumes (MetS, 2.68 ± 0.31 versus non-MetS, 2.91 ± 0.35 mL; t[90] = −2.93, P < .01, d = 0.63 [medium to large effect size]) and larger ICV-adjusted overall CSF volume (MetS, 42.08 ± 20.51 versus non-MetS, 31.35 ± 13.75 mL; t[91] = 3.08, P < .01, d = 0.73 [medium to large effect size]). The groups did not differ in the ICV-adjusted DLPFR volume (MetS, 235.62 ± 29.18 versus non-MetS, 239.61 ± 35.00 mL; t[85] = 0.04, P = .97, d = 0.01).

No participant had clinically relevant WM hyperintensities. VANOVA analyses assessing WM microstructural integrity and utilizing conservative statistics with a cluster size of 100 contiguous voxels and a false discovery rate of 1% identified a total of 14 clusters (overall, 4.90 mL in volume), all showing reduction in FA, an indication of diminished fiber organization, among adolescents with MetS (P < .001). The significant clusters were located in major fiber tracts such as the corpus callosum, optic radiations, and medial longitudinal fasciculi. See Fig 1 where we present the 10 largest clusters. Given that 20% of participants with and 6% without MetS were hypertensive and that hypertension is associated with WM disease,26 we confirmed that the group differences remained when also controlling for mean arterial BP (data not shown).

Our non-MetS (control) group also had varying degrees of metabolic dysregulation (see Table 1); thus, we also contrasted our adolescents with MetS (n = 49) with those without any positive MetS components or “completely healthy” (n = 21); this more restricted control group also had similar demographic characteristics to the MetS group. We found the hippocampal volume reductions and increased CSF volumes remained significant and that the cognitive group differences were more dramatic, with 10 of the 17 (up from 7/17) cognitive measures now showing at least a statistical trend, all with larger effect sizes (data not shown). In addition, the VANOVA analysis (P < .005, also at false discovery rate = 1%), revealed a total of 16 clusters (overall, 4.47 mL in volume) all demonstrating FA reductions in the adolescents with MetS.

Impact of Higher MetS Burden on the Cognitive and Brain Findings

Of the 111 participants included, 18 did not have MRI data (most of them could not be accommodated by the scanner because of their large body size), which resulted in 93 adolescents with MR

flexibility (completion time on the Trails B Test), but no other test of executive function was affected. The groups did not differ on memory performance or psychomotor efficiency.

Although the groups did not differ in self-ratings of obstructive sleep apnea or depressive symptoms, to err on the side of caution, we confirmed that the significant cognitive differences were largely unchanged after controlling for those ratings.

Imaging Results

Of the 111 participants included, 18 did not have MRI data (most of them could not be accommodated by the scanner because of their large body size), which resulted in 93 adolescents with MR

scans (34 MetS and 59 non-MetS adolescents). We found no significant differences in ICV volume (MetS, 1178.65 ± 102.76 mL; non-MetS, 1202.74 ± 116.17 mL; t[91] = −1.00, P = .32, d = 0.22). Adolescents with MetS had significantly smaller ICV-adjusted hippocampal volumes (MetS, 2.68 ± 0.31 versus non-MetS, 2.91 ± 0.35 mL; t[90] = −2.93, P < .01, d = 0.63 [medium to large effect size]) and larger ICV-adjusted overall CSF volume (MetS, 42.08 ± 20.51 versus non-MetS, 31.35 ± 13.75 mL; t[91] = 3.08, P < .01, d = 0.73 [medium to large effect size]). The groups did not differ in the ICV-adjusted DLPFR volume (MetS, 235.62 ± 29.18 versus non-MetS, 239.61 ± 35.00 mL; t[85] = 0.04, P = .97, d = 0.01).
TABLE 2 Cognitive Data

<table>
<thead>
<tr>
<th>Measures</th>
<th>MetS (n = 49)</th>
<th>Non-MetS (n = 62)</th>
<th>Effect Size</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intellectual functioning and academic achievement</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated full-scale IQ</td>
<td>102.00 ± 11.63</td>
<td>105.95 ± 12.35</td>
<td>0.17</td>
<td>.09</td>
</tr>
<tr>
<td>WRAT reading standard score</td>
<td>106.44 ± 11.57</td>
<td>107.64 ± 10.74</td>
<td>0.02</td>
<td>.83</td>
</tr>
<tr>
<td>WRAT spelling standard score</td>
<td>101.22 ± 15.04</td>
<td>105.93 ± 10.94</td>
<td>0.21</td>
<td>.04</td>
</tr>
<tr>
<td>WRAT arithmetic standard score</td>
<td>93.02 ± 13.25</td>
<td>102.09 ± 13.04</td>
<td>0.89</td>
<td>.001</td>
</tr>
<tr>
<td>Memory function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WRAML general memory index</td>
<td>103.05 ± 14.14</td>
<td>106.37 ± 13.59</td>
<td>0.24</td>
<td>.24</td>
</tr>
<tr>
<td>WRAML verbal memory index</td>
<td>105.60 ± 13.20</td>
<td>104.76 ± 12.45</td>
<td>0.001</td>
<td>.99</td>
</tr>
<tr>
<td>WRAML visual memory index</td>
<td>99.05 ± 13.13</td>
<td>101.35 ± 13.03</td>
<td>0.06</td>
<td>.55</td>
</tr>
<tr>
<td>WRAML working memory index</td>
<td>101.02 ± 16.19</td>
<td>105.00 ± 15.76</td>
<td>0.25</td>
<td>.22</td>
</tr>
<tr>
<td>Executive function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WCST – perseverative errors</td>
<td>10.57 ± 8.17</td>
<td>9.81 ± 6.21</td>
<td>0.05</td>
<td>.63</td>
</tr>
<tr>
<td>TOL – excess moves</td>
<td>15.66 ± 14.78</td>
<td>12.92 ± 8.92</td>
<td>0.02</td>
<td>.87</td>
</tr>
<tr>
<td>Stroop interference score</td>
<td>-1.24 ± 8.92</td>
<td>-1.57 ± 8.24</td>
<td>0.04</td>
<td>.82</td>
</tr>
<tr>
<td>COWAT total score</td>
<td>37.20 ± 12.03</td>
<td>37.67 ± 12.58</td>
<td>0.03</td>
<td>.79</td>
</tr>
<tr>
<td>Trails B time (s)</td>
<td>69.39 ± 31.41</td>
<td>58.18 ± 30.80</td>
<td>0.2</td>
<td>.04</td>
</tr>
<tr>
<td>Attention and psychomotor efficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVT total time</td>
<td>403.91 ± 118.96</td>
<td>354.95 ± 83.27</td>
<td>0.23</td>
<td>.02</td>
</tr>
<tr>
<td>WRAML attention-concentration index</td>
<td>102.40 ± 14.48</td>
<td>108.13 ± 16.06</td>
<td>0.37</td>
<td>.07</td>
</tr>
<tr>
<td>Trails A time (s)</td>
<td>28.68 ± 6.71</td>
<td>25.84 ± 8.02</td>
<td>0.19</td>
<td>.06</td>
</tr>
<tr>
<td>DSST total score</td>
<td>60.37 ± 10.54</td>
<td>63.61 ± 11.28</td>
<td>0.3</td>
<td>.14</td>
</tr>
</tbody>
</table>

Normally distributed continuous variables were evaluated with the t test (effect size Cohen d), unless indicated otherwise. COWAT, Controlled Oral Word Association Test; DVT, Digit Vigilance Test; DSST, Digit Symbol Substitution Test; TOL, Tower of London Test; WCST, Wisconsin Card Sorting Test; WRAML, Wide Range Assessment of Memory and Learning; WRAT, Wide Range Achievement Test.

* Mann-Whitney U test was used (effect size r: 0.1, small; 0.3, medium; 0.5, large). Effect sizes are expressed as absolute values.

those with 4 and 5 components. As seen on Fig 2 A–D, linear regression analyses revealed that, for an increasing number of MetS component criteria met, adolescents had progressive reductions in performance for WRAT Arithmetic (β = −4.11, r² = 0.153, F[1,96] = 17.31, P < .001) and Spelling Standard Scores (β = −2.18, r² = 0.057, F[1,97] = 5.82, P = .02). Trails B total time (β = 7.52, r² = 0.146, F[1,98] = 16.60, P < .001) and DVT total time (β = 18.50, r² = 0.087, F[1,100] = 9.58, P < .01). The same pattern existed for smaller ICV-adjusted hippocampal volumes (β = −0.07, r² = 0.081, F[1,90] = 7.91, P = .01) and increased overall CSF volume (β = 4.38, r² = 0.123, F[1,90] = 12.58, P = .001) (no figure shown). Inflammation as indicated by fibrinogen (β = 33.23, r² = 0.261, F[1,78] = 27.51, P < .001) and CRP levels (β = 0.81, r² = 0.192, F[1,90] = 21.32, P < .001) also increased with increasing number of MetS components present (no figure shown).

In stepwise regression analyses as to ascertain which of the 5 MetS components best predicted the brain volumes, we found that after controlling for age and gender (βage = −0.02, βgender = 0.006, Δr² = 0.005, ΔF[2,88] = 0.22, ΔP = .80), IR as estimated by the QUICKI score was the only significant MetS component associated with ICV-adjusted hippocampal volumes (β = 2.60, Δr² = 0.110, ΔF[1,87] = 10.84, ΔP = .001; see Fig 3A). Similarly, after adjusting for age and gender (βage = 1.96, βgender = −2.98, Δr² = 0.043, ΔF[2,89] = 2.01, ΔP = .14), IR was the only significant MetS component associated with ICV-adjusted CSF volume (β = −157.96, Δr² = 0.133, ΔF[1,88] = 14.18, ΔP < .001; see Fig 3B).

To ensure that the associations above (Fig 3 A and B) between IR and brain volumes were independent of overall obesity, as reflected by BMI (or waist circumference), we conducted hierarchical regression analyses confirming that the QUICKI score, after accounting for age, gender, and BMI (or waist circumference), still predicted a significant proportion of the variance for both hippocampal volume (β = 2.70, Δr² = 0.073, ΔF[1,87] = 7.29, ΔP = .01) and overall CSF volume (β = −157.91, Δr² = 0.084, ΔF[1,88] = 8.92, ΔP < .01) (no figure shown).

DISCUSSION

To the best of our knowledge, this is the first report demonstrating brain abnormalities among obese nondiabetic adolescents with MetS. In addition to lower scores on cognitive measures, we also demonstrated that adolescents with MetS have reduced hippocampal volumes, increased overall CSF volume, and compromised WM microstructural integrity. These findings are conservative, because many of our control adolescents met criteria for some of the MetS components, just not 3/5 (see Table 1); when we used a subset of controls not meeting criteria for any MetS component, the group differences became more pronounced.

Overall, nondiabetic adolescents with MetS, although still performing in the normal range, scored lower across all the cognitive domains assessed than those without MetS; they had significantly lower academic achievement (ie, spelling and arithmetic), attention, and mental flexibility and trended to have lower estimated intellectual functioning. This suggests that these obesity-associated medical abnormalities, short of T2DM, may have a dampering effect on academic performance, which may impact professional potential and perhaps lifelong learning. We have recently reported that obese adolescents with T2DM also have cognitive dysfunction, but more prominent than those reported here and also included memory difficulties.

Hippocampal volume reductions have been described in adults and obese adolescents with T2DM. The current
finding of smaller hippocampal volumes among nondiabetic adolescents with MetS was unexpected. These data suggest that among obese adolescents the hippocampus may already be affected in the prediabetic stages of metabolic disease. In addition, we observed an increase in overall CSF volume among adolescents with MetS. Given that our study groups did not differ in ICV volume, whose volume is likely determined by the early growth of the brain, this suggests that the increased overall CSF volume among obese adolescents with MetS is likely caused by brain parenchyma volume loss rather than differences in early brain development.

We found lower WM microstructural integrity among adolescents with MetS than what we had found among obese adolescents with T2DM. However, in the current study, we had nearly 4 times the sample size, and
the more extensive current findings are likely a function of more robust statistics and not disease. Consistent with findings in adults with MetS, we found compromised WM microstructural integrity in major fiber tracts involved in interhemispheric or corticosubcortical communications. In the future, prospective studies should clarify whether these WM microstructural changes represent a delay in the WM maturation, which is still ongoing during adolescence, or actual damage.

We found that IR was the most significant predictor of brain volume changes, and the only one that was a significant predictor in a multivariate stepwise analysis. IR is thought to be central to MetS, and this was confirmed by the fact that it remained significantly associated to smaller hippocampal volumes and increased CSF volumes even after accounting for BMI (or waist circumference). Nevertheless, the other MetS components also contributed to the brain abnormalities, in that the more MetS components reached threshold, the smaller the hippocampal volumes and greater overall CSF volumes.

We found worsening cognitive performance with increasing number of MetS components present, with variance explained ranging from 5.7% (small to medium effect size) to 15.3% (medium to large effect size), which is consistent with adult data. However, unlike the associations of brain volume changes with IR, cognition was not significantly associated with IR. These findings suggest that, to impair cognitive performance, obesity or hypertension may be sufficient, but that to affect structural brain changes such as hippocampal volume reductions or increased overall CSF volume, further metabolic dysregulation,
such as marked fasting hyperinsulinemia, may be required. These somewhat speculative conclusions will need to be confirmed in a larger prospective longitudinal study.

The current study has significant strengths. The groups were moderate in size and had distributions that minimized socioeconomic and education bias. In addition, we used reliable and validated brain volume measurements as well as sensitive WM microstructural assessment methods with rigorous thresholds for statistical significance. Moreover, our results are likely conservative, because the control group was not totally free of metabolic dysregulation. These cognitive and brain findings in adolescents with MetS are in line with our previous reports on adolescents with T2DM, just showing smaller effect sizes. Taken together, these data suggest that there may be dose effects in brain complications as we move along the spectrum linking obesity to T2DM.

Study participants were recruited from the community and evaluated at the medical center. Although not a clinical population, our participants are not representative of the general population. In addition, given that we used IR, rather than hyperglycemia, as one of our MetS components, the results presented here may not be directly comparable to those of other studies. Although other studies of adolescents have used fasting glucose level \( \geq 110 \) mg/dL, all of those studies included adolescents with diabetes. Had we used a fasting glucose of \( \geq 110 \) mg/dL, only one of our obese adolescents would have met that criterion. Nevertheless, had we had sufficient participants with impaired fasting glucose level, the brain abnormalities would have likely been worse, because that group is closer to T2DM than our group with IR but normal glucose levels. Insulin sensitivity has been shown to worsen during pubertal progression, and, thus, the use of the QUICKI score cutoff of \( \leq 0.350 \) to indicate IR could potentially bias against younger children being included in the MetS group. However, in these data we did not have such potential bias, because QUICKI scores rose, nonsignificantly, with age.

Another possible limitation of the current study is that we did not control for multiple comparisons when testing for group differences in cognitive performance. However, given that all the cognitive measures were lower in the MetS group, with 7 of 17 cognitive measures showing at least a statistical trend, we felt justified for this first report of brain and cognitive impairments in adolescents with MetS in not controlling for multiple comparisons.

CONCLUSIONS

Although obesity may not be enough to stir clinicians or even parents into action, these results among youth with MetS strongly argue for an early and comprehensive intervention. We propose that brain function be introduced among the parameters that need to be evaluated when considering early treatment of childhood obesity. Future work should also ascertain whether the reductions in cognitive performance and structural brain abnormalities are reversible with significant weight loss and reversal of the obesity-associated MetS components.

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SESSION 4
Influences on the Development of Children's Eating Behaviours: From Infancy to Adolescence

Leann Birch. Dr. Leann Birch is Distinguished Professor of Human Development and Nutritional Sciences at the Pennsylvania State University, and Director of the Center for Childhood Obesity Research. Dr. Birch is a developmental psychologist, and she is internationally recognized for her research on how early experience and family environments shape children's developing food preferences, eating behaviors, and weight status. She obtained her Ph.D. in Psychology from the University of Michigan, Ann Arbor.

Jennifer S. Savage, and
Jennifer S. Savage is a research assistant in the Center for Childhood Obesity Research at the Pennsylvania State University. She completed her M.S. in Nutritional Sciences as well as a dietetic internship from the Department of Nutritional Sciences at Penn State University, University Park. Jennifer is interested in how parenting practices influence children's food preferences and weight status.

Alison Ventura
Alison Ventura is a research assistant in the Center for Childhood Obesity Research at the Pennsylvania State University. She has degrees in both Nutrition & Human Development and Family Studies, and is interested in health promotion in families with infants and young children.

Introduction
Eating behaviours evolve during the first years of life; children learn what, when, and how much to eat through direct experiences with food and by observing the eating behaviours of others. In light of the increasing prevalence of overweight and obesity in North America among all age groups, including very young children, an understanding of the factors that influence eating behaviours during childhood is needed to improve the dietary patterns and health status of this age group. In this review, we will describe behavioural factors that shape the development of food acceptance, including food selection and food preferences, as well as the regulation of food intake in young children. Although a range of environmental factors may directly influence the development of child eating behaviours, the primary focus of this review will be on ways in which caregivers influence children's eating environments and eating behaviours.

The Current State of Children's Diets
Across human history, undernutrition and food scarcity have been major threats to children's survival, and parental feeding practices have evolved in response to these threats. These feeding practices, which include behaviours such as providing large portions of palatable foods and encouraging children to eat, are still pervasive in most cultures, despite the fact that in many regions the balance has shifted from food scarcity to food excess and over-consumption has become a new threat. The impact of these ongoing practices on children's dietary intake can be seen in several recent dietary surveys.

The Feeding Infants and Toddlers Study (FITS), which provided data on the dietary patterns of 3022 infants and toddlers, revealed that 4 to 24 month old children typically consumed...
significant amounts of developmentally inappropriate, energy-dense, nutrient poor foods. Of particular concern was the finding that 18% to 33% of infants and toddlers consumed no distinct servings of vegetables on a typical day and when vegetables were consumed the most common choice was french fries. Additionally, reported energy intakes exceeded requirements by 10 to 30%. Unfortunately, there is also evidence that these patterns tend to persist throughout childhood and into adolescence, and that diet quality tracks and declines from early childhood through adolescence. The Canadian Community Health Survey (CCHS) suggests that seven out of ten children aged 4 to 8 years fail to meet the minimum number of servings for vegetables and fruit in Canada's Food Guide to Healthy Eating. These children also fall short of reaching the recommended servings for grains and milk products, thereby suggesting that poor eating habits among children are endemic.

The transition into late childhood and adolescence can also be characterized by undesirable changes in eating behaviours such as increased consumption of sugar sweetened beverages (e.g., soda), calorie-dense, nutrient poor snacks and food away from home (e.g., fast food) and a decline in the consumption of milk and other nutrient-dense foods. Meal patterns also tend to change, as teenagers are more likely to skip breakfast and less likely to participate in family dinners. All of these trends are associated with decreased diet quality and may partially explain the fact that most adolescents are failing to meet the majority of dietary recommendations.

Influences during Infancy and the Toddler Years

The first year of life is a period of rapid physical, social and emotional growth, during which eating patterns also develop. During this first year, infants transition from consuming a single food (i.e., breast milk or formula) to consuming a variety of foods more characteristic of an adult diet. This transition allows infants to learn about food through direct experience, as well as through observation of others' eating behaviours.

Data indicate that breastfeeding and parental modeling in the toddler years play significant roles in establishing longer-term eating behaviours. As reviewed below, children who are not breastfed still derive a significant benefit from the behaviours that their parents impart as they grow and develop. Breastfeeding is recommended as the optimal feeding method for the first 6 months of life, in part because of the mounting evidence that breastfeeding has a positive impact on the development of a child's later eating behaviours.

Breastfeeding plays a role in the development of a child's response to internal hunger and satiety cues, and may foster the development of self-regulatory abilities during feeding. Variations in the composition of breast milk during a single feed, as well as differences in composition across the first months of life, foster this heightened sensitivity to energy intake. Emerging scientific evidence also supports the role of breastfeeding in early metabolic imprinting, which partially accounts for later differences in eating behaviours. Breastfeeding also has a positive impact on later eating behaviour because it may promote acceptance of flavours in the maternal diet that are passed through breast milk. As a result, breastfed infants are exposed to a more varied flavour experience, depending on the variety of the mother's diet and this exposure may affect food acceptance during the transition to solid foods and later in life.

Infants are born with a preference for sweet and salty taste, thus sweet and salty foods have a greater likelihood of being accepted by infants when compared to foods with bitter flavours, such as certain vegetables. Both infants and young children can learn to accept a greater variety of foods and flavours through repeated exposure. Thus, in a sense, breastfeeding gives the infant early, repeated exposure to the flavours of the mother's diet, providing a flavour bridge that promotes the infant's acceptance of familiar flavours when they appear in solid foods.
As a result, breastfed infants may be more accepting of new foods and likely to consume a more varied diet later in life, depending on the variety of the mother's diet during breastfeeding. 26,29

**Parental Feeding Practices: Parents as Providers, Models, and Regulators**

Parents influence children's eating behaviour in a variety of ways: parents actively make food choices for the family, serve as models for dietary choices and patterns, and use feeding practices to reinforce the development of eating patterns and behaviours that they deem appropriate. Parenting practices are also influenced by the child's characteristics, including age, gender, weight status and eating behaviour. 12,30 Thus, parent and child affect and react to one another's eating behaviour. Additionally, parenting practices are often a response to parents' perceived threats to their children's health and development.31 As discussed above, food scarcity has historically been the major threat to children's health and development and traditional feeding practices have developed accordingly. In this context, many societies perceive larger infants as healthy and a sign of successful parenting.32 Therefore, feeding strategies in these societies are designed to increase children's intake, reduce distress and promote weight gain. However, when these strategies persist in environments with over-abundance of food, they tend to promote unhealthy diets, accelerated weight gain, and obesity.

With respect to the foods parents select for their children, the FITS study6 suggests that the “bigger is better” mentality may also be influencing parental feeding practices regarding the portion sizes and energy density of foods offered to children, both of which can increase children's total energy intake. Parents participating in FITS reported serving large portions of energy dense foods7, which may negatively impact children's eating behaviour and weight status.33 The few studies that have investigated the influence of portion size on children's eating behaviours reveal that it is positively associated with increased energy intake and body weight.33,34

Children also learn about food by observing the eating behaviours modeled by others. For example, research reveals that children's intake of fruits, vegetables, and milk increased after observing adults consuming the foods.35 When children observed the eating behaviour of their peers, the effect was similar such that selection and consumption of vegetables increased.36 Thus, positive social modeling is an indirect, yet effective practice for promoting healthier diets in children.

Parents who are concerned about their child's diet may attempt to limit what and how much food is eaten, pressure their child to eat a healthier diet, or reward their child for eating healthy foods, practices which may all lead to unintended consequences.27,37,38 Excessive restriction of children's access to, and intake of, highly palatable foods can promote increased preference for, and over consumption of, those restricted foods when they are readily available.37,38 Highly restricted children have poorer self regulation of energy intake, which is associated with greater weight gain across childhood.27,37,38 Similarly, research indicates that encouraging or pressuring children to consume more fruits and vegetables is associated with lower fruit and vegetable intakes39, and higher intakes of dietary fat.40 In addition, using food as a reward may also have inadvertent effects in that rewarding children for consuming healthy foods actually results in decreased preference for those foods.41 These findings indicate that, regardless of parents' rationale for controlling their child's eating behaviours, excessive control may have negative impacts on child food intake and weight status.

With respect to parenting styles, an authoritarian style of feeding, in which eating demands placed on children are high and responsiveness to children's needs are low, promote overeating, overweight, food rejection and picky eating.42 In contrast, an authoritative style of feeding, characterized by placing high demands on eating behaviour while also being highly responsive

*Can J Diet Pract Res. Author manuscript; available in PMC 2009 May 7.*
to eating cues, may promote healthier eating behaviours. In combination with what is known about the effect of parental modeling on children's eating behaviours there is consistent evidence that the responsive “do as I do” approach has a stronger positive effect on children's consumption patterns than the unresponsive “do as I say” approach to parenting.

### Children's Eating Away From Home: Childcare and School

A higher percentage of mothers are entering or re-entering the workforce than ever before and as a consequence young children are routinely being fed by someone else. Child care settings should provide appropriate food to meet one half to two thirds of children's daily energy and nutrient requirements. However, evaluation of actual intakes at childcare centers in the United States reveals that children often fail to consume recommended intakes of energy, iron, zinc, and magnesium. Furthermore, a recent study comparing dietary intakes of U.S. children who attend childcare with recommendations in the Food Guide Pyramid found that only 5% of 4-year-old and 25% of 5-year-old children met two thirds of their estimated energy requirements, and their intake of grains, vegetables, and dairy were inadequate. These findings indicate that there exists a tremendous opportunity to improve the role of childcare centers in serving as a venue for children to learn to accept and consume healthy foods.

Similarly, the school environment can also help to teach children about dietary patterns and eating behaviours. Almost 50% of school children in the United States participate in the National School Lunch Program (NSLP) which requires the meals served to be consistent with the Dietary Guidelines for Americans and adhere to the RDAs for protein, vitamin A, vitamin C, iron, calcium, and calories. Yet, students' measured intakes from the NSLP often do not meet recommended intakes of energy, vitamin A, and iron. Furthermore, schools also provide access to competitive food sources (such as vending machines), which may contribute to poor diet quality depending on the nature of the foods sold.

### Changing Eating Behaviours: What Works?

Many school based interventions have attempted to modify children's eating behaviour, diet, nutrition knowledge, and television viewing. A recent examination of the progress made since the release of the 2004 Institute of Medicine report on the Progress in Prevention of Childhood Obesity by school-based obesity prevention initiatives in the United States revealed that many of the current interventions are focusing on improving the nutritional quality and portion sizes of foods and beverages available in schools. However, this report highlighted two major limitations to these efforts: (1) comparison of the effectiveness of different interventions is difficult because schools vary widely in their resources, commitment to improvement, and intervention evaluation efforts; and (2) insufficient attention is being placed on improving preschool and childcare environments.

Despite these limitations, several recent reviews summarizing the effectiveness of school-based nutrition interventions show some success has been achieved in changing eating behaviour, but less success has been achieved in changing indicators of obesity. It should be noted that the majority of these interventions only lasted 3 to 6 months; the few studies that do report long term follow-up data reveal that changes in eating behaviour and weight status are less pronounced over longer periods of time. Moreover, a study examining the effectiveness of Canadian school-based programs indicates that rates of overweight are significantly lower among students participating in the Annapolis Valley Health Promoting Schools Project (see Table 1 for details on the project), than among students from schools without a nutrition program. However, rates of overweight did not differ between students attending schools that provided healthy menu alternatives and students from schools without programs. Thus, more work is needed to improve the effectiveness of the school-based interventions currently available.
There are several strengths and weaknesses associated with school-based intervention strategies. One strength is that most U.S. children attend school and are estimated to eat between 19% and 50% of their daily energy intake at school. Schools also provide a host of resources such as gyms, tracks, fields, physical education, health curriculum, food service systems, and program implementers such as teachers and nurses. As well, schools also provide ready access to a variety of racial and ethnic groups of varying age and socioeconomic status. Studies also indicate that school-based interventions are cost-effective. One major limitation of school-based prevention is that interventions are typically administered at the local level, making wide-scale evaluation of prevention strategies difficult. In addition, teachers and administrators report that time-constraints, other curriculum priorities, and lack of financial resources are obstacles to effective implementation and evaluation. Another limitation is that by the time a child enters school, they already have many preferences for food and eating. Additionally, programs targeting younger children have been shown to be more successful than those with adolescents. Given these findings, and the fact that approximately 20% of children entering school are already overweight waiting until children are in school overlooks what may be our best opportunity to prevent obesity: the first few years of life.

Suggestions for Healthy Eating Interventions

Conclusions from several comprehensive reports examining the modifiable determinants of childhood obesity (including eating behaviours) suggest that early intervention, i.e., prenatal and the first years of a child's life, may be the optimal window for promoting the development of healthy eating behaviours in children. As reviewed above, experiences with food and food preferences begin in infancy and continue to develop as children transition to solid foods. During this time, children's food preferences are also influenced by availability, accessibility, and familiarity to foods as well as parental modeling. Thus, if children are to learn to prefer and select healthy foods, they need early, positive, repeated experiences with those foods.

Table 2 highlights several aspects of parent and caregiver behaviour related to child feeding practices that should be targeted by education, prevention and intervention efforts. The evidence reviewed above also supports the importance of parents and caregivers as primary influencers and gatekeepers of children's eating behaviours. It is not surprising that caregivers play a critical role in developing children's food preferences, but the poor diet quality of North American children suggests that parents need guidance in this area. Interventions targeting parent and caregiver attitudes and behaviours may prove most effective for the promotion of healthy dietary habits in children.

Even with an emphasis on early prevention, healthy eating behaviours will also need to be taught and reinforced in family, school and community environments throughout childhood and adolescence, as these contexts continually have influences on and interactions with characteristics and behaviours of both parents and children. Early intervention alone is not enough; effective prevention requires consistent, continuing and age appropriate strategies. Additionally, many of the interventions used today to modify eating behaviours adopt the “kitchen sink” approach: a large number of intervention components are included with the hope that at least one or two will have an effect on the desired outcome.

However, as discussed above, to date there have been few successful interventions to improve children's dietary patterns. New approaches to developing effective interventions, such as the multiphase optimization strategy (MOST) proposed by Collins and colleagues, where program components are individually tested before inclusion into an intervention, may provide insights into more efficient ways to design and implement more effective interventions to establish and promote optimal eating behaviours during childhood.
In conclusion, children's eating behaviours are susceptible to many external influences within their families, schools and communities. Currently, many of these influences promote dietary patterns that predispose to obesity. Fortunately, these influences can also act to promote healthy dietary practices. Incorporation of the current knowledge concerning the multifaceted influences on children's eating behaviours into evidence-based prevention and intervention efforts is needed to help improve the diet quality and eating behaviours of youth.

References


# Annapolis Valley Health Promoting Schools Project Details

<table>
<thead>
<tr>
<th><strong>Goal:</strong></th>
<th>To enable children to make healthy food and physical activity choices on a daily basis and provide them with skills to develop healthy eating and activity behaviours for life.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scope:</strong></td>
<td>A 2½ year project evaluated in 8 schools in the Annapolis Valley Regional School Board in Nova Scotia.</td>
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</table>

<table>
<thead>
<tr>
<th><strong>Strategy:</strong></th>
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</thead>
<tbody>
<tr>
<td>• Student involvement and family inclusion</td>
</tr>
<tr>
<td>• Community cooperation</td>
</tr>
<tr>
<td>• Healthy menu choices</td>
</tr>
<tr>
<td>• Affordable food pricing</td>
</tr>
<tr>
<td>• School food policies</td>
</tr>
<tr>
<td>• Daily physical activity</td>
</tr>
<tr>
<td>• Fitness equipment; affordable and accessible physical activity opportunities</td>
</tr>
<tr>
<td>• Health and nutrition curriculum</td>
</tr>
<tr>
<td>• Healthy environments</td>
</tr>
<tr>
<td>• Modeling healthy attitudes and behaviours</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Results:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• Better diets</td>
</tr>
<tr>
<td>• More physically active</td>
</tr>
<tr>
<td>• Less screen time</td>
</tr>
<tr>
<td>• 59% decrease in prevalence of overweight</td>
</tr>
<tr>
<td>• 72% decrease in prevalence of obesity</td>
</tr>
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Table 2
Recommendations for parents and caregivers to be included in early prevention and intervention efforts

<table>
<thead>
<tr>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Encourage breastfeeding when possible for the first 6 months of life</td>
</tr>
<tr>
<td>• Eat a varied diet during pregnancy and lactation to create for your infant</td>
</tr>
<tr>
<td>a “flavour bridge” to the modified adult diet</td>
</tr>
<tr>
<td>• Practice responsive parenting by discriminating hunger from other distress</td>
</tr>
<tr>
<td>cues and avoiding always using food to comfort your child</td>
</tr>
<tr>
<td>• Provide positive, repeated exposure to novel foods (especially typically</td>
</tr>
<tr>
<td>rejected foods, such as vegetables) to promote acceptance of and preference</td>
</tr>
<tr>
<td>for those foods</td>
</tr>
<tr>
<td>• Offer developmentally appropriate and healthy foods to your child during</td>
</tr>
<tr>
<td>the transition to solids</td>
</tr>
<tr>
<td>• Serve portion sizes that are developmentally appropriate for your child’s</td>
</tr>
<tr>
<td>age and nutrient needs</td>
</tr>
<tr>
<td>• Choose when and what your child should eat, but let your child decide how</td>
</tr>
<tr>
<td>much to eat</td>
</tr>
<tr>
<td>• Trust a child of normal weight status to self-regulate his own intake</td>
</tr>
<tr>
<td>• Make a wide variety of nutrient-dense rather than energy-dense, nutrient</td>
</tr>
<tr>
<td>poor foods available and accessible to your child</td>
</tr>
<tr>
<td>• Use your own behaviours and attitudes to model healthy dietary patterns</td>
</tr>
<tr>
<td>• Create a positive feeding environment by initiating regular family meals</td>
</tr>
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</table>
Relationship between Portion Size and Energy Intake among Infants and Toddlers: Evidence of Self-Regulation

MARY KAY FOX, MEd; BARBARA DEVANEY, PhD; KATHLEEN REIDY, DrPH, RD; CAROL RAZAFINDRAKOTO, MS; PAULA ZIEGLER, PhD, RD

ABSTRACT

Objectives To assess whether dietary intakes of infants and young toddlers show evidence of energy self-regulation.

Design Data from 24-hour recalls collected in the 2002 Feeding Infants and Toddlers Study were analyzed. Multivariate regressions were used to explore the relationship between portion size and usual energy intake as well as the relationship between portion size, number of eating occasions, number of unique foods, and energy density.

Subjects/setting A national random sample of 3,022 US infants and toddlers 4 to 24 months of age.

Statistical analyses performed To measure variability in portion size, an average portion size z score was computed for each child in the sample, across 45 different food groups. The number of eating occasions was defined as the total number of times a child had anything to eat or drink during the day, excluding eating occasions that included only water and/or supplements. The total number of unique foods in a day was defined as the number of unique food codes included in the 24-hour recall, and energy density was computed as kilocalories/gram, including all foods, beverages, and water. Linear regression models were used to assess the effect of portion size and other self-regulation mechanisms on energy intake and to assess the effect of these self-regulation mechanisms on portion size. Separate analyses were performed for three age groups: 4 to 5 months, 6 to 11 months, and 12 to 24 months.

Results A significant negative association was found for all age groups between the number of eating occasions and average portion size z-scores, indicating that children who eat less often during the day consume larger-than-average-portion sizes and children who eat more often during the day consume smaller-than-average portions. For infants (11 months and younger), a significant negative association was noted between energy density and average portion size z-scores, indicating that, as the energy density of the diet goes down, infants consume larger-than-average portions and, as the energy density of the diet goes up, they consume smaller-than-average portions. Among infants 6 to 11 months, there was a significant positive relationship between portion size and the number of unique foods consumed. For toddlers, there was no association between average portion size z-scores and energy density, suggesting that energy self-regulation mechanisms are diminished in this age group.

Conclusions Our findings confirm the presence of energy self-regulation among infants and young toddlers. These findings can be used to assure parents and caregivers that infants have an innate ability to regulate energy intake. At the same time, it is important to educate parents and caregivers about the potential for environmental cues to diminish natural hunger-driven eating behaviors, even among young toddlers. Dietetics professionals should emphasize the potential adverse effects that coercive feeding behaviors can have on children’s innate ability to regulate energy intake. This includes not only admonitions to “clean your plate,” but overrestriction of intake that may be motivated by concerns that children are overeating.

J Am Diet Assoc. 2006;106:S77-S83.

Recently, several researchers have hypothesized that the increasing propensity for Americans to consume larger portions of food could be contributing to the ongoing obesity epidemic (1,2). Several studies among adults have demonstrated that larger portions can lead to increased energy intakes (3-6). Fewer studies have looked at this issue among children. In a laboratory setting, Rolls and colleagues found that portion size influenced the energy intakes of 5-year-old children, but not 3-year-olds (7). In contrast, McConahy and colleagues analyzed...
data from the 1994 to 1996 and 1998 Continuing Survey of Food Intakes by Individuals (CSFII) and reported a positive association between portion size and energy intake among 1- and 2-year-olds and among preschool children (8,9).

On one hand, it seems tautological that larger portion sizes lead to higher energy intakes. However, total energy intake is influenced not only by portion size, but by the number of eating occasions in a day, the number of foods consumed, and the energy density of the foods consumed. The overall effect of portion size on energy intake may vary depending on how these other determinants of intake change as portion size changes. Studies in both controlled and free-living environments have demonstrated that infants have an innate ability to self-regulate energy intake, with intake being driven primarily by responses to hunger and satiety cues (7,10-13). Other research has shown, however, that this innate ability to self-regulate deteriorates over time as eating becomes more influenced by external cues, such as palatability, schedule/routine, and social context (7,14,15).

Total energy intake is influenced not only by portion size, but by the number of eating occasions in a day, the number of foods consumed, and the energy density of the foods consumed.

An important question to ask is: how early in children’s development does the deterioration of energy self-regulation begin? To examine this issue, we analyzed data from the 2002 Feeding Infants and Toddlers Study (FITS), which collected dietary intake data on a stratified, random sample of infants and toddlers 4 to 24 months of age. The ideal analysis would assess the relationship between portion size and weight-for-height. We were unable to look at this relationship, however, because of questionable reliability of the heights reported by caregivers. As an alternative, we assessed relationships between portion size, usual energy intake, and other factors that may be used in self-regulating energy intake. These include the number of eating occasions, the total number of unique foods consumed, and energy density. We looked at the effects of portion size, number of eating occasions, number of unique foods, and energy density on energy intake and then examined how portion size varied with the other predictors of energy intake.

METHODS
Sample Design and Subjects
The 2002 FITS was sponsored by Gerber Products Company to update our knowledge of the food and nutrient intakes of infants and toddlers in the United States (16). FITS included a stratified random sample of infants between 4 and 24 months of age. The sample was drawn from Experian’s New Parents Database, February to May 2002, Experian (Lincoln, NE) because it was judged to provide the greatest coverage of infants and toddlers. Infants and toddlers were sampled in six age groups: 4 to 6 months, 7 to 8 months, 9 to 11 months, 12 to 14 months, 15 to 18 months, and 19 to 24 months. Infants 4 to 6 and 9 to 11 months of age were oversampled because these two age groups typically experience significant transitions in infant feeding patterns and practices (from liquid diets to the addition of complementary foods, and from complementary foods to the addition of table foods, respectively). Sample weights were developed to adjust for oversampling, nonresponse, and undercoverage of some subgroups of children not included in the sample frame. A detailed overview of the FITS study design and sample is available elsewhere (16,17).

In this article (and others in this supplement), we have modified the reporting categories for the two youngest age groups, relative to the age groups that have been used in previous reports of FITS data (18-25). This change was made in response to recent clarifications about the intended age groups for infants in the Dietary Reference Intakes (26-31). The life-stage groups used in the Dietary Reference Intakes define infancy as the period from birth through 12 months of age and “divide [it] into two 6-month intervals.” Thus, although some text and tables in the Dietary Reference Intake reports refer to 0 to 6 months and 7 to 12 months, the actual intent is 0 through 5 months (0-5.99)—the first six months of life—and 6 through 11 months (6.0-11.99)—the second six months of life (Janice Rice Okita, PhD, RD, senior program officer, Food and Nutrition Board, Institute of Medicine, personal communication, June 7, 2005). For this reason, this article limits the youngest age group to infants 4 and 5 months old and includes infants 6 months of age in the second age group (6-11 months). The sample includes 3,022 infants, in the following age groups: 4 to 5 months (n=624), 6 to 8 months (n=708), 9 to 11 months (n=687), 12 to 14 months (n=371), 15 to 18 months (n=312), and 19 to 24 months (n=320).

Data Collection Methods
FITS data were collected by Mathematica Policy Research, Inc (Princeton, NJ). All data collection instruments and procedures were reviewed and approved by Mathematica Policy Research, Inc’s institutional review board compliance officer and quality assurance system. Parents or primary caregivers of sampled infants and toddlers completed a single 24-hour dietary recall. The 24-hour recall collected information on water intake (tap water and bottled water) as well as use of dietary supplements. All recalls were completed between March and July 2002. Trained interviewers conducted the 24-hour recalls over the telephone using the Nutrition Data System for Research (version 4.03, 2001, University of Minnesota Nutrition Coordinating Center, Minneapolis). An information packet was mailed to respondents a week to 10 days prior to the interview. The packet included a detailed two-dimensional booklet for use in reporting portion sizes. The booklet was designed specifically for FITS and was pilot-tested with mothers of infants and toddlers. It included graphic depictions, drawn to scale, of common infant feeding utensils, including eight popular “sippy” cups, four different spoons, and two different bowls. Also included were circles from 1 to 8 inches in diameter; for
use in reporting the size of round foods, such as cookies, pancakes, and hamburger patties, and a 5×5-inch grid for use in reporting square and rectangular foods, such as crackers and cheese, and for estimating thickness. A random subsample of 703 respondents completed a second 24-hour recall 3 to 10 days after the first recall, on a different day of the week. This second recall was used to generate estimates of usual energy and nutrient intake. Additional details about collection, processing, and quality control of 24-hour recall data are reported elsewhere (16,17).

**Analytic Methods**

Average portion sizes were estimated for 45 different food groups (Figure) for three age groups: infants 4 to 5 months, infants 6 to 11 months, and toddlers 12 to 24 months. In estimating average portion sizes, we followed procedures used in US Department of Agriculture reports that report population estimates of average portion sizes based on the CSFII (32,33). Details about this process are reported in another article in this supplement (34). Portions were assessed in common household measures. To compare portion sizes across children, we adapted the approach used by McConahy and colleagues (8,9) and computed, for each child, an average portion size \( z \) score. Because foods have different units of measurement, the use of \( z \) scores standardized food portions and allowed us to incorporate foods with different units of measurement into a single variable. For an individual food group, \( z \) scores express each portion size in terms of standard deviations from the sample mean. For example, let \( PS_j \) denote a child’s portion size for food group \( j \), and let \( SD_j \) denote the standard deviation of portion size. The \( z \) score for that child for food group \( j \) is defined as:

\[
z_{scorej} = \frac{PS_j - PS}{SD_j}
\]

An overall average portion size \( z \) score for a child was then constructed by averaging the individual food group \( z \) scores across all food groups consumed by that child, as follows:

\[
z_{score} = \frac{\sum z_{scorej}}{J}
\]

\( Z \) scores were computed within age group. If fewer than 25 children in an age group consumed a food group, the food group was not included in computed \( z \) scores for that age group.

Other regulation variables were defined as follows. The total number of eating occasions was defined as the total number of times a child ate during the day, excluding occasions that included only water and/or supplements. The number of unique foods was defined as the number of unique food codes included in the 24-hour recall. Energy density was computed as kilocalories/gram, including all foods, beverages, and water. The energy intake variable used in these analyses reflects the usual energy intake of each child. Estimates were computed using the second 24-hour recalls and the personal computer version of the Software of Intake Distribution Estimation (version 1.02, 2001, Iowa State University, Ames). This software adjusts for day-to-day variation in intake. Although it is generally used to adjust intake distributions, it includes a function that calculates usual intakes at the individual level. These estimates are acknowledged to be less precise than estimates of usual intake distributions (35); however, in our judgment, they are preferable to estimates based on a single 24-hour recall because they include some adjustment for day-to-day variation in intake.

We used multivariate regressions to model the relationship between the full set of regulation variables (portion size, number of eating occasions, number of unique foods, and energy density) and energy intake. Next, we modeled the relationship between the other regulation variables and portion size. Results of the latter model tell us how variations in the other regulation variables affect portion size. We used Statistical Analysis Software (version 8.2, 2001, SAS Institute, Inc. Cary, NC) in preparing data. Data were analyzed using SUDAAN (version 9.0, 2004, Research Triangle Institute, Research Triangle Park, NC), incorporating appropriate sample weights and design effects.

**RESULTS**

Results of the multivariate regressions for usual energy intake are summarized in Table 1. Results are consistent across age groups and indicate that each of the regulation variables is positively associated with usual energy intake. This indicates that children’s energy intakes are influenced by all of the hypothesized regulation mechanisms: consuming larger-than-average portions, eating more often, eating more unique foods, and consuming a more energy-dense diet.

The results of this model are limited, however, by the fact that they reflect the impact of portion size on energy intake with all other regulation variables held constant. In this context, a positive association between portion size and energy intake is not surprising. Our second model, which examines the relationships between the other regulation variables and portion size...
provides a better understanding of the potential impact of portion size on energy intake. Results, summarized in Table 2, reveal a significant negative association, for all age groups, between the number of eating occasions and average portion size $z$ scores. This indicates that, other regulation variables held constant, children who eat less often during the day consume larger-than-average-portion sizes and children who eat more often during the day consume smaller-than-average portions. Results for the other regulation variables vary across age groups. For both groups of infants, a significant negative association is noted between energy density and average portion size $z$ scores. This indicates that, as the energy density of the diet goes down, infants consume larger-than-average portions and, as the energy density of the diet goes up, they consume smaller-than-average portions. Among infants 4 and 5 months old, there is no relationship between the number of unique foods and average portion size $z$ scores; among infants 6 through 11 months old, there is a significant positive relationship between these two variables. The lack of an association among the youngest infants probably reflects the fact that the diets consumed by these infants are very homogeneous, relative to other age groups, and largely milk-based (19). The positive association between the number of unique foods and average portion size $z$ scores for infants 6 through 11 months old indicates that infants in this age group who are consuming a more varied diet are also consuming larger-than-average portions. For toddlers, there is no association between average portion size $z$ scores and either the number of unique foods consumed or energy density.

### DISCUSSION

Our findings indicate that the energy intakes of infants and toddlers are influenced by their portion sizes, the number of times they eat throughout the day, the number of unique foods consumed in a day, and the energy density of the foods consumed. These results are consistent with findings reported by McConahy and colleagues for preschool children, but contradictory to their results for 1-year-olds (8,9). In this younger age group, McConahy and colleagues reported a positive association between portion size and energy intake, but found no association between energy intake and either the number of eating occasions or number of unique foods (8). Our analysis of interactions between portion size and the other regulation variables indicates that infants and toddlers compensate for variation in the number of eating occasions by adjusting their portion sizes. This is consistent with findings from previous research in both controlled and free-living environments (10,12). In addition, the negative association between energy density and average portion size $z$ scores among infants indicates that infants com-

#### Table 1. Results of multivariate regressions of regulation variables as predictors of energy intake

<table>
<thead>
<tr>
<th>Predictor</th>
<th>4 and 5 Months</th>
<th>6 to 11 Months</th>
<th>12 to 24 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>104.99</td>
<td>86.83</td>
<td>51.04</td>
</tr>
<tr>
<td>Coefficient</td>
<td>13.87</td>
<td>21.12</td>
<td>38.42</td>
</tr>
<tr>
<td>Average portion size $z$</td>
<td>SE b</td>
<td>SE b</td>
<td>SE b</td>
</tr>
<tr>
<td>score</td>
<td>1.95</td>
<td>2.35</td>
<td>2.35</td>
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<tr>
<td>No. of eating occasions</td>
<td>3.62</td>
<td>4.92</td>
<td>4.92</td>
</tr>
<tr>
<td>SE b</td>
<td>0.90</td>
<td>0.62</td>
<td>0.58</td>
</tr>
<tr>
<td>No. of unique foods</td>
<td>2.01</td>
<td>2.36</td>
<td>3.25</td>
</tr>
<tr>
<td>SE b</td>
<td>0.61</td>
<td>0.28</td>
<td>0.30</td>
</tr>
<tr>
<td>Energy density</td>
<td>5.73</td>
<td>20.82</td>
<td>40.12</td>
</tr>
<tr>
<td>SE b</td>
<td>1.25</td>
<td>4.17</td>
<td>4.76</td>
</tr>
<tr>
<td>T ratio</td>
<td>7.11***</td>
<td>8.97***</td>
<td>16.29***</td>
</tr>
<tr>
<td>**P&lt;.001.</td>
<td>**P&lt;.001.</td>
<td>**P&lt;.001.</td>
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</table>

#### Table 2. Results of multivariate regressions of other regulation variables as predictors of portion size

<table>
<thead>
<tr>
<th>Predictor</th>
<th>4 and 5 Months</th>
<th>6 to 11 Months</th>
<th>12 to 24 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.21</td>
<td>−0.45</td>
<td>−0.32</td>
</tr>
<tr>
<td>Coefficient</td>
<td>−0.13</td>
<td>−0.07</td>
<td>−0.04</td>
</tr>
<tr>
<td>No. of eating occasions</td>
<td>0.02</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>SE b</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>No. of unique foods</td>
<td>−0.13</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>SE b</td>
<td>0.05</td>
<td>0.07</td>
<td>0.10</td>
</tr>
<tr>
<td>Energy density</td>
<td>−7.68***</td>
<td>−3.88***</td>
<td>1.53</td>
</tr>
<tr>
<td>SE b</td>
<td>−6.02***</td>
<td>−6.54***</td>
<td>−3.71***</td>
</tr>
<tr>
<td>**P&lt;.001.</td>
<td>**P&lt;.001.</td>
<td>**P&lt;.001.</td>
<td>**P&lt;.001.</td>
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</table>
pensate for changes in energy density by adjusting the amount of food they consume. This is consistent with research that has demonstrated that infants have an innate ability to adjust food intake in response to changes in the energy content of food (11,36). The lack of a relationship between energy density and portion size among toddlers suggests a diminished responsiveness to changes in energy density. Birch and colleagues report that young children’s ability to adjust intake in response to alterations in energy density can be readily disrupted by the imposition of controlling child-feeding practices that attempt to regulate what and how much children eat (37).

It is important to educate parents and caregivers about the potential for environmental cues to diminish natural hunger-driven eating behaviors, even among young toddlers.

While our findings provide evidence of energy self-regulation among young infants and toddlers, they do not tell us how successful these compensatory mechanisms are in keeping infants, and especially toddlers, in energy balance. There is evidence that by the time children are 3 or 4 years old, eating may be less controlled by natural self-regulation than by responses to a variety of environmental influences (37). It is not clear how early interference with natural self-regulation may start. Several studies have suggested that bottle feeding may promote overconsumption of energy because caregivers using this feeding method may be less responsive to infants’ cues about hunger and satiety than breastfeeding mothers (38,39). Tavares and colleagues found that mothers who breastfed their infants in early infancy and who breastfed for longer periods of time reported less restrictive child feeding practices at 1 year (40). Future research should assess the relationships between energy intake, self-regulation mechanisms, and the weight status of infants and toddlers.

LIMITATIONS

This study has some limitations that should be acknowledged. First, all of our data were self-reported. Caregivers may have over- or underreported intakes. There is reason to believe that overreporting was more common than underreporting (18). However, mean energy intakes of FITS toddlers are consistent with mean energy intakes reported for 1-year-olds in CSFII 1994 to 1996 and 1998 (41) and in the Third National Health and Nutrition Examination Survey (42) and the portion sizes reported in FITS are consistent with the portion sizes reported in CSFII 1994 to 1996 (8,34). Thus, if overreporting is present, it appears to be comparable to that observed in national nutrition monitoring surveys. Second, analyses are based on intake of foods during a single 24-hour period rather than “usual” food intake. Methods for estimating usual food intake are limited and cannot be applied to detailed food groups. Moreover, there is some evidence to suggest that there is less day-to-day variation in dietary intake among infants and toddlers than among older children and adults (43).

CONCLUSIONS

Our findings confirm the presence of energy self-regulation among infants and young toddlers. These findings can be used to assure parents and caregivers that infants have an innate ability to regulate energy intake. At the same time, it is important to educate parents and caregivers about the potential for environmental cues to diminish natural hunger-driven eating behaviors, even among young toddlers. Dietetics professionals should emphasize the potential adverse effects that coercive feeding behaviors can have on children’s innate ability to regulate energy intake. This includes not only admonitions to “clean your plate,” but overrestriction of intake that may be motivated by concerns that children are overeating (14). Satter summed up the situation quite well when she said: “Effective feeding demands a division of responsibility: The parent is responsible for what the child is offered to eat; the child is responsible for how much and even whether to eat” (44).

This research project was funded by Gerber Products Company. This research project was a collaborative effort among Mathematica Policy Research, Inc staff (authors Devaney and Razafindrakoto), consultant Fox, and staff (authors Ziegler and Reidy) for the Gerber Products Company.

The opinions or views expressed in this supplement are those of the authors and do not necessarily reflect the opinions or recommendations of Gerber.

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Anthropometric measures in middle age after exposure to famine during gestation: evidence from the Dutch famine

Aryeh D Stein, Henry S Kahn, Andrew Rundle, Patricia A Zybert, Karin van der Pal–de Bruin, and LH Lumey

ABSTRACT
Background: Few studies in humans have related maternal undernutrition to the size of the adult offspring.
Objective: The objective was to assess whether reductions in food intake by pregnant women during the Dutch famine of 1944–1945 were related to offspring length, weight, and indexes of adiposity in middle age.
Design: We recruited 1) exposed persons born in western Netherlands between January 1945 and March 1946 whose mothers experienced famine during or immediately preceding pregnancy, 2) unexposed persons born in the same 3 institutions during 1943 or 1947 whose mothers did not experience famine during this pregnancy, and 3) unexposed same-sex siblings of persons in series 1 or 2. Anthropometric measurements (n = 427 males and 529 females) were obtained between 2003 and 2005. We defined 4 windows of gestational exposure (by ordinal weeks 1–10, 11–20, 21–30, and 31 through delivery) on the basis of exposure to a ration of <900 kcal/d during the whole 10-wk interval.
Results: Exposure to reduced rations was associated with increased weight and greater indexes of fat deposition at several tissue sites in women but not in men (P for interaction <0.01). Measures of length and linear proportion were not associated with exposure to famine.
Conclusion: Reduced food availability may lead to increased adiposity later in life in female offspring. Am J Clin Nutr 2007;85:869–76.

KEY WORDS Anthropometric measures, body composition, body mass index, body size, famine, maternal and infant health, Netherlands, nutrition, obesity

INTRODUCTION

Adult body mass is a function of height, girth, and tissue mass and distribution. Each of these measures has independent associations with risk of disease and may have specific associations with early development. Attained height, which is inversely associated with risk of cardiovascular disease (1), is strongly associated with birth length (2). Variations in body proportions, such as the ratio of the leg to trunk lengths, may have their origin in childhood (3) and are independent predictors of the risk of later morbidity and mortality (4). Little is known about the role, if any, of prenatal nutrition in the ontogeny of body proportions.

Birth weight, especially when adjusted for birth length, is positively associated with measures of body size in later life (2). Even so, and despite the consistent association between adult overweight and type 2 diabetes or cardiovascular disease (5), an increased birth weight is also associated with a decreased risk of major chronic diseases (6). An explanation for this apparent paradox might come from information on the sources of variation in size at birth (7), but few studies of humans can document the complex relations extending from maternal nutrition through fetal development and risk of adult disease.

The Dutch famine of 1944–1945 provides a rare opportunity to study the long-term consequences of maternal undernutrition in defined stages of gestation (8, 9). The Dutch famine affected the western Netherlands (10–12). Official rations, which by the end of the famine consisted almost exclusively of bread and potatoes, fell below 900 kcal/d by 26 November 1944 and were as low as 500 kcal/d by April 1945. The famine ceased immediately after liberation. This extraordinary period of deprivation affected fertility, weight gain during pregnancy, maternal blood pressure, and infant size at birth (13–15). The reduction in fertility was greater among manual than among nonmanual occupational classes (8). The decline in mean birth weight of 300 g was restricted to exposure to maternal undernutrition during the third trimester (16, 17).

An earlier investigation of Dutch men aged 19 y found a doubling of the prevalence of overweight with maternal exposure to famine in midgestation (18). A second study, with data collected when the famine-exposed birth cohort was aged 50 y, reported increased body mass index (BMI; in kg/m²) in women (but not in men) who were exposed to famine in early gestation (19). To date, no studies have reported on other anthropometric indexes of adiposity after gestation during the Dutch famine. The present study was conducted to replicate the earlier findings, extend follow-up through age 59 y, and analyze a wider array of measures of tissue distribution. We also accounted statistically


1 From the Rollins School of Public Health, Emory University, Atlanta, GA (ADS); the Division of Diabetes Translation, Centers for Disease Control and Prevention, Atlanta, GA (HSK); the Mailman School of Public Health, Columbia University, New York, NY (AR, PAZ, and LHL); and TNO Quality of Life, Leiden, Netherlands (KvdP).
2 The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.
3 Supported by grant ROI HL067914 (Principal Investigator: LHL), National Institutes of Health, Bethesda, MD.
4 Address reprint requests to LH Lumey, Department of Epidemiology, Mailman School of Public Health, 722 West 168th Street, New York, NY 10032. E-mail: lumey@columbia.edu.
Received July 6, 2006.
Accepted for publication October 12, 2006.
for familial determinants of growth and tissue distribution by including same-sex siblings as control subjects.

SUBJECTS AND METHODS

Population source and tracing

We identified 3307 live singleton births (probands) at 3 institutions in famine-exposed cities (midwifery training schools in Amsterdam and Rotterdam and the university hospital in Leiden) in 1945 and early 1946 (100% sample) and in 1943 and 1947 (the first 30 births/no across the 3 institutions). At the time of the famine, a large majority of deliveries (≥70%) in the Netherlands were scheduled to occur at home. The client mix at the 2 midwifery training schools consisted of low-risk pregnancies of women of lower socioeconomic status whose home environment was unsuitable for delivery. The client mix in Leiden included such deliveries as well as women with higher-risk pregnancies identified during prenatal care and emergency admissions after complications of home labor. We extracted personal identifiers, including name and maternal address, birth weight, and other information from the admission logs and delivery progress charts. To trace the adult offspring, we provided the names and addresses at birth of all 3307 persons to the population register in the municipality of birth. Of these named persons, 308 (9.5%) were reported to have died and 275 (8.3%) to have migrated. For 294 persons (8.9%) a current address could not be located, and the population registry in Rotterdam declined to trace 130 persons born out of wedlock. Thus, a current address was obtained for 2300 persons (70% of the institutional birth cohort).

Recruitment and examination

Traced persons were mailed a letter of invitation signed by the current director of the institution in which they were born, a brochure describing the study, and a response card. We mailed one reminder letter to nonresponders. Initially, our study design called for the recruitment of same-sex sibling pairs; hence, the lack of an available sibling was a reason for ineligibility. We received some reply from 1767 persons, of whom 347 (19.6%) expressed willingness to participate together with a sibling. Of those who declined, 67% reported not having a same-sex sibling available for study. To increase the overall number of participants, therefore, we attempted to enroll persons who had indicated ineligibility because of the lack of an available sibling.

We conducted a telephone interview, which was followed by a clinical examination at the Leiden University Medical Center. Most of the clinical examinations were conducted within 6 wk of the telephone interview. All study protocols were approved by the human subjects committees of the participating institutions, and participants provided verbal consent at the start of the telephone interview and written informed consent at the start of the clinical examination. We obtained anthropometric measurements from 971 subjects (437 men and 534 women): 311 proband-sibling pairs, 2 siblings whose matching proband did not complete the clinical examination, and 347 additional probands.

Anthropometric measures

All anthropometric measures were obtained by experienced research nurses, who were provided specific training in the methods by one of us (HSK); only trivial differences in means or in the variability of measures across nurses were observed. Weight was obtained to the nearest 100 g with the participant standing on a portable digital scale (SECA, Hamburg, Germany). Standing height was measured to the nearest 1 mm with a portable stadiometer (SECA), and seated height was obtained to the nearest 1 mm with the participant seated on a hard stool of known height with the use of the same stadiometer. Right arm length (tip of acromion to the distal tip of the third metacarpal bone) and waist (at level of iliac crests, intersection with midaxillary line), hip (buttocks at the point of maximum extension), and right midthigh (supine with hip flexed at 45°, between lateral inguinal crease and proximal patella) circumferences were obtained to the nearest 1 mm with the use of a nonextensible measuring tape (Hoechstmass, Sulzbach, Germany). The supine sagittal abdominal diameter (SAD) at the level of the iliac crests was obtained to the nearest 1 mm with a sliding-beam caliper (Holtain, Dyfed, Wales, United Kingdom). Tricipital, subscapular, and anterior midthigh skinfold thicknesses were obtained to the nearest 0.2 mm with calipers with a maximal spread of 40 mm (Holtain). These calipers were calibrated daily. A single measurement was taken for height and weight. Two measurements were taken for other anthropometric outcomes, and the mean was used for the analysis. To ensure independence of the replicate measures, all markings of measurement points were erased before the second measurement was obtained. If the first 2 measurements were not sufficiently close (arm length, waist circumference, midthigh circumference 1.0 cm; sagittal abdominal diameter 0.5 cm; subscapular or triceps skinfold thickness 2.0 mm) a third and fourth measurement were taken and the 3 measures closest together from the 4 available measures were averaged.

Derived measures

Trunk length was calculated by subtracting the height of the stool from seated height, and leg length was obtained by subtracting trunk length from standing height. As indexes of body proportion, we computed the ratios of the right arm to leg lengths and the leg to trunk lengths. We computed the BMI. As additional indexes of mass distribution, we computed the ratios of waist-to-hip circumference, waist-to-midthigh circumference, and SAD-to-midthigh circumference. We excluded from the analysis individuals for whom any of the above anthropometric measures were missing (n = 12), for whom the ratio of trunk to leg length exceeded 1.10 (n = 2), and one man with polio-related atrophy of a lower limb; the analytic sample consisted of 956 subjects. Because several of the participants had one or more skinfold thicknesses that exceeded the capacity of the calipers, we categorized the skinfold thicknesses into empirical quartiles. We then developed a 3-level indicator of the relative distribution of subcutaneous fat between the triceps and subscapular regions by cross-tabulating the quartile distributions for these 2 regions. We coded this indicator as −1 if the triceps value was in a higher quartile of the distribution than was the subscapular value, as +1 if the reverse was true, and 0 if both were in the same quartile.

Categorizing exposure to famine

We defined the start of each gestation by the date of the mother’s last menstrual period (LMP), as noted in the original prenatal record, unless it was missing or the resulting gestational age was implausible (12.4%). In these cases, we approximated the date of LMP from the unambiguous date of birth and estimates of gestational age recorded on the birth record or from a gestational age
exposed to an official ration of <900 kcal/d (the 24 wk included the period from 26 November 1944 to 12 May 1945). We defined the mother as exposed in specific periods if gestational weeks 1–10, 11–20, 21–30, or 31 to delivery were entirely included in this time window. Thus, pregnancies with an LMP between 26 November 1944 and 4 March 1945 (n = 74) were considered exposed in weeks 1–10, between 18 September 1944 and 24 December 1944 (n = 124) exposed in weeks 11–20, between 10 July 1944 and 15 October 1944 (n = 140) exposed between weeks 21–30, and between 2 May 1944 and 24 August 1944 (n = 128) exposed between week 31 and delivery. Because these time windows overlap, the participants could be considered exposed during one or (at most) two 10-wk periods; those exposed in at least one 10-wk period (n = 350) were considered to have some exposure to famine. In this formulation, the estimate for the variable “any exposure to famine” is not necessarily an average of the estimates for the four 10-wk periods, because these may have independent and additive or counteractive associations with adult size.

Statistical methods

We computed means and SDs or categorical distributions, as appropriate. We developed independent linear regression models for all models that did not include skinfold thicknesses. Skinfold thicknesses and their ratios were analyzed by using logistic regression, with the highest category compared against all others. Because humans are sexually dimorphic and previous research has identified associations of famine with body composition in one or the other sex (18, 19), we tested for heterogeneity of associations by sex using F tests. We considered a P value <0.10 to indicate an interaction and conducted sex-stratified analyses where indicated.

We considered a set of models and regressed each outcome variable separately on exposure to famine. Exposure to famine was characterized by using an indicator for any gestational exposure, with the reference category being no exposure, and by using the four 10-wk intervals described, which we entered as a set of 4 indicator variables. We used the combined population of control subjects (unexposed births in the 3 hospitals: n = 296; siblings of the birth series: n = 310) as the reference, and we adjusted for age at examination. We used the xtreg and xtabit commands in STATA 8 (Stata Corp, College Station, TX) to control for clustering within families. We assessed whether associations with exposure to famine were mediated through birth weight or length by entering these terms and comparing the changes in coefficients. These analyses were run on the institutional birth series alone (birth weight: n = 297 men and 348 women; birth length: n = 278 men and 325 women; we lacked information on size at birth for the sibling control subjects). Estimates of the effect of exposure to famine were similar in models that included the birth series and those that included the birth series and the siblings.

We examined whether measurement error in seated height because of excess adiposity in the buttocks might affect relations by adding hip circumference to the relevant models. In practice, this adjustment had no effect on any observed associations (data not shown), and models without this adjustment are presented. Models for circumferences, the SAD, and their resulting ratio measures included a term for standing height to account for allometric scaling; we also tested whether these associations were affected by body proportion by including a term for the leg-to-trunk ratio. Although the outcome measures were generally associated with both height and the leg-to-trunk ratio, addition of these terms did not alter the observed associations between the measures and exposure to famine (data not shown), and we present results without this adjustment. Models for indexes of mass distribution included adjustment terms describing adult measures that might be causally related to adiposity, including smoking status, intake of alcohol, intake of energy as estimated from a food-frequency questionnaire, physical activity level in the year before the examination as assessed by the SQUASH questionnaire (21), and, for women, parity. In practice, control for these factors did not affect the estimates (data not shown). We did not consider these variables relevant for analyses of lengths and body proportion because these outcomes are established by early adulthood.

RESULTS

Differences between traced and untraced persons

The proportion of participants identified as deceased was highest among probands born in 1943 (10.4%) and lowest among probands born in 1947 (6.0%). Status as an emigrant or other reasons why a current address was not found did not differ by year of birth or period of exposure to famine. When we compared the birth records of participants traced to a current address with those who had either died, emigrated, or had not been located we found no clinically significant differences in mean birth weight (3350 compared with 3314 g) or length (50.4 compared with 50.2 cm), placental weight (601 compared with 592 g), maternal age at delivery (28.2 compared with 27.4 y), or birth order (2.3 compared with 2.3).

Differences between interviewed and noninterviewed persons

Of the 2300 persons who were invited to join the study, we found no significant differences between those interviewed to those who were not interviewed in mean birth weight (3374 compared with 3339 g) or length (50.5 compared with 50.3 cm), placental weight (600 compared with 601 g), maternal age at delivery (28.6 compared with 28.1 y), or birth order (2.4 compared with 2.2). The response to our invitation, however, was lower for those born in 1947 (25%) than in all others (35%). Eleven percent of those who were interviewed lived within 5 km of the examination site compared with 10% of those who were not interviewed, and 34% of those interviewed lived >45 km from the examination site compared with 29% of those who were not interviewed.

Final sample for analysis

We analyzed anthropometric data (except for skinfold thicknesses) from 956 persons. On the basis of their behavior and anthropometric measures, these persons appeared unremarkable for Dutch populations of this age (Table 1 and Table 2).
Exposure to famine and measures of length and body proportions

There was no evidence of a statistical interaction by sex in the association of maternal exposure to famine with measures of offspring length or their ratios (data not shown). There was no overall association between exposure to famine and these measures when famine was considered as a whole (Table 3); when considered as 4 periods of exposure, the ratio of the arm to leg lengths showed gestation-period—specific associations, which increased ($P < 0.10$) after exposure in weeks 21–30 and decreased ($P < 0.05$) after exposure in weeks 31 through delivery.

Skinfold thicknesses

In sex-pooled analyses (Table 6), the odds of being in the highest quartile of the subscapular skinfold thickness and the ratio of the subscapular to tricipital skinfold thickness were modestly elevated with any exposure to famine ($P < 0.10$). The test for interaction by sex was not significant ($P > 0.10$ in age-adjusted models) for any skinfold thickness. There was no strong indication of association with specific periods of exposure to famine.

Analyses on birth series alone

We repeated all analyses using the 645 participants with measures of birth weight and the 603 participants with measures of birth length. Results were very consistent with those reported for the whole sample (data not shown). In these groups, the results did not change when birth weight or birth length were included in the model (data not shown).

DISCUSSION

In a follow-up study of persons exposed during gestation to the Dutch famine of 1944–1945, we observed that maternal exposure to acute famine is associated with increases in several indexes of body mass and mass distribution among female offspring at age 59 y. We did not observe any strong independent association of prenatal exposure to famine with adult lengths or body proportions.

The circumstances of the Dutch famine provide a model to test for isolated effects of undernutrition at defined stages of development and do not speak to the situation in which inadequate prenatal nutrition is followed by continued undernutrition, as...
was until recently common in many developing countries. Exposure to famine, as we defined it in relation to official rations, is an ecologic measure of undernutrition; we lacked individual dietary intake data. However, evidence of the severity of the famine was abundant, including evidence that during the height of the famine pregnant women actually lost weight over the second half of their pregnancy (15). Thus, our data support the notion that maternal undernutrition in gestation, if postnatal nutrition and infections are not limiting, neither programs a person for an altered trajectory of linear growth if it occurs in early pregnancy nor results in a wider range of anthropometric dimensions and indexes. We observed some suggestion that the heterogeneity of associations between famine exposure and adult body mass and mass distribution between men and women is established only after the first 10-wk period of gestation. This may reflect the increasing importance of a wider range of anthropometric dimensions and indexes. We earlier studies considered only weight and height; we examined for chronic disease (24-26). Although BMI is widely used, it does

Two earlier studies of persons exposed to the Dutch famine in utero have yielded mixed results. Among men examined at age 18 y, the absolute risk of obesity (defined as ≥120% of the ideal weight for height according to the Metropolitan Life Insurance Company tables) was elevated from 1.5% to 2.8% with exposure to famine (18). Our study lacked the power to detect an effect of that small a magnitude. A study similar in design to ours found an elevated BMI in women aged 50 y whose mothers were exposed to the famine early in gestation, but there was no association with other periods of exposure to famine or among men (19). Our results are broadly consistent with that study insofar as we also observed a marked difference in associations between men and women, but we did not identify early gestation as being the critical window for effects in adulthood. A third study of the siege, or born in an area not subject to the siege (22). That study was unable to assess the timing of exposure to maternal undernutrition because the Leningrad siege lasted >2 y. All of the earlier studies considered only weight and height; we examined a wider range of anthropometric dimensions and indexes. We observed some suggestion that the heterogeneity of associations between famine exposure and adult body mass and mass distribution between men and women is established only after the first 10-wk period of gestation. This may reflect the increasing importance of sex-specific growth factors in fetal development (23). There is ongoing debate about the relative utility of the available indexes of body mass distribution in predicting risk for chronic disease (24-26). Although BMI is widely used, it does

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**TABLE 2**

Selected body measurements and ratios for Dutch men and women examined between 2003 and 2005, by famine exposure and sex

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposed to famine during gestation (n = 160)</td>
<td>Time control subjects (n = 137)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>177.4 ± 6.2‡</td>
<td>178.3 ± 6.3</td>
</tr>
<tr>
<td>Trunk length (cm)</td>
<td>92.6 ± 3.2</td>
<td>93.0 ± 3.2</td>
</tr>
<tr>
<td>Leg length (cm)</td>
<td>84.7 ± 4.3</td>
<td>85.3 ± 4.2</td>
</tr>
<tr>
<td>Arm length (cm)</td>
<td>66.8 ± 3.3</td>
<td>67.0 ± 3.7</td>
</tr>
<tr>
<td>Ratio of arm to leg lengths (× 100)</td>
<td>78.9 ± 3.1</td>
<td>78.6 ± 3.0</td>
</tr>
<tr>
<td>Ratio of leg to trunk lengths (× 100)</td>
<td>91.5 ± 4.5</td>
<td>91.7 ± 4.2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>87.6 ± 12.1</td>
<td>88.8 ± 13.4</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>100.5 ± 10.1</td>
<td>101.4 ± 10.5</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>102.7 ± 6.6</td>
<td>103.1 ± 6.9</td>
</tr>
<tr>
<td>Supine sagittal abdominal diameter (cm)</td>
<td>23.8 ± 3.0</td>
<td>23.9 ± 3.3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.8 ± 3.6</td>
<td>27.9 ± 4.0</td>
</tr>
<tr>
<td>Midshigh circumference (cm)</td>
<td>52.1 ± 3.8</td>
<td>52.5 ± 4.1</td>
</tr>
<tr>
<td>Ratio of waist to hip circumferences (× 100)</td>
<td>91.7 ± 5.5</td>
<td>93.8 ± 5.5</td>
</tr>
<tr>
<td>Ratio of supine sagittal abdominal diameter to midshigh circumference (× 100)</td>
<td>45.6 ± 4.8</td>
<td>45.5 ± 4.7</td>
</tr>
<tr>
<td>Ratio of waist to midshigh circumferences (× 10)</td>
<td>19.3 ± 1.5</td>
<td>19.3 ± 1.5</td>
</tr>
<tr>
<td>Subscapular skinfold thickness (mm)†‡</td>
<td>21.0 ± 8.3 [151]</td>
<td>19.0 ± 10.3 [136]</td>
</tr>
<tr>
<td>Triceps skinfold thickness (mm)†‡</td>
<td>12.9 ± 4.3</td>
<td>13.0 ± 6.6</td>
</tr>
<tr>
<td>Anterior midshigh skinfold thickness (mm)†‡</td>
<td>14.2 ± 7.5 [145]</td>
<td>15.4 ± 12.1 [127]</td>
</tr>
</tbody>
</table>

‡ Sample sizes for skinfold thicknesses include subjects in whom skinfold thicknesses were measured but for whom the skinfold thickness exceeded the caliper capacity.

§ All values are medians and interquartile intervals; n in brackets.

The 75th percentile for this group exceeded the maximum caliper capacity of 40 mm. The 25th percentile was 23.9 mm.
TABLE 3
Association of exposure to the Dutch famine overall or in the specified period of gestation with adult measures of length and with indexes of proportion for 956 persons examined between 2003 and 2005

<table>
<thead>
<tr>
<th>Period of gestational exposure</th>
<th>Overall (n = 350)</th>
<th>Weeks 1–10 (n = 74)</th>
<th>Weeks 11–20 (n = 124)</th>
<th>Weeks 21–30 (n = 140)</th>
<th>Week 31 to delivery (n = 128)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
</tr>
<tr>
<td>Length</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>-0.39, -1.11, 0.33</td>
<td>-0.30, -1.72, 1.13</td>
<td>-0.35, -1.51, 0.82</td>
<td>-1.01, -2.13, 0.11</td>
<td>0.51, -0.62, 1.63 NS</td>
</tr>
<tr>
<td>Trunk (cm)</td>
<td>-0.03, -0.41, 0.35</td>
<td>0.11, -0.62, 0.85</td>
<td>-0.12, -0.73, 0.49</td>
<td>-0.28, -0.87, 0.30</td>
<td>0.17, -0.41, 0.76 NS</td>
</tr>
<tr>
<td>Leg (cm)</td>
<td>-0.40, -0.90, 0.10</td>
<td>0.11, -1.45, 0.52</td>
<td>-0.26, -1.07, 0.55</td>
<td>-0.72, -1.50, 0.06</td>
<td>0.29, -0.49, 1.07 NS</td>
</tr>
<tr>
<td>Arm (cm)</td>
<td>-0.23, -0.65, 0.19</td>
<td>0.41, -1.21, 0.40</td>
<td>-0.13, -0.80, 0.54</td>
<td>-0.07, -0.71, 0.57</td>
<td>-0.13, -0.77, 0.51 NS</td>
</tr>
<tr>
<td>Indexes of proportion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio of arm to leg lengths (× 100)</td>
<td>0.10, -0.28, 0.49</td>
<td>0.07, -0.65, 0.79</td>
<td>0.26, -0.34, 0.87</td>
<td>0.58, 0.00, 1.16</td>
<td>-0.65, -1.22, -0.08 &lt;0.05</td>
</tr>
<tr>
<td>Ratio of leg to trunk lengths (× 100)</td>
<td>0.44, 1.00, 0.12</td>
<td>0.75, -1.83, 0.34</td>
<td>0.26, -1.16, 0.64</td>
<td>-0.53, -1.39, 0.34</td>
<td>0.30, -0.56, 1.17 NS</td>
</tr>
</tbody>
</table>

Values represent differences from control group (n = 606). Estimates were obtained by linear regression and were adjusted for sex, age, and clustering of siblings. Models for each specific 10-wk period of gestational exposure were also adjusted for exposure in overlapping 10-wk periods. Estimates for any exposure may reflect additive effects of exposure in specific periods. Tests for interaction by sex were not significant (P > 0.25 for each outcome).

Values reflect the overall test of association of all 4 periods of exposure considered as a group (Wald test, 4 df).

TABLE 4
Association of exposure to the Dutch famine overall or in the specified period of gestation with weight, circumferences, and indicators of body composition in adulthood for 427 men measured between 2003 and 2005

<table>
<thead>
<tr>
<th>Period of gestational exposure</th>
<th>Overall (n = 160)</th>
<th>Weeks 1–10 (n = 35)</th>
<th>Weeks 11–20 (n = 59)</th>
<th>Weeks 21–30 (n = 69)</th>
<th>Week 31 to delivery (n = 59)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
<td>Estimate 95% CI</td>
</tr>
<tr>
<td>Weight and circumferences</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.98, -1.22, 3.18</td>
<td>3.37, -0.73, 7.46</td>
<td>-1.63, -5.19, 1.93</td>
<td>2.08, -1.31, 5.47</td>
<td>-1.17, -4.49, 2.16 NS</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>0.51, -1.40, 2.42</td>
<td>1.82, -1.73, 5.37</td>
<td>-1.28, -4.37, 1.81</td>
<td>1.88, -1.06, 4.82</td>
<td>0.37, -3.25, 2.51 NS</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>0.73, -0.63, 2.90</td>
<td>1.94, -0.90, 4.15</td>
<td>-0.48, -2.38, 1.43</td>
<td>0.93, -0.89, 2.76</td>
<td>-0.43, -2.21, 1.36 NS</td>
</tr>
<tr>
<td>Supine sagittal abdominal diameter (cm)</td>
<td>0.20, -0.38, 0.79</td>
<td>0.84, -0.26, 1.93</td>
<td>-0.42, -1.37, 0.54</td>
<td>0.37, -0.54, 1.28</td>
<td>0.01, -0.88, 0.90 NS</td>
</tr>
<tr>
<td>Midchigh circumference (cm)</td>
<td>0.11, -0.64, 0.87</td>
<td>1.04, -0.34, 2.42</td>
<td>0.22, -0.99, 1.43</td>
<td>-0.07, -1.22, 1.07</td>
<td>0.73, -1.85, 0.40 NS</td>
</tr>
<tr>
<td>Indexes of mass distribution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.32, -0.37, 1.01</td>
<td>1.06, -0.23, 2.34</td>
<td>-0.49, -1.61, 0.63</td>
<td>0.66, -0.41, 1.72</td>
<td>-0.35, -1.40, 0.69 NS</td>
</tr>
<tr>
<td>Ratio of waist to hip</td>
<td>-0.27, -1.34, 0.80</td>
<td>0.01, -1.97, 1.98</td>
<td>-0.86, -2.59, 0.86</td>
<td>0.82, -0.82, 2.46</td>
<td>0.11, -1.50, 1.72 NS</td>
</tr>
<tr>
<td>circumferences (× 100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio of supine sagittal</td>
<td>0.31, -0.61, 1.23</td>
<td>0.70, -1.00, 2.40</td>
<td>-0.86, -2.34, 0.62</td>
<td>0.66, -0.75, 2.06</td>
<td>0.76, -0.62, 2.14 NS</td>
</tr>
<tr>
<td>abdominal diameter to</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>midchigh circumference (× 100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio of waist to midchigh</td>
<td>0.50, -2.47, 3.47</td>
<td>-0.17, -5.63, 5.29</td>
<td>-2.91, -7.68, 1.86</td>
<td>3.43, -1.10, 7.96</td>
<td>2.20, -2.25, 6.64 NS</td>
</tr>
<tr>
<td>circumferences (× 100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent differences from control group (n = 267). Estimates were obtained by linear regression and were adjusted for age, height, and clustering of siblings. Estimates for specific 10-wk periods of gestational exposure were also adjusted for exposure in overlapping 10-wk periods. Estimates for any exposure may reflect the additive effects of exposure in specific periods.

Values reflect the overall test of association of all 4 periods of exposure considered as a group (Wald test, 4 df).
TABLE 5
Association of exposure to the Dutch famine overall or in the specified period of gestation with weight, circumferences, and indexes of adiposity in adulthood for 529 women measured between 2003 and 2005

<table>
<thead>
<tr>
<th>Period of gestational exposure</th>
<th>Overall (n = 190)</th>
<th>Weeks 1–10 (n = 39)</th>
<th>Weeks 11–20 (n = 65)</th>
<th>Weeks 21–30 (n = 71)</th>
<th>Week 31 to delivery (n = 69)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight and circumferences</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>4.83</td>
<td>2.51, 7.14</td>
<td>3.98</td>
<td>0.53, 8.48</td>
<td>3.71</td>
<td>0.04, 7.39</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>4.69</td>
<td>2.66, 6.72</td>
<td>1.96</td>
<td>-1.93, 5.85</td>
<td>2.83</td>
<td>0.64, 7.02</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>4.37</td>
<td>2.58, 6.15</td>
<td>2.86</td>
<td>-0.66, 6.38</td>
<td>3.81</td>
<td>0.96, 6.66</td>
</tr>
<tr>
<td>Supine sagittal abdominal diameter (cm)</td>
<td>1.52</td>
<td>0.91, 2.13</td>
<td>1.00</td>
<td>-0.18, 2.18</td>
<td>1.13</td>
<td>0.17, 2.10</td>
</tr>
<tr>
<td>Midthigh circumference (cm)</td>
<td>1.61</td>
<td>0.65, 2.57</td>
<td>1.01</td>
<td>-0.86, 2.88</td>
<td>1.40</td>
<td>-0.12, 2.92</td>
</tr>
<tr>
<td>Indexes of mass distribution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1.85</td>
<td>1.01, 2.69</td>
<td>1.44</td>
<td>-0.21, 3.09</td>
<td>1.45</td>
<td>0.11, 2.80</td>
</tr>
<tr>
<td>Ratio of waist to hip circumferences (× 100)</td>
<td>0.89</td>
<td>-0.15, 1.83</td>
<td>0.09</td>
<td>-1.80, 1.99</td>
<td>0.33</td>
<td>-1.22, 1.88</td>
</tr>
<tr>
<td>Ratio of supine sagittal abdominal diameter to midthigh circumference (× 100)</td>
<td>1.54</td>
<td>0.69, 2.39</td>
<td>1.28</td>
<td>-0.35, 2.91</td>
<td>0.90</td>
<td>-0.44, 2.23</td>
</tr>
<tr>
<td>Ratio of waist to midthigh circumferences (× 100)</td>
<td>3.34</td>
<td>0.38, 6.29</td>
<td>1.84</td>
<td>-3.82, 7.50</td>
<td>1.79</td>
<td>-2.85, 6.44</td>
</tr>
</tbody>
</table>

1 Values represent differences from control group (n = 339). Estimates were obtained by linear regression and were adjusted for age, height, and clustering of siblings. Estimates for specific 10-wk periods of gestational exposure were also adjusted for exposure in overlapping 10-wk periods. Estimates for any exposure may reflect the additive effects of exposure in specific periods.
2 Values reflect the overall test of association of all 4 periods of exposure considered as a group (Wald test, 4 df).
3 P < 0.01.
4 P < 0.10.
5 NS
6 P < 0.05.

It is possible that participation bias may have led to our findings if heavy women with famine exposure were more likely to not associated with overall mortality (33); all these conditions have shown associations with adiposity. Thus, future research needs to consider how differences in adiposity consequent to exposure to famine during gestation, including differences in the distribution of lean and adipose tissue throughout the body, might mediate any effect of the famine on risk of disease.

TABLE 6
Association of exposure to the Dutch famine overall or in the specified period of gestation with selected skinfold thicknesses in adulthood for persons measured between 2003 and 2005

<table>
<thead>
<tr>
<th>Period of gestational exposure</th>
<th>Overall</th>
<th>Weeks 1–10</th>
<th>Weeks 11–20</th>
<th>Weeks 21–30</th>
<th>Week 31 to delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio</td>
<td>95% CI</td>
<td>Odds ratio</td>
<td>95% CI</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>Subscapular (n = 929)</td>
<td>1.38</td>
<td>0.95, 1.99</td>
<td>0.87</td>
<td>0.43, 1.76</td>
<td>1.35</td>
</tr>
<tr>
<td>Tricipital (n = 935)</td>
<td>1.30</td>
<td>0.89, 1.89</td>
<td>1.50</td>
<td>0.75, 3.00</td>
<td>1.16</td>
</tr>
<tr>
<td>Anterior midthigh (n = 760)</td>
<td>1.21</td>
<td>0.80, 1.81</td>
<td>1.18</td>
<td>0.57, 2.47</td>
<td>1.10</td>
</tr>
<tr>
<td>Ratio of subscapular to tricipital (n = 916)</td>
<td>1.55</td>
<td>1.08, 2.22</td>
<td>1.07</td>
<td>0.55, 2.08</td>
<td>1.67</td>
</tr>
</tbody>
</table>

1 Estimates were obtained by logistic regression and were adjusted for sex, age, height, and clustering of siblings. Tests for interaction by sex were not significant (P > 0.10). Estimates for specific 10-wk periods of gestational exposure were also adjusted for exposure in overlapping 10-wk periods.
2 Odds ratios are for the highest quartile compared with all others.
3 P < 0.01.
4 NS
5 Odds ratios are for the group in which the subscapular skinfold is in a higher quartile than is the tricipital skinfold compared with all others.
6 P < 0.05.
participate in our study than were heavy women with no famine exposure. We have no method to test for this potential bias, however. We note that participation rates did not differ by sex or by distance from the examination site. It is also possible that parental characteristics associated with offspring adiposity differed by period of maternal exposure to famine. The effect of such bias was minimized in our study because we selected control subjects from among siblings born outside of the famine period (thus controlling for genetic sources of variation in adult adiposity) and among births in the same institutions (thus minimizing social class differences between exposed and unexposed persons). Adjustment for several variables that are themselves predictors of adiposity, including measures of energy balance and, in women, parity, did not affect our measures of association between famine exposure and body mass distribution of the offspring.

In conclusion, exposure to the Dutch famine was strongly associated with a wide range of indexes of body mass distribution in middle-age women, and it was not associated with these indexes in men or with measures of length or body proportions in either men or women. These data suggest sex-specific, long-lasting effects of maternal undernutrition during pregnancy.

We thank the Vroedvrouwenscholen of Amsterdam and Rotterdam and the Maternity-Department of the Leiden University Medical Center for their help in accessing their archives. The clinical examinations were carried out at the study center of Gerontology & Geriatrics, Leiden University Medical Center, under supervision of L. de Man (head of study center).

LHL, ADS, and HSK developed the study hypothesis and study protocols, designed the study, and developed and coordinated all data collection activities. LHL obtained the major funding. ADS conducted the data analysis and designed the study, and developed and coordinated all data collection activities. AR participated in the data analysis and interpretation. KvdP participated in the development of the data collection protocols, and managed the files and data cleaning and participated in the data interpretation. All authors reviewed and approved the final version of the manuscript. None of the authors declared any financial conflict of interest.

REFERENCES

Prenatal Famine and Adult Health

L.H. Lumey,1 Aryeh D. Stein,2 and Ezra Susser1,3

1Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, New York 10032; email: lumey@columbia.edu
2Hubert Department of Global Health, Rollins School of Public Health, Emory University Atlanta, Georgia 30322
3New York State Psychiatric Institute, New York, New York 10032

Keywords
maternal nutrition, prenatal exposure delayed effects, Dutch famine, Great Leap Forward famine, Siege of Leningrad

Abstract
We review human studies on the relation between acute exposures to prenatal famine and adult physical and mental health. These studies are observational and include exposures to a famine environment by natural or man-made causes or, more commonly, from the interplay between natural and human factors. These natural experiments provide an opportunity to examine long-term outcomes after famine exposures by comparing exposed and nonexposed individuals.

The studies show consistent associations between prenatal famine and adult body size, diabetes, and schizophrenia. For other measures of adult health, findings are less robust. A relation between prenatal famine and some reported epigenetic changes may provide a potential mechanism to explain specific associations. Much progress can be made if current separate studies are further analyzed with comparable definitions of exposures and outcomes and using common analytic strategies.
INTRODUCTION

A comprehensive narrative of famines over time and of society’s responses to them was recently published by O’Grada (80). He provides a wealth of empirical data to understand better their manifold causes and outcomes. Although the short-term consequences of famine are abundantly clear, scholars are also increasingly interested in assessing potential long-term consequences for which systematic reviews are still lacking.

In this review we focus on nonchronic exposures to prenatal famine and their potential effects on adult physical and mental health. Some experimental studies have considered the immediate effects of (semi) starvation in young adult volunteers (9, 52), but for obvious ethical reasons, we have no such studies on the long-term effects of prenatal starvation of the growing fetus. The studies we review have all been observational. They generally include an involuntary exposure to famine from natural or man-made causes or, more commonly, from the interplay between natural and human factors. These natural experiments offer the opportunity to learn much from the follow-up of exposed and nonexposed individuals. Here we do not review studies of chronic or sustained conditions of pregnancy undernutrition and their relation to adult health. A poor diet may indeed contribute to low birth weights in many parts of the world (16, 44, 75, 124), but only in a quasi-experimental setting will it be possible to separate with some confidence the effects of social, economic, and family conditions on adult health outcomes from the effects of nutrition itself (69).

In Table 1, we summarize the settings that have been used to study long-term effects of prenatal famine. These include nineteenth-century crop failures in Sweden and Finland, the Siege of Leningrad of 1941–1944, the Dutch Hunger Winter of 1944–1945, seasonal famines in the Gambia between 1949 and 1994, the Chinese Great Leap Forward famine of 1959–1961, and recent seasonal famines in Bangladesh. Famines that may still allow a systematic follow-up include the Soviet (and Ukraine) famine of 1931–1933 as well as severe undernutrition in Greece and the Channel Islands during German Occupation in WWII, in Germany itself at the end of WWII, and in the early postwar period. In other famine settings, such as in the Warsaw ghetto in WWII, only a small number of people survived (132). Tables 1 and 2 provide descriptions of the study settings (Table 1) and of the study populations and outcomes studied to date (Table 2).

The simplest way of classifying individuals by likely famine exposure is by establishing their presence in a famine setting in well-defined periods. Personal interviews regarding past events usually will not be reliable enough to assess the degree of famine exposure and will often not even be possible. Historical studies usually cannot provide estimates of individual food intake in a famine environment but may help to estimate food intake at the group level. This between-group comparison can be accurate enough to differentiate populations by degree of famine exposure.

Our main interest is in the study of the long-term consequences of exposure to famine during gestation. We also note that reports from the Nordic countries during WWII (3), the Siege of Leningrad (54, 55, 107), and the Dutch Hunger Winter (26–28, 30, 128) describe individuals with postnatal famine exposure (e.g., during infancy, childhood, adolescence, or young adulthood), but these groups fall outside the scope of our review. We also exclude the study of cancers among Israeli Jewish survivors of WWII (50) because in this study prenatal famine exposure was defined by residence in one of the countries under a Nazi regime alone.

DESIGN CONSIDERATIONS

Studies of the long-term consequences of famine during gestation have looked at total mortality, at the incidence and prevalence of morbidity and of risk factors for morbidity, and
Table 1  Prenatal famine study settings

<table>
<thead>
<tr>
<th>Place</th>
<th>Famine years</th>
<th>Circumstances that led to famine</th>
<th>Estimated number of deaths from undernutrition</th>
<th>Key references</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1800, 1809, 1812, 1821, 1829–1837, 1831, and 1856</td>
<td>Overkalix parish crop failures</td>
<td>Not estimated</td>
<td>Kaati et al. 2002 (45)</td>
</tr>
<tr>
<td>Finland</td>
<td>1866–1868</td>
<td>Three successive crop failures</td>
<td>~8% of 1.7 million population</td>
<td>Kannisto et al. 1997 (49)</td>
</tr>
<tr>
<td>Soviet Union</td>
<td>1931–1933</td>
<td>Natural causes with poor economic policies and political neglect</td>
<td>~4–6 million?</td>
<td>Wheatcroft 2004 (131)</td>
</tr>
<tr>
<td></td>
<td>1941–1944</td>
<td>Siege of Leningrad, WWII blockade by German army</td>
<td>0.7–1.2 million?</td>
<td>Salisbury 1969 (101), Barber &amp; Dzeniskevich 2005 (6)</td>
</tr>
<tr>
<td>Greece</td>
<td>1941–1944</td>
<td>German occupation in WWII</td>
<td>Not estimated</td>
<td>Valaoras 1946 (40), Hionidou 2002 (127)</td>
</tr>
<tr>
<td>Western</td>
<td>1944–1945</td>
<td>Dutch Hunger Winter; WWII blockade by German army and national railway strike</td>
<td>20,000–30,000?</td>
<td>Dols &amp; van Arcken 1946 (12), Burger et al. 1948 (29), Stein et al. 1975 (121), Trienekens 2000 (126)</td>
</tr>
<tr>
<td>Netherlands</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Channel</td>
<td>1940–1945</td>
<td>WWII German occupation</td>
<td>Not estimated</td>
<td>Ellison et al. 2005 (31)</td>
</tr>
<tr>
<td>Islands</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>1944–1949</td>
<td>Food shortages during civil and military disorganization and Allied bombings at the end of WWII. Also during early postwar Allied occupation, from increased food needs of returning military and civil populations</td>
<td>Over 100,000?</td>
<td>Medical Research Council 1951 (74), Grontzki &amp; Niewerth 2007 (35)</td>
</tr>
<tr>
<td>The Gambia</td>
<td>1949–1994</td>
<td>West Kiang region births in the hungry versus the harvest season</td>
<td>Not estimated</td>
<td>Moore et al. 1997 (77)</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>1974–2000</td>
<td>Matlab region births in the hungry versus the harvest season</td>
<td>Not estimated</td>
<td>Moore et al. 2004 (78)</td>
</tr>
<tr>
<td>Setting</td>
<td>Source of study sample</td>
<td>Outcomes studied</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>--------------------</td>
<td></td>
</tr>
</tbody>
</table>
| Sweden, nineteenth-century crop failures | Skelleftea parish births 1805–1849 alive at age 40,  
\( n = 7,572 \)                                                                 | 2,715 deaths from all causes and 312 sudden deaths ("stroke") from cerebrovascular and cardiac causes between ages 40 and 70                                                                                                             | Bygren et al. 2000 (13) |
| Finland, 1866–1868 crop failures | Finnish live births 1861–1876,  
\( n = 816,977 \)                                                                 | Mortality between ages 0 and 17, through age 40, 60, and 80 years and mean lifetime after age 17 and 80 years comparing 331,932 individuals born before, 161,744 born during, and 323,321 born after the famine using Finnish national vital statistics data. All subjects followed until death. | Kannisto et al. 1997 (49) |
| Siege of Leningrad, 1941–1944    | Local registers,  
\( n = 169 \) individuals exposed in utero in Leningrad based on date and place of birth,  
\( n = 192 \) individuals exposed before the rationing began, and  
\( n = 188 \) place controls born in the same time period but outside the area of the siege | Glucose tolerance, blood pressure, lipids, anthropometry, and other measures at age \( \sim \) 52 years                                                                                                                                   | Stanner et al. 1997 (109) |
| Dutch Hunger Winter, 1944–1945   | Births 1944–1946 in selected famine cities (\( n = 146,347 \)) and control cities (\( n = 60,979 \)). Exposure defined by place and date of birth and based on historical records of the famine. Some outcomes were restricted to male births in famine cities (\( n = 74,927 \)). | Mental performance, obesity, antisocial personality disorder, and a schizophrenia spectrum (ICD-9 schizoid) personality disorder in national military examination records for males aged \( \sim \) 18 years. Time and place controls (mental performance, obesity) and time controls (antisocial, schizoid) based on place and date of birth 1944–1946. | Stein et al. 1972 (120)  
Stein et al. 1975 (121)  
Ravelli et al. 1976 (94)  
Hoek et al. 1996 (41)  
Neugebauer et al. 1999 (79) |
Brown et al. 2000 (11) |
|                               | Wilhelmina Gasthuis (WG) hospital, Amsterdam, delivery records 1960–1984 of mothers born between 1944 and 1946 in the Netherlands,  
\( n = 1,808 \) infants                                                | Birth weight and gestation in the offspring, exposure defined by mother’s place and date of birth                                                                                                                                    | Lumey 1992 (59)       |
<table>
<thead>
<tr>
<th>Location</th>
<th>Sample Description</th>
<th>Methodology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilhelmina Gasthuis (WG) hospital, Amsterdam, delivery records 1944–1946, ( n = 1,116 ) live-born singleton girls</td>
<td>683 traced survivors were interviewed at home at age ( \sim 43 ) years and provided reproductive outcomes on 1,299 offspring</td>
<td>Lumey et al. 1993 (61)</td>
<td></td>
</tr>
<tr>
<td>Wilhelmina Gasthuis (WG) hospital, Amsterdam, births 1943–1947, ( n = 2,414 ) live-born singletons</td>
<td>741 traced survivors had a clinical examination at age ( \sim 50 ) years and 810 had a home visit and a clinic examination at age ( \sim 58 ) years. Cohort incorporates births from previous WG follow-up (Lumey et al. 1993) (61).</td>
<td>Ravelli et al. 1998 (92) de Rooy et al. 2006 (23)</td>
<td></td>
</tr>
<tr>
<td>Amsterdam and Rotterdam midwife training schools and Leiden University hospital, births 1944–1946, ( n = 3,307 ) live-born singletons</td>
<td>751 traced survivors had a telephone interview and 658 had a clinical examination at age ( \sim 59 ) years. Additionally, 324 same-sex siblings of survivors had a telephone interview and 313 had a clinic examination.</td>
<td>Lumey et al. 2007 (68)</td>
<td></td>
</tr>
<tr>
<td>The Gambia, seasonal famines 1949–1994</td>
<td>West Kiang region, ( n = 3,162 ) births</td>
<td>1,077 deaths until 1994, and 1,103 until 2000, comparing births in the annual harvest versus the hungry season. Subset of 1,842 births and 58 deaths followed beyond the age of 15 years. Case-control analysis with 61 adult deaths matched for sex and year of birth to two controls from cohort.</td>
<td>Moore et al. 1997 (77) Moore et al. 2004 (78)</td>
</tr>
<tr>
<td>Two-per-Thousand National Fertility Survey (1988) of ever married women aged 15–57 years, women’s reproductive histories relating to infants born between 1954 and 1967, ( n = 122,352 ) women with ( n = 343,973 ) births</td>
<td>Mortality through age 35 estimated by birth cohort, using women’s reproductive histories to estimate birth counts and survival and census information to estimate aggregate population counts</td>
<td>Cai et al. 2005 (14) Song 2009 (105)</td>
<td></td>
</tr>
<tr>
<td>China National Health and Nutrition panel survey (1989–2002) providing individuals born before, during, and after the famine period, ( n \sim 3,400–3,800 ) households</td>
<td>Attained height and income or body mass index (BMI: kg/m(^2)) of 25 or more or obese (BMI 30 or more) and attained height and income</td>
<td>Chen &amp; Zhou 2007 (15) Luo et al. 2006 (70) Yang et al. 2008 (134) Li et al. 2010 (56)</td>
<td></td>
</tr>
</tbody>
</table>

(Continued)
Table 2 (Continued)

<table>
<thead>
<tr>
<th>Setting</th>
<th>Source of study sample</th>
<th>Outcomes studied</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese National Disability Survey (1987) births 1955–1965 provide cohorts born immediately before, during, and immediately after the famine period, n = 294,365</td>
<td>Individuals with disabilities (including mental disorders) were identified in the first step of a household interview. A separate questionnaire was administered to those identified with a disability for further identification. 494 individuals aged 22–32 years were classified with schizophrenia</td>
<td>Song et al. 2009 (106)</td>
<td></td>
</tr>
<tr>
<td>Women recruited into the China-U.S. Collaborative Project for Neural Tube Defect Protection (1993–1996) to evaluate efficacy of periconceptional folic acid supplementation to prevent neural tube defects in new born infants. Selected births 1957–1963, n = 35,757</td>
<td>Womens’ information (predelivery height, weight, education, occupation, hypertension, and county of birth) was derived from the medical examination at registration and newborn information (sex, birth order, birth length, and birth weight) and mother’s age at delivery immediately after birth</td>
<td>Huang et al. 2010 (43) Hunag et al. 2010 (42)</td>
<td></td>
</tr>
<tr>
<td>Bangladesh, seasonal famines 1974–2000 Matlab region, n = 172,228 births</td>
<td>24,697 deaths until 2000 comparing births in the harvest versus hungry season, and subset of 59,834 births and 252 deaths followed beyond the age of 15 years</td>
<td>Moore et al. 2004 (78)</td>
<td></td>
</tr>
</tbody>
</table>
Population-based registries can also be helpful to study outcomes among exposed and unexposed groups. Military records were used, for instance, to study mental performance of Dutch conscripts examined at age 18 years (120), and psychiatric registers were used for the study of schizophrenia risk up to midlife (122). In China, researchers used psychiatric hospital records from specific birth regions to classify individuals with and without likely famine exposure during critical periods in life. This method was appropriate because migration was strictly limited during the study period, and the regions had no alternative psychiatric services for this condition (108, 133). The use of registries assumes that censoring because of death or migration has not created selection bias, a reasonably safe assumption that was explicitly examined in the Dutch conscript study (121). In the Chinese studies, mortality was examined and appeared unlikely to have been a source of bias, but the assumption rests on somewhat weaker ground.

Another way to identify exposed and unexposed study subjects is through historical birth records in selected clinics. Individuals can then be followed over time through the present date for collection of new data and also for future studies. In some settings—such as the Netherlands, with its exceptional population registers—the pattern of deaths among cohort members up to the time of new data collection can also be examined (23, 61, 68, 92). Study power in these follow-up studies is limited by the number of births at the selected institutions. In some settings, a relatively large number of deaths had already taken place at the time of follow-up, and these can then be examined. Examples are the mortality patterns after seasonal famines in the Gambia and Bangladesh (77, 78).

Cross-sectional designs are also feasible. In a survey (usually of adults) at a given point in time, each individual is classified as exposed or unexposed to prenatal famine (e.g., by region and date of birth) and as having or not having the outcome of interest at the time of the survey. National surveys in China conducted between 1987 and 2002 (14, 70, 105) and the study of pregnant women recruited into the China-U.S. Collaborative Project for Neural Tube Defect Protection (1993–1996) have been used to study long-term health effects of prenatal exposure to the Great Leap Forward famine of 1959–1961 (42, 43). The China-U.S. Collaborative project was originally set up to evaluate the efficacy of periconceptional folic acid supplementation to prevent neural tube defects in newborns.

In a cross-sectional design, it is not possible to relate the numerator (people with the outcome) to a defined denominator (study cohort at risk for the outcome), and cause-effect relations between some study measures may be unclear because they are not observed over time. In the study of the consequences of prenatal famine exposure during the Siege of Leningrad (109), the exposed and unexposed individuals were even sampled using different approaches with different but unknown underlying denominators. This variation further complicates interpretation of the study findings. Despite these limitations, cross-sectional designs can be informative.

**ANALYTIC STRATEGIES**

As noted earlier, the most common analytic strategy is to compare famine-exposed individuals with people born earlier or later in the same location. The estimates are typically adjusted for risk factors related to the outcome (age, gender, parental and own social class, etc.), and the implicit assumption is that changes in a woman’s ability to conceive during a famine have not introduced spurious associations. This notion is important because ovulation and fecundity are clearly reduced under famine conditions.

Several investigators have used a difference-in-difference analytic approach to strengthen the time-based approach (43, 70, 121). In this analysis, outcomes among multiple populations, which vary in exposure intensity, are compared over time. In Holland, for example, patterns over time in the exposed west are contrasted with patterns over time in the unexposed north and south (121). In China, outcomes for selected regions or counties exposed to severe
and less severe famine were compared over time (43, 70). Outcome differences by famine exposure period are then more likely to be related to the famine itself rather than to other reasons. The difference-in-difference approach may still be biased, however, because it still assumes that other relevant characteristics of the comparison populations have not changed in relation to the famine and nonfamine periods.

Sibling designs can also strengthen causal inference in epidemiologic studies and have been effective in ruling out or detecting confounding in many contexts (5, 95, 123). In a study of the Dutch famine, Lumey et al. (68) recruited same-sex full-sibling pairs with one sibling exposed to famine and the other not exposed. This design is facilitated by adequate family sizes in Holland, but strictly speaking, the results apply only to families with sibling pairs. Because mortality increases with age, an available sibling is also on average more likely to be somewhat younger rather than older compared with the exposed member of the birth series. This notion might be of concern for studies of health outcomes that are age-related, but it will not affect characteristics that are fixed across the life course, such as fingerprint patterns (47, 48) or perhaps indices of DNA methylation (39, 125). In the studies by Lumey et al., about one-half of the birth series were recruited with a sibling and the other half as individuals without a sibling. This method provides for many sibling pairs with one exposed and one unexposed individual, where the possible effect of individual maternal characteristics is controlled by design. With this hybrid model, it is possible in theory to separate maternal (selection) effects from famine effects when evaluating offspring health outcomes. In practice, however, the relatively small sample sizes in this setting seldom provide enough study power to do this effectively.

**Exposure Definitions**

Every study to date has considered famine exposure as an ecological measure because there are no data on individual food intakes. This measure has been extensively validated, however, through its strong correlation with fertility (119), pregnancy weight gain (113), size at birth (4, 102, 104, 116, 118), and mortality (49). In the Dutch famine, the well-defined famine period lasted approximately six months. Within this window, conditions deteriorated over time with further decreases in the rations and a progressive depletion of body stores until liberation in early May 1945 (12, 126). This window of time provides an opportunity to look at outcomes in terms of cumulative exposures and in relation to the stage of pregnancy itself.

In most studies of the Dutch famine, prenatal famine exposure was defined relative to date of birth, assuming a gestation of 40 weeks for each pregnancy. In some instances, by using births from specific institutions where birth records were still available, the timing of famine exposure could be more precisely characterized, using mothers’ reported last menstrual period (LMP) instead of the child’s birth date to estimate the time of conception (68). As illustrated elsewhere in more detail (68), there is some variation in the exact timing of the early, middle, and late gestation periods by date of birth or mother’s LMP in different studies of the famine. Lumey et al. (68), for instance, used LMP to define four partially overlapping 10-week periods in gestation to classify the timing of exposure. In some other studies, the focus has been on exposure at the extreme end of the famine or during the periconceptional period (39, 122). These approaches show a substantial overlap in the assignment of broad categories of exposure.

Although the primary exposure during famine is food restriction, other exposures are likely to have been present as well. The Dutch Famine, for example, took place in the setting of war, precipitated by interactions between the German occupation forces and Dutch activities in support of the Allied forces. It also occurred during a particularly cold winter. Toward the end of the famine, some people resorted to food substitutes (e.g., tulip bulbs) that can be potentially toxic. The range of exposures, therefore, includes the stresses of war and occupation,
extremes in cold temperatures, food substitutes, and undernutrition. These conditions were also present during the Siege of Leningrad, but the city was also under artillery fire (6). In the Gambia, the pattern of undernutrition is different. It is seasonal and coincides with a period of increased energy expenditure (77). Although the changes in nutrition remain a key factor, the pattern in the Gambia may be associated with lower levels of stress for the affected individuals, which could provide better opportunities for individuals to make some advance preparations. The Gambia findings, therefore, will not always be comparable to the Dutch Hunger Winter.

So far, no study has been able to assess the independent contribution of any of the co-existing conditions mentioned above or evaluate prospectively the role of postnatal circumstances in shaping adult health outcomes. Where postnatal information is available, it usually comes from interviews with respondents during the follow-up assessment, and it is not based on independent contemporary observations. We believe, however, that the consistent findings across different famines—where the nature of the associated conditions and any toxic food substitutes are likely to be so very different—provide strong support for a dominant contribution of starvation itself to the relevant exposure.

Studies in Finland and Sweden represent alternative approaches to defining famine exposures. In Finland, the investigators linked mortality data to historical records of crop failures, of which the year 1867 represented the most severe in a long cycle (49). Life-table methods were used to relate survival of individuals born in that year to those born in surrounding years. Strengths of this design include the large, national sample, all of whom had died by the time of the study, which avoids any data censoring. Limitations include the lack of information on morbidity or on selected behaviors that might explain differences in mortality. Investigators also assumed that migration from Finland was not associated with an individual’s year of birth. In Sweden, the regional Overkalix study (45) represents a multigenerational genealogical investigation. Although limited by the available data (church records of births, deaths, and crop failures in the paternal and grand paternal generations) and small study size, the study presents an intriguing approach to evaluate sex-specific transmission of effects experienced not during gestation but in previous generations.

**STUDY OUTCOMES**

In the sections below, we summarize the findings on adult physical and mental health after prenatal famine in chronological order, starting with markers that find their origin in the gestation period but may still be present in adulthood, such as fingerprints, selected epigenetic changes, and sex-ratio at birth. We then examine specific adult health outcomes, a woman’s fertility, and for men and women we consider adult height and weight, glucose metabolism, blood pressure, lipid profiles, the metabolic syndrome, cardiovascular outcomes, self-reported health, mental performance and cognition, and mental disorders. We review studies of adult mortality and possible inter-generational effects and, for completeness, also note studies in other health areas not previously mentioned. We then come to our conclusions.

**Markers from Gestation: Fingerprints and Epigenetic Changes**

Fingerprints and fingertip ridge counts have a significant genetic component and also reflect the nongenetic environment of early pregnancy because they are permanently configured before the twentieth week of gestation. There is a relation between prenatal famine exposure and the fingerprint pattern in Dutch adults (48). In a further study in this population, diabetes mellitus diagnosed at age 50+ years was associated with fingerprints, irrespective of birth weight (47).

As a working hypothesis, epigenetic changes during gestation might explain longer-term effects on phenotype (130), but empirical data on associations with prenatal famine are still
scarce. Recent data showed that individuals with periconceptual exposure to the Dutch famine at age \(\sim 59\) years had less DNA methylation of the imprinted \(IGF2\) gene compared with their unexposed, same-sex siblings. These findings suggest that early-life environmental conditions can cause epigenetic changes in humans that persist throughout life. No such changes were seen in individuals who were exposed at the end of pregnancy, when a decrease in birth weights is seen (39). Further evaluations indicate that epigenetic changes may be common even if the effects at individual loci are small. Effects may also greatly depend on the timing of the exposure in relation to trimester of gestation and may be gender specific (125). These findings provide a strong rationale to describe these relations in other study samples and to carry out well-designed epigenome-wide studies to discover and catalog epigenomic regions that are sensitive to the prenatal environment. Developmental influences on common human diseases can then be better evaluated (38).

### Sex Ratio at Birth

Temporal variations in the sex ratio or the ratio of boys over girls at birth have been widely studied and variously attributed to social changes, conditions of war, and environmental changes (34, 129). Specific claims have also been made about direct effects on the sex of the newborn of maternal nutrition in general or during pregnancy (2, 33, 73). None of these reported associations can be replicated with data from the wartime famine in Holland in 1944/1945 (18, 115). Thus a causal link is highly improbable.

### Women’s Fertility

In the Dutch famine birth cohorts, no reduction in cumulative fertility to age 43 years was found by Lumey et al. (62) among 700 women exposed in pregnancy, but a higher next-generation mortality rate was seen among offspring of women exposed in late gestation. In an update of the study, conducted by Painter et al. (89) among 473 women when the cohort was age 50 years with slightly different time windows to characterize exposure, women exposed to famine at any time in gestation showed increased fertility. There is no clear explanation for the discrepancies in the estimates from the two studies. They may have resulted from minor differences in the selected study populations and in the definitions of famine exposure (64). If the ability to conceive has a familial component, women with a higher ability to conceive are likely to be overrepresented among famine births, which will then also show as higher fertility in their daughters. This mechanism should be more fully explored.

### Height and Weight

In Dutch recruits examined at age 18 years, G.P. Ravelli et al. (94) reported that 2.8% of recruits with prenatal famine exposure in early pregnancy were obese. The term obese was defined as having a weight to height ratio of more than 120% compared with a reference population. The overall prevalence of obesity in the cohort as so defined was 1.7%. Further information on height, weight, or body mass index (BMI) of the recruits was not provided in the report. Pregnant women recruited for the U.S.-China folic acid supplementation study who themselves had been exposed to the Great Famine during gestation or in very early childhood show some decrease (\(\sim 1.5\) cm) in height, but only when exposed as infants (43).

A.J.C. Ravelli et al. (93) reported an increase in body weight, BMI, and waist circumference at age 50 years in women, but not in men with prenatal famine exposure. The increase was largest in women exposed in early gestation. Other birth cohorts from clinics in Amsterdam, Rotterdam, and Leiden examined at age 59 show a similar pattern, with differences in women but not in men after famine exposure at any stage of pregnancy. There was no statistical difference in measures of body size or composition by stage of gestation. Measures of length and linear proportions were not affected in either men or women (110).
Women but not men born during the famine years of the Chinese Great Leap Forward and surveyed in the 1990s were more likely to be overweight (here defined as having a BMI of 25 kg/m² or more) compared with individuals born thereafter (70, 134). In these studies, the overall prevalence of being overweight among the women was 20%–30%. Women from the U.S.-China folic acid supplementation study also showed some increase in prepregnant BMI compared with unexposed controls (43).

These studies suggest that prenatal famine exposure may be associated with a higher body weight, BMI, and waist circumference in women, but not in men. In the study of military male recruits aged ∼18 years in the Netherlands (94), investigators saw an increase of individuals with an extreme weight/height ratio, but relations with more common measures of obesity defined by BMI increases have not yet been reported in this population. Further work is needed, therefore, to compare findings across studies using uniform definitions for obesity and other outcomes. It is also important to explore further the role of exposures at different times in pregnancy using uniform definitions and to refine possible gender-specific effects.

Glucose Metabolism

In the cross-sectional study of men and women exposed in utero to the Siege of Leningrad (1941–1944), individuals born in Leningrad just before the rationing began did not differ by measures of fasting glucose or 2-h glucose after a glucose challenge compared with individuals born outside the siege area (109).

In Dutch famine birth cohorts from the Wilhelmina Gasthuis hospital in Amsterdam, mean 2-h glucose concentrations after a standard glucose load were elevated in famine-exposed men and women aged ∼50 years, compared with unexposed controls, especially when exposure occurred in late gestation. Investigators found no differences in baseline fasting glucose levels, in the 30-min glucose response, or in the diagnosis of type 2 diabetes mellitus (92). At age ∼58 years, 2-h glucose levels were equally elevated in individuals with early, mid, and late gestation exposure compared with controls (23). Combining individuals with impaired glucose tolerance and diabetes mellitus into a single disease category, the prevalence of this condition differed by PPAR-gamma 2 genotype status but only for individuals with mid-gestation famine exposure (22). In a subset of participants, investigators could evaluate changes over time in the glucose responses, comparing the findings at ages 50 and 58 years in the same individuals. Although the mean 2-h glucose values had increased over time, there was no association between the rate of progression and famine exposure (23).

Further examinations by a multisample intravenous glucose tolerance test in a subset of the cohort showed differences in some but not all measures of insulin secretion or insulin sensitivity after famine exposure in mid-gestation, after adjustment for sex and BMI (21).

In Dutch famine births cohorts from Amsterdam, Rotterdam, and Leiden examined at age 59 years, prenatal famine exposure was associated with a higher prevalence of type 2 diabetes but not impaired fasting glucose (IFG; 5.6–7.0 mmol/L) or impaired glucose tolerance (IGT: fasting glucose <7.0 mmol/L and 2-h glucose 7.8–11.1 mmol/L after a glucose challenge) (66).

Chinese men and women examined in the 2002 National Nutrition and Health Survey showed a similar pattern, with an increased prevalence of hyperglycemia (defined as an increase in fasting glucose, a decrease in 2-h glucose tolerance, or an increase in type 2 diabetes mellitus) among participants born in the more severely, compared with less severely, affected famine areas (56).

Overall, the above studies suggest an association between prenatal famine and the response to a glucose challenge, and there could also be an association between prenatal famine exposure and diabetes mellitus. Further work is needed to compare findings across studies using uniform exposure and outcome definitions. This work may also promote a better
understanding of the role of famine exposure at different times in pregnancy.

**Blood Pressure**

In the Leningrad study, no differences in blood pressure were seen in the various exposure groups (109).

In Dutch birth cohorts from the Wilhelmina Gasthuis hospital in Amsterdam, no differences were found in systolic or diastolic blood pressure at age ∼50 years, comparing men and women born during the famine with unexposed controls born before or after the famine. Measurements were made in the study participants’ homes and also during examinations in the clinic (99). Investigators found no association between adult blood pressure and the protein, carbohydrate, or fat content of food rations distributed at any time during the famine (100). Whereas small variations in adult blood pressure were reported in relation to the protein/carbohydrate balance of the maternal food rations during the third trimester of gestation, data for other trimesters were not provided (100). When the subjects were reexamined at age ∼58 years, the absence of an association between prenatal famine and current blood pressure was confirmed (83). An increase in systolic blood pressure, but not in diastolic blood pressure or heart rate, was seen in response to a challenge designed to induce psychological stress. These findings were limited to subjects exposed in early gestation (83).

In the Dutch birth cohorts from Amsterdam, Rotterdam, and Leiden examined at age 59 years, Lumey et al. (67) showed that exposure to famine at any time in gestation was associated with an increase in the level of total cholesterol and triglycerides in women, but not in men. The increases in total cholesterol and LDL cholesterol were independent of BMI, waist circumference, and midthigh circumference. The increase in triglycerides was independent of midthigh circumference but was attenuated by the control for either BMI or waist circumference. In these cohorts, associations between prenatal famine exposure and the current dietary intake of proteins, carbohydrates, and fat were weak, and most

**Lipid Profile**

In birth cohorts from the Wilhelmina Gasthuis hospital in Amsterdam examined at age 50 years, Roseboom et al. (96) reported an association between famine exposure in early gestation and an increase in the ratio of low-density to high-density lipoproteins (LDL/HDL ratio). In this group, LDL tended to be higher and HDL lower, but neither of these outcomes was statistically different from unexposed controls (96). Upon reexamination of the cohort at age 58 years, there was no longer an association of HDL cholesterol with early famine exposure, but associations with LDL cholesterol and with the LDL/HDL ratio persisted with attenuation, adjusting for sex, BMI, socioeconomic status, and lipid-lowering medication. At age 58 years, but not at age 50 years, dietary patterns were also compared between individuals of the different exposure groups, and no differences were found in the mean percentage of dietary intake of proteins, carbohydrates, or fat. The proportion of individuals in the highest quartile of fat intake was higher, however, in the group with famine exposure in early gestation compared with controls born before or conceived after the famine (71). In Dutch famine births followed from Amsterdam, Rotterdam, and Leiden examined at age 59 years, Lumey et al. (67) showed that exposure to famine at any time in gestation was associated with an increase in the level of total cholesterol and triglycerides in women, but not in men. The increases in total cholesterol and LDL cholesterol were independent of BMI, waist circumference, and midthigh circumference. The increase in triglycerides was independent of midthigh circumference but was attenuated by the control for either BMI or waist circumference. In these cohorts, associations between prenatal famine exposure and the current dietary intake of proteins, carbohydrates, and fat were weak, and most
estimates were sensitive to the choice of controls (114).

On the basis of the reported findings, the available studies on adult lipid profiles after prenatal famine exposure are difficult to compare because of differences in the methods of analysis and in exposure and outcome definitions. In one of the Dutch birth cohorts, famine was associated with an increase in the LDL/HDL ratio in adults. The increase was attenuated when the study participants were examined a few years later. In a highly selected subgroup, famine was also associated with macronutrient density. In the other Dutch cohorts, famine was associated with an increase in total cholesterol and triglyceride levels and very weakly with the intake of macronutrients, but only in women. A further comparison of findings across available studies using uniform exposure and outcome definitions may provide a better understanding of the relation between prenatal famine exposures and adult lipid outcomes.

**Metabolic Syndrome**

In the Dutch famine cohorts, there is an association between the metabolic syndrome (MS) with prenatal famine exposure, but the pattern is rather confusing. The metabolic syndrome is currently defined by meeting three out of a cluster of five risk factors for cardiovascular disease and diabetes mellitus that often occur together (1). The cluster includes elevated blood pressure, waist circumference, or blood glucose levels, as well as abnormal blood lipids with elevated triglyceride or reduced HDL levels. In the past, the National Cholesterol Education Program (NCEP), the International Diabetes Federation (IDF), and other agencies including the World Health Organization (WHO) and the American Diabetes Association (ADA) have proposed syndrome definitions. All definitions use similar elements and are broadly overlapping. In the Dutch birth cohorts from the Wilhelmina Gasthuis (19), there was no association between prenatal famine and MS as defined by either NCEP or IDF criteria. But in the Amsterdam, Rotterdam, and Leiden birth cohorts, there was an increase in MS by either NCEP or other well-established criteria but not by IDF criteria. The findings from the Amsterdam, Rotterdam, and Leiden birth cohorts are based on preliminary communications (19, 65). No published data are available from other settings.

**Cardiovascular Outcomes**

In individuals from the Wilhelmina Gasthuis followed through age 50 years, a diagnosis of coronary artery disease (CAD) was given to 24 people, on the basis of angina symptoms as reported in the medical interview, Q-waves on the echocardiogram (ECG), or a history of coronary revascularization (97). By age 58 years, this number had increased to 83. Eleven out of 138 individuals with famine exposure in early gestation were diagnosed with CAD at age 58 compared with 49 out of 590 individuals who had no famine exposure in pregnancy (84). The difference is not statistically significant ($p = 0.18$).

In individuals with exposure to famine at any time during gestation, the intima media thickness (IMT) of the carotid artery, a measure of CAD risk, was reported to be thinner in persons exposed to famine during gestation compared with nonexposed individuals (85). This finding is contrary to expectations because thicker vessels typically point to a higher degree of atherosclerosis. Maternal famine exposure was not associated with carotid artery stiffness or carotid artery size (81).

In Leningrad, prenatal famine exposure was not associated with coronary heart disease outcomes (109).

The current studies of CAD outcomes after prenatal famine are inconclusive but have only been reported for one of the available birth cohorts in the Netherlands. It will be of interest to compare the results from additional study populations as they become available.

**Self-Reported Health**

At age 50 years, men and women born in the Wilhelmina Gasthuis cohort were asked the
question, “How do you rate your health?” The response was scored on a five-point scale with categories ranging from “excellent,” “very good,” “good,” “fair,” to “poor” (5% of all respondents). Nine of 87 individuals who had been exposed to famine in early gestation rated their health as poor compared with 27 of 548 unexposed controls (98). A more comprehensive evaluation was carried out at age 59 years in the birth cohorts from Amsterdam, Leiden, and Rotterdam, using the 36-question SF-36 quality-of-life questionnaire and the 20-question Center for Epidemiologic Studies–Depression (CES-D) scale of depressive symptoms (112). In these individuals, only a mother’s exposure to famine in the 10 weeks prior to conception, but not during pregnancy itself, was associated with lower self-reported measures of mental health and quality of life in her offspring.

The current studies of perceived health and quality of life after prenatal famine have used different outcome measures and are therefore not directly comparable. The reported findings in middle-aged men and women examined at the same age, an association was reported between prenatal famine and a selective attention task. No association was seen, however, with three measures, including the Alice Heim general intelligence test, a memory task (paragraph recall), and a perceptual motor-learning task (mirror drawing). The finding on the attention task, although based a rather small subgroup in the cohort, was interpreted as an early manifestation of accelerated cognitive aging in the cohort (25).

The available measures of cognitive functioning in middle age, therefore, do not suggest a long-term association with prenatal famine exposure, but potential differences in selective attention should be further investigated.

Mental Disorders

Studies on the relation of prenatal famine to mental disorders began with the observation by Z.A. Stein et al. (121) that there was an excess of congenital nervous system anomalies in individuals with periconceptional famine exposure toward the end of the famine. Later, it became clear that these anomalies were mainly neural tube defects, and in hindsight, it seemed likely that this excess could be due to maternal periconceptional folate deficiency. In this context, E. Susser et al. (122) reanalyzed the original data to define better the birth cohort with excess CNS anomalies and investigated whether there was also an excess of schizophrenia—which many consider to be a neurodevelopmental disorder—in the birth cohort so defined. Using data from the Dutch psychiatric registry, they found a twofold increased risk
of schizophrenia in adult men and women in that cohort. In subsequent work, the authors reanalyzed the original data on male military recruits at age 18 years and found an excess of “schizoid personality” (in today’s terms, this would be most similar to schizophrenia spectrum personality disorders) in the same birth cohort (41). These findings are complementary and support the hypothesis that famine exposure in early gestation is related to an increase in schizophrenia risk. Because only date of birth and not date of conception was available in these studies, the results do not precisely identify the timing of exposure. They do point to a window, however, that may extend up to eight weeks of gestation or possibly further. The neural tube defects were likely related to exposure up to four weeks of gestation, when the neural tube closes, but the same cannot be assumed for schizophrenia.

The same hypothesis was tested in two later studies in China (108, 133). Because only the year of birth was available for the studied individuals, the relation between early gestational exposure and offspring schizophrenia was examined by identifying the year(s) in which there was a dramatic drop in the birth rate and by assuming that most of the births in those year(s) had been exposed in early gestation. In both studies, investigators noted a twofold increase in schizophrenia after early prenatal famine exposure. The much larger numbers in these Chinese studies, the consistency across diverse settings, and other features such as rural-urban differences that matched famine conditions add a great deal of support to the schizophrenia hypothesis. Although the mechanism is still not known, the totality of evidence strongly suggests that early prenatal famine is linked to an increase in the risk of schizophrenia in adults. Alternative explanations such as toxic food substitutes cannot be ruled out entirely but are highly unlikely, given the consistent results across different settings with prenatal famine exposure.

A cross-sectional study of schizophrenia based on the Chinese National Disability sample of 1987 in urban and rural populations compared this outcome among prefamine, famine, and postfamine births, on the basis of ecological data across provinces in China (106). In the urban population, the risk of schizophrenia as diagnosed in the survey was increased among individuals who were conceived or born during the 1959–1963 famine, compared with individuals born before or after the famine. In the rural population, by contrast, the postfamine births had an increased risk. The authors suggest that an excess of infant mortality could explain the rural findings. It is difficult, however, to compare this survey with the cohort studies from China described above because this study was not designed to test the same hypothesis of an early gestational effect of famine on schizophrenia.

In the Dutch studies, prenatal famine exposure has also been related to other psychiatric disorders in adulthood. At age 18 years, antisocial personality disorder increased among those recruits who were exposed during the first and second (but not third) trimesters (79). And using the national psychiatric hospital registry, investigators found an increase in affective psychoses (many of which would today be classified as mood disorders but not psychoses) among those exposed in the second and third (but not first) trimesters (10, 11). Thus far, neither of these findings has been replicated.

**Adult Mortality**

In the Finnish historical cohort study of births before, during, and after the 1866–1868 crop failures, survival from 17 to 80 years and beyond was very similar in cohorts born before, during, and after the famine (49). In the Wilhelmina Gasthuis Dutch famine cohort, there is no relation between prenatal famine exposure and deaths through age 57 years (87).

The lack of long-term effects on adult survival is also suggested by the Chinese national fertility survey, which includes information from maternal interviews on deaths among their children. No differences in long-term

The absence of an association between prenatal famine and long-term mortality does not exclude the possibility that there is a real effect if this effect is hidden by differences in early mortality. As noted by Song et al. (106), famine births have a lower long-term mortality compared with postfamine births. This lower mortality would generally not be expected in view of the usual secular trend of mortality decline over time across the world. One possible explanation is selection bias because weaker individuals may have experienced an excess of spontaneous abortions or early postnatal deaths. Song et al. (106) describe the paradox that for a famine to have an effect it needs to be severe, but the more severe the famine, the more distorted the population structure can become because of excess differential mortality and the more difficult it will be to detect a true effect among survivors. This dilemma is not easily resolved.

In Bangladesh births between 1974 and 2000, individuals born in the hungry season had an increased mortality in the first year of life compared with births in the harvest season, but there was no excess in deaths at ages >15 years (78). In the Gambia, deaths for those born in the hungry season increased from age 15 years (77), related mainly to non-HIV/AIDS infectious diseases (76).

Overall, the available studies, except for the study from the Gambia, suggest no relation between prenatal famine and adult mortality.

Intergenerational Effects

Kaati et al. (45, 46), in their study of mortality in the Swedish Overkalix parish in relation to parents’ and grandparents’ nutrition in critical growth periods, have proposed a feed-forward process in which undernutrition of one or more of the grandparents during their slow-growth period before the adolescent growth spurt imprints on subsequent generations, possibly through epigenetic mechanisms. Replication of these findings will be critical to assess to what extent these findings could be explained as chance observations.

Lumey (59) reported that mothers prenatally exposed to the Dutch famine during the first and second trimesters in gestation themselves had firstborn offspring with lower birth weights compared with unexposed controls. By contrast, birth weights of the children of mothers prenatally exposed in the third trimester were not affected. Mothers born after third-trimester famine exposure had lower birth weights themselves, however.

Later, Stein & Lumey (111) reported that the usual correlation of mother-child birth weights weakened in offspring of women with famine exposure late in gestation. They had observed earlier that the expected increase in birth weights with parity was not seen among offspring of women with famine exposure early in gestation (63).

Painter et al. (86) also interviewed famine-exposed women about the health of their children and found that gestational famine exposure was associated with reduced offspring length in the next generation but not with reduced birth weight, resulting in an increase in the ponderal index (kg/m$^3$). Neither were there any differences in the prevalence of congenital disorders or of cardiovascular/metabolic, psychiatric, or other conditions comparing gestation-exposed mothers with controls. The authors report, however, that children of famine-exposed mothers were more likely to be in poor health from “other” causes, a rather heterogeneous condition defined as accidental, acquired neurological, auto-immune, respiratory, infectious, neoplastic, or dermatological problems (86).

In China, offspring of babies born to women survivors of the 1959–1961 famine appeared to be heavier at birth (42). This finding might be explained by the selective fertility of larger mothers during a famine.

In the aggregate, the reported findings on maternal prenatal famine exposure and birth
Table 3  Prenatal famine and associations with adult health

<table>
<thead>
<tr>
<th>Condition</th>
<th>Measures used</th>
<th>Reported association</th>
<th>Number of independent study populations</th>
<th>Quality of evidence for a positive association</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>Selected markers set during early gestation</td>
<td>Fingerprint ridge counts at age 58 years</td>
<td>The ridge-count difference between digits 1 and 5 (Md15) is associated with prenatal famine in early pregnancy (48). Ridge-count differences are also associated with type 2 diabetes mellitus in late middle age (47).</td>
<td>1</td>
<td>+</td>
<td>Potential for permanent effects on selected characteristics that are determined in early pregnancy.</td>
</tr>
<tr>
<td></td>
<td>DNA methylation on selected genes at age 58 years</td>
<td>Decrease in DNA methylation of imprinted IGF2 gene (39). Variable effects on other loci, which may be gender specific (125).</td>
<td>1</td>
<td>+</td>
<td>Positive timing-specific association using matched sibling design. Further studies are needed to establish a systematic pattern. Effect of prenatal famine may be sex specific and may depend on timing of insult.</td>
</tr>
<tr>
<td>Sex ratio at birth</td>
<td>Births by sex</td>
<td>No changes in sex ratio in relation to prenatal exposure to the Dutch famine (18, 115).</td>
<td>3</td>
<td>−</td>
<td>No causal association.</td>
</tr>
<tr>
<td>Women’s fertility</td>
<td>Reported age at menarche and menopause and reproductive history from interviews</td>
<td>No reduction in number of children reported among women interviewed at age 43 (62, 64). Increase in number of children reported at age 50 (89).</td>
<td>2</td>
<td>±</td>
<td>Inconsistent findings from interviews at two time points in the same study population. Selective fertility in famine could bias long-term outcomes.</td>
</tr>
<tr>
<td>Anthropometry</td>
<td>Height and weight</td>
<td>Increase in extreme upper tail of weight/height ratio in men at age 18 (94). Increase of BMI in women but not in men aged ~40 years (70, 134) and of body weight, BMI, and waist circumference in women but not in men at age 50–58 years (93, 110).</td>
<td>4</td>
<td>++</td>
<td>Increase in body weight, BMI, and waist circumference in women after prenatal famine. No such pattern in men established. Need for common data-analytic approach across comparable studies.</td>
</tr>
</tbody>
</table>

(Continued)
Table 3 (Continued)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Measures used</th>
<th>Reported association</th>
<th>Number of independent study populations</th>
<th>Quality of evidence for a positive association</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose metabolism</td>
<td>Fasting glucose, 2-h glucose, and type 2 diabetes mellitus</td>
<td>Increase in 2-h glucose on OGTT in men and women 50–58 years in Holland (23, 92). Increase in type 2 diabetes mellitus in men and women at age 59 years in Holland (66). Similar patterns seen in men and women age ~40 years in China (56).</td>
<td>3</td>
<td>++</td>
<td>Association between prenatal famine and glucose metabolism and type 2 diabetes mellitus in adults. No association with fasting glucose. Need for common data-analytic approach across comparable studies.</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>Systolic and diastolic blood pressure</td>
<td>No association at age 40 in China in women (43) or at age 50–58 years in men or women in Holland (83, 99, 117).</td>
<td>3</td>
<td>−</td>
<td>No significant associations. Very small effects cannot be excluded.</td>
</tr>
<tr>
<td>Lipid profile</td>
<td>HDL, LDL, HDL/LDL ratio, triglycerides</td>
<td>Some changes in the LDL/HDL ratio in individuals at age 50 but not age 58 (71, 96) and an increase in total and HDL cholesterol in women but not in men (114).</td>
<td>2</td>
<td>±</td>
<td>Reported findings are difficult to interpret owing to the lack of uniform methods of analysis and differences in exposure and outcome definition across comparable studies.</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>The cluster of elevated blood pressure, waist circumference, blood glucose, and abnormal blood lipids (elevated triglycerides or reduced HDL)</td>
<td>No consistent associations with the various metabolic syndrome classifications in two Dutch studies (19, 65) of men and women aged 50–58 years.</td>
<td>2</td>
<td>−</td>
<td>Associations may be mediated by body size.</td>
</tr>
<tr>
<td>Cardiovascular outcomes</td>
<td>Coronary artery disease (angina pectoris symptoms, Q-waves on ECG, history of coronary revascularization); intima media thickness; carotid artery stiffness and size</td>
<td>CAD increase in Holland birth cohort at age 50–58 but subgroup numbers are small. Paradoxical decrease in intima media thickness in same individuals. No change in carotid artery stiffness or size (81, 84, 85). No association in Leningrad study (109).</td>
<td>2</td>
<td>−</td>
<td>Inconsistent findings across ages 50 and 58 and across cardiovascular risk measures in one study. No association in a second study.</td>
</tr>
</tbody>
</table>
### Table: Prenatal Famine and Adult Health

<table>
<thead>
<tr>
<th>Self-reported health</th>
<th>Quality of life (SF-36) questionnaire, CES-D depression scale, open question on health rating</th>
<th>No famine-related differences in self-reported health at age 50–58 years using comprehensive questionnaires (112). Positive association with a single measure of an individual’s health rating (98).</th>
<th>2</th>
<th>–</th>
<th>Studies of perceived health and quality of life after prenatal famine have used different outcome measures.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mental performance and cognition</td>
<td>Raven progressive matrices.</td>
<td>No association between prenatal famine and mild or severe mental retardation in 18-year-old men (120).</td>
<td>1</td>
<td>–</td>
<td>Raven measures from national male birth cohort examined at age 18.</td>
</tr>
<tr>
<td>Cognitive test batteries</td>
<td>Cognitive test batteries show no association with prenatal famine among men and women ages 50–58 years except for one item in one study (25, 36)</td>
<td></td>
<td>2</td>
<td>±</td>
<td>Studies of cognition after prenatal famine have used different test batteries.</td>
</tr>
<tr>
<td>Adult mortality</td>
<td>Death registers or mother’s report of children’s deaths</td>
<td>No relation of prenatal famine with mortality over age 18 in Finnish national birth cohort 1866–1868 (49) or with mortality among children born to mothers in Chinese famine (105). Excess deaths seen in early life but not thereafter (77, 87).</td>
<td>4</td>
<td>±</td>
<td>True effects may be hidden by selective differences in early mortality.</td>
</tr>
<tr>
<td>Intergenerational effects</td>
<td>Mortality in later generations in relation to measures of parent’s and grandparent’s nutrition at different ages.</td>
<td>Relation between slow-growth period when grandparents were 11–14 years old and mortality in their grandchildren (45, 46). Offspring birth weights are related to maternal prenatal famine exposure (59, 63, 111)</td>
<td>2</td>
<td>±</td>
<td>Findings are inconclusive. Issues related to selective fertility and survival under famine conditions and their impact on long-term health outcomes need further exploration.</td>
</tr>
<tr>
<td>Reproductive outcomes in women with prenatal famine exposure</td>
<td></td>
<td></td>
<td>1</td>
<td>+</td>
<td>Need for replication using common data-analytic approach in comparable studies.</td>
</tr>
<tr>
<td>Other outcomes</td>
<td>Asthmatic conditions, micro-albuminuria, breast cancer, HPA-axis activity, sexual orientation, irritable bowel syndrome</td>
<td>Various conditions reported after prenatal exposure to the Dutch famine</td>
<td>1</td>
<td>–</td>
<td>Exploratory reports based on very small subgroups.</td>
</tr>
</tbody>
</table>

---

*Abbreviations: BMI, body mass index; CAD, coronary artery disease; CES-D scale, Center for Epidemiologic Studies—Depression scale; ECG, echocardiogram; HDL, high-density lipoprotein; HPA-axis, hypothalamic–pituitary–adrenal axis; LDL, low-density lipoprotein; OGTT, oral glucose tolerance test.*
size in a mother’s own offspring appear to be inconclusive, and associations with offspring poor health in the next generation are observed only in the cluster of apparently unrelated “other” conditions. Issues related to selective fertility and survival under famine conditions and their impact on long-term health outcomes need further exploration.

Other Outcomes
In Dutch famine birth cohorts interviewed and examined at age 50 years, men and women with prenatal famine exposure in midgestation more commonly reported that a doctor had ever told them that they had asthma, chronic bronchitis, emphysema, or chronic nonspecific lung disease compared with unexposed controls. The overall prevalence of this condition among the study participants was 18%. There were no differences by famine exposure in clinical markers for allergic and respiratory function such as IgE concentrations (whether IgE specific for cat, house dust mite, pollen, a combination of these three, or total IgE) or lung-function tests (57).

The proportion of participants with microalbuminuria (defined as an albumin/creatinine ratio ≥2.5 g/mol) was also higher (12%) in the 104 participants with prefamine exposure in midgestation compared with 6% in the 429 unexposed controls (88), although this difference does not reach statistical significance.

In a follow-up of 475 women, 10 out of 205 participants with prenatal exposure to famine reported that they had ever been diagnosed with breast cancer compared with five out of 275 without famine exposure. The difference was not statistically significant (82).

Hypothalamic-pituitary-adrenal (HPA) axis activity, sexual orientation, and irritable bowel syndrome have been examined in the Dutch Wilhelmina Gasthuis hospital cohort, with no reported differences by famine exposure (20, 24, 53).

A summary of the study outcomes discussed above is provided in Table 3.

CONCLUSIONS
Ten years ago there was little information on the role of maternal nutrition in pregnancy in relation to long-term disease outcomes. Reviews of the available evidence gave mixed evaluations, either accepting “a small but growing set of data providing direct evidence” for changes in adult health (37) or viewing the available data as providing “only minimal support” for a specific role of maternal nutrition before or during pregnancy (91). In addition, the lack of “well-articulated, testable causal sequences” (91) and of systematic attempts to examine specific hypotheses critically was identified as a problem (60, 90), as were common pitfalls in the interpretation of models relating early exposures to later health outcomes (58).

The current review shows that some progress has been made over the past decade. A pattern is emerging for relations between prenatal famine and adult health for some outcomes, on the basis of compatible findings from different studies and a priori formulations of testable hypotheses. The more consistent patterns apply to body size, diabetes, and schizophrenia. Further progress is possible using currently available data with a systematic data analytic approach across comparable studies. This method has not been attempted to date.

The finding of epigenetic markers of prenatal famine exposure is intriguing and opens the window to explore specific biological mechanisms linking prenatal events and adult health.

For many other outcomes, study findings are still diffuse and conflicting, hampered by limited sample size and chance observations, and should still be considered exploratory and hypothesis generating. Also in these areas, common analytic strategies across comparable studies to explore specific hypotheses further are likely to be very informative.

The current insights are based mostly on reports from the Dutch famine of 1944–1945 and the Chinese Great Leap Forward famine of 1959–1961. These settings will continue to
offer a special opportunity for the study of nutrition in pregnancy and adult disease in humans. For the many questions on programming for which these settings provide no answer, closely coordinated animal studies need to be considered (17).

ACKNOWLEDGMENTS
L.H.L. and A.D.S. were supported in part by NIH grants RO1-HL67914 and RO1-AG028593 (PI: L.H.L.). E.S. was supported in part by a NARSAD Distinguished Investigator award.

LITERATURE CITED


From folk medicine to popular culture, there is an abiding fascination with how the experiences of pregnant women imprint on their descendants. The latest wave in this discussion flows from studies of epigenetics — analyses of heritable changes to DNA that affect gene activity but not nucleotide sequence. Such DNA modification has been implicated in a child’s future risk of obesity, diseases such as diabetes, and poor response to stress.

Headlines in the press reveal how these findings are often simplified to focus on the maternal impact: ‘Mother’s diet during pregnancy alters baby’s DNA’ (BBC), ‘Grandma’s Experiences Leave a Mark on Your Genes’ (Discover), and ‘Pregnant 9/11 survivors transmitted trauma to their children’ (The Guardian). Factors such as the paternal contribution, family life and social environment receive less attention.

Questions about the long shadow of the uterine environment are part of a burgeoning field known as developmental origins of health and disease (DOHaD). For example, one study revealed that 45% of children born to women with type 2 diabetes develop diabetes by their mid-twenties, compared with 9% of children whose mothers developed diabetes after pregnancy.

DOHaD would ideally guide policies that support parents and children, but exaggerations and over-simplifications are making scapegoats of mothers, and could even increase surveillance and regulation of pregnant women. As academics working in DOHaD and cultural studies of science, we are concerned. We urge researchers, press officers and journalists to consider the ramifications of irresponsible discussion.

ALARMING PRECEDEENTS

There is a long history of society blaming mothers for the ill health of their children. Preliminary evidence of fetal harm has led to regulatory over-reach. First recognized in the 1970s, fetal alcohol syndrome (FAS) is a collection of physical and mental problems in children of women who drink heavily during pregnancy. In 1981, the US Surgeon General advised that no level of alcohol consumption was safe for pregnant women. Drinking during pregnancy was stigmatized and even criminalized. Bars and restaurants were required to display warnings that drinking

Don’t blame the mothers

Careless discussion of epigenetic research on how early life affects health across generations could harm women, warn Sarah S. Richardson and colleagues.
causes birth defects. Many moderate drinkers stopped consuming alcohol during pregnancy, but rates of FAS did not fall.

Although those who drink heavily during pregnancy can endanger their children, the risks of moderate drinking were overstated by policy-makers—a point recently reaffirmed by the Danish National Birth Cohort study, which did not find adverse effects in children whose mothers drank moderately during pregnancy. Nonetheless, warnings about alcohol during pregnancy made in inappropriate contexts still cause pregnant women to suffer social condemnation and to agonize over an occasional sip.

In the 1980s and 1990s, surging use of crack cocaine (a smokable form of the drug) in the United States led to media hysteria around ‘crack babies’ — those who had been exposed to cocaine in the womb. Pregnant women who took drugs lost social benefits, had their children taken away and were even sent to prison. More than 400 pregnant women, mostly African American, have been prosecuted for endangering their fetuses in this way. Exposed infants were stigmatized as a biologically doomed underclass. Today, fetal exposure to crack or cocaine is considered no more harmful than exposure to tobacco or alcohol, but criminal prosecution of pregnant women who take such drugs continues.

Previous generations found other ways to blame women. As late as the 1970s, ‘refrigerator mothers’ (a disparaging term for a parent lacking emotional warmth) were faulted for their children’s autism. Until the nineteenth century, medical texts attributed birth deformities, mental defects and criminal tendencies to the mother’s diet and nerves, and to the company she kept during pregnancy.

Although it does not yet go to the same extremes, public reaction to DOHaD research today resembles that of the past in disturbing ways. A mother’s individual influence over a vulnerable fetus is emphasized; the role of societal factors is not. And studies now extend beyond substance use, to include all aspects of daily life.

CONTEXT IS KEY
A 2013 story on the health-information website WebMD demonstrates the sort of responsible reporting that we would like to see more of (see go.nature.com/p2krhs). The story reported findings of a four-fold increased risk of bipolar disorder in adult offspring if a mother had influenza during pregnancy, but it emphasized that the overall risk observed was small and that bipolar disorder is treatable. It stated that the study considered only one of many possible risk factors and did not establish cause and effect. Furthermore, the headline did not lead with the scary number.

Much less context was given in coverage of a 2012 paper showing that second-generation offspring of rats eating a high-fat diet during pregnancy had an 80% chance of cancer, compared with 50% of control rats. ‘Why you should worry about grandma’s eating habits’, read one headline. “Think twice about that bag of potato chips because you are eating for more than two,” warned another story. These articles did not state that the rats were bred for high cancer rates. Nor did they include inconsistent results: third-generation offspring of female rats on high-fat diets actually had lower incidences of tumours than their control peers.

“We urge scientists, educators and reporters to anticipate how this work is likely to be interpreted in popular discussions.”

Inadequately supported and poorly contextualized statements are also found in well-intentioned educational materials. The website beginbeforebirth.org, put together by researchers at Imperial College London, advocates ways to “support and look after pregnant women”. A video on the website portrays a 19-year-old released from prison after a stint for looting (see go.nature.com/wnfzw). “Perhaps his problems stretch right back to the womb,” the narrator says. “Could better care of pregnant women be a new way of preventing crime?” At best, such suggestions overstate conclusions of current research.

BEYOND THE MATERNAL IMPRINT
Today, an increasing segment of DOHaD research recognizes that fathers and grandparents also affect descendants’ health. Studies suggest that diet and stress modify sperm epigenetically and increase an offspring’s risk of heart disease, autism and schizophrenia. In humans, the influence of fathers over mothers’ psychological and physical state is increasingly recognized. So are effects of racial discrimination, lack of access to nutritious foods and exposure to toxic chemicals in the environment.

Viewed from this broader perspective, DOHaD provides a rationale for policies to improve the quality of life for women and men. It must not be used to lecture individuals, but rather to anticipate how DOHaD work is likely to be interpreted in popular discussions. Although no one denies that healthy behaviour is important during pregnancy, all those involved should be at pains to explain that findings are too preliminary to provide recommendations for daily living.

Caveats span four areas. First, avoid extrapolating from animal studies to humans without qualification. The short lifespans and large litter sizes favoured for lab studies often make animal models poor proxies for human reproduction. Second, emphasize the role of both paternal and maternal effects. This can counterbalance the tendency to pin poor outcomes on maternal behaviour. Third, convey complexity. Intrauterine exposures can raise or lower disease risk, but so too can a plethora of other intertwined genetic, lifestyle, socioeconomic and environmental factors that are poorly understood. Fourth, recognize the role of society. Many of the intrauterine stressors that DOHaD identifies as having adverse intergenerational effects correlate with social gradients of class, race and gender. This points to the need for societal changes rather than individual solutions.

Although remembering past excesses of ‘mother-blame’ might dampen excitement about epigenetic research in DOHaD, it will help the field to improve health without constraining women’s freedom.

Sarah S. Richardson is associate professor of the history of science and of studies of women, gender and sexuality at Harvard University in Cambridge, Massachusetts, USA. Cynthia R. Daniels is professor of political science at Rutgers University in New Brunswick, New Jersey, USA. Matthew W. Gillman is professor of population medicine and director of the Obesity Prevention Program at Harvard Medical School in Boston, Massachusetts, USA. Janet Golden is professor of history at Rutgers University in Camden, New Jersey, USA. Rebecca Kukla is professor of philosophy at Georgetown University in Washington DC, USA. Christopher Kuzawa is professor of anthropology at Northwestern University in Evanston, Illinois, USA. Janet Rich-Edwards is associate professor of medicine at the Connors Center for Women’s Health and Gender Biology at Harvard Medical School in Boston, Massachusetts, USA.

e-mail: srichard@fas.harvard.edu
Mothers as smoking guns: Fetal overnutrition and the reproduction of obesity

Megan Warin, Tanya Zivkovic, Vivienne Moore and Michael Davies
University of Adelaide, Australia

Abstract
Mothers are expected to monitor their children's dietary intakes and physical activities and are blamed for over feeding their children if they are obese. Women are also urged to manage their own weight in preparation for conception and during pregnancy in order to reduce complications associated with maternal obesity at childbirth. Through a theoretical lens of maternal blame, we argue that Australian media representations of scientific studies of the fetal overnutrition hypothesis extend behavioural maternal blame to the interiority of women's bodies. Women's intrauterine environments are positioned in the media as central to the intergenerational transmission of obesity, with women portrayed as responsible for passing obesity on to their children (and grandchildren) via biology and ill-informed ‘lifestyle choices’. Linking in with historical and contemporary discourses of maternal bodies and individual responsibility, the implications of the ‘double damage’ caused by women entails a concerning return to essentialism in which women's bodies are being largely blamed for producing and reproducing obesity across generations.

Keywords
Fetal origins hypothesis, maternal obesity, mother blame, pregnancy, reproduction

In March 2009 an article in the daily State newspaper of South Australia (The Advertiser) featured a large photograph of a smiling mother and her newborn baby, warning that ‘health problems are passed on through generations’ (Stewart, 2009). The headline to the story – ‘Mothers’ smoking gun’ – referred to a cohort study
conducted by researchers at the University of Adelaide (including two authors of this article) which explicitly states that overweight, pregnant women are more likely to have children, even grandchildren, who are overweight. A similar news item in Australia’s national circulation paper (The Australian) in the previous month claimed that ‘obese women are more likely to have children with a range of birth defects’ (Taor, 2009). The warnings are clear – obesity in pregnancy is potentially damaging and it is mothers who are held responsible for their children’s ill health.

The media conflation of women’s reproductive bodies with smoking guns reflects recent paradigmatic developments in scientific research about the origins of health and disease, and how adult chronic disease might be determined by the ‘womb environment’. This new paradigm, termed Barker’s hypothesis (or the fetal origins hypothesis), has led to maternal obesity now being understood to contribute to obesity in children through intra-uterine factors that alter fetal metabolism regarding growth, fat deposition, and insulin regulation (Oken and Gillman, 2003). As a result, the interiority of women’s reproductive bodies is brought sharply into the media limelight not only as a causal agent in the obesity ‘epidemic’, but also the (potential) solution.

We critically examine how the fetal origins hypothesis is reported in popular print media, arguing that reproduction (and more specifically women’s reproduction) is now a key discursive site in which intergenerational cycles of obesity are being culturally produced and reproduced. To provide evidence of these new discourses in which the bodies of obese, pregnant women are being implicated we examined the reporting of scientific research in Australian print media. In addition to mothers being held legally and morally culpable for overfeeding and neglect of fat children, we found that the reporting of scientific research compounds blame by suggesting that women are responsible for ‘programming’ their baby for a lifetime of obesity. A new and powerful meta-discourse has emerged in which women are blamed for both their reproductive physiology and their social role as mothers, thus constructing women as potentially contaminating future generations by creating obesity lineages.

Of course this discourse of blame did not appear out of thin air; blaming the bodies and behaviours of pregnant women for misshapen fetuses lingers on from our historical understandings of disease causation. Mother blame is not a new phenomenon and historians (Ladd-Taylor and Umansky, 1998) and feminist scholars (Litt, 2000; Singh, 2004) have noted how women have long been accused of smothering children and causing all manner of ‘ills’ such as homosexuality, schizophrenia, autism, and anorexia to name a few. While the concept of mother blame has ‘extraordinary elasticity’ (Blum, 2007: 203), we argue that the shifting historical discourses of maternal appetites, the scientific location of obesity through the fetal origins of disease, and the popularisation of this ‘new science’ now provide a singular space for the overweight, maternal body to take centre stage. Fat, pregnant bodies are constructed as bio-cultural anxieties, distilling biological and social causes into the one embodied location. Coupled with a neoliberal agenda that emphasises self-governance and individual responsibility, this powerful meta-discourse (Nerlich, 2009) provides a compelling web of individual and gendered blame for the obesity ‘epidemic’.
The obese, pregnant body

Demi Moore’s infamous *Vanity Fair* cover in 1991 was a sensational prelude to the idea that good mothers are closely aligned with the ideal neoliberal citizen. Since her cover, photos and stories of pregnant and post-partum celebrities have proliferated in various popular media. Although the consumption of celebrity pregnancies (Danni Minogue, Britney Spears, Angelina Jolie, Heidi Klum, and Nicole Richie) – with their neat bumps, well supported breasts, glowing skin, and radiantly energised appearance – communicates a distorted image of expectant mothers and emphasises the social controls that ordinarily discipline mothers’ bodies, a fetishisation of pregnant celebrities (and ‘yummy mummies’) has helped to shape new standards of bodily deportment and appearance toward which the pregnant woman is expected to aspire. Foucauldian issues of surveillance and pregnancy policing have been well documented in feminist literature (see Fox et al., 2009; Longhurst, 2008; Ussher, 2006), and the price of mothers-to-be not complying with the dominant ideals of ‘good motherhood’ are high. Women who don’t self-regulate or ignore dominant pregnancy practices are especially beholden to public scathing, as they present what Skeggs (2005: 968) calls a ‘constitutive limit to propriety’ within both celebrity culture and wider social life.

Once considered healthy, the storage of fat was an acceptable and ‘natural’ part of pregnancy. Indeed, pregnancy, as understood in recent ‘western’ history, was a period in which a woman could, albeit temporarily, guiltlessly gain weight; ‘eat for two’ and rest from exercise. In recent times, however, anxieties about the spread of the ‘obesity epidemic’ has led fat to be demonised and pathologised as a disease (see Campos et al., 2005; Moffat, 2010; Murray, 2008; Orbach, 2009) no matter which body it appears on. In a climate where obesity has become a potent signifier for neglect of self (and others), pregnant women are no longer encouraged to eat for two (Bell et al., 2009; Keenan and Stapleton, 2010) and a new regime of dietary practices and recommended weights for mothers-to-be and pregnant women has been promoted by clinical, public health, and biomedical experts.

Good mothering practices now begin *before* conception (Fox et al., 2009; Lupton, 1996), and preconception care is promoted as ‘the most loving and responsible choice you and your partner can make together, not only for you and your child’s health, but also for future generations’ (McDowell 2005, cited in Possamai-Inesedy, 2006). Obese, even overweight, mothers-to-be should exercise and lower their calorie intake in order to reduce their Body Mass Index (BMI). Additionally, and like all women, they are expected to ensure the optimal conditions for fertility by avoiding substances such as caffeine, alcohol, and nicotine, and supplementing their diets with folic acid and other vitamins and minerals. Vigilant attention to their dietary requirements should continue throughout pregnancy, with a regular intake of folic acid in the first trimester and scheduled blood tests to detect nutritional deficiencies at routine intervals. Still abstaining from alcohol and other toxins and consuming a well-balanced diet, the mother should ideally breastfeed, then, when her baby is eventually weaned, nutritious meals should be prepared.
Effectively, the ‘good mother’, responds to a discourse that requires her to act responsibly (Goodwin and Huppatz, 2010: 5) and to perform labour-intensive (Hays, 1996) food preparation practices to avoid any potential risks and to nourish the body of her child.

‘Biological postcards’: The popularisation of Barker’s hypothesis

These disciplinary regimes reflect not only an obsession with the management of ‘healthy’ bodies, but are now linking ‘pre-pregnancy appropriate weight, weight gain and nutrition in pregnancy with satisfactory fetal outcomes and increasingly, with infant health over the life-course’ (Keenan and Stapleton, 2010: 371). This new attention to the fetus not only reflects a concern for fetal personhood (Ruddick, 2007) but is supported by scientific developments in the early origins of disease that trace chronic disease in adults back to the intrauterine environment.

From the late 1980s, research conducted by UK physician and epidemiologist David Barker and his colleagues advanced the theory that chronic disease originated, at least in part, in early life. Barker and his colleagues’ work (Barker, 1990; Barker and Osmond, 1986) led to a profound paradigmatic shift in medical understanding and knowledge, as low birth weight and intra-uterine growth retardation (caused by under-nutrition during fetal development) are presented as important indicators or signals of an elevated risk for many adult diseases (see Moore and Davies, 2008). In other words, many chronic adult diseases (especially diabetes and heart disease) are seen as having origins in the intra-uterine environment or early infancy (as well as being influenced by later environmental and life style factors). Throughout the 1990s, evidence of associations (statistical connections) between low birth weight and increased risk of chronic disease in adulthood accumulated. In 1995 the British Medical Journal named this ‘discovery’ the ‘Barker Hypothesis’, an expression that Barker rejected in favour of ‘the fetal origins hypothesis’ (Warin et al., 2011). In the science community this new insight became the focus of a major international research effort, and in 2010 Time Magazine called Barker’s hypothesis a ‘pioneering New Science’ that turned ‘pregnancy into a scientific frontier’ (Paul, 2010).

Although the main focus of the field now known as ‘developmental origins of adult health and disease’ has been on the effects of poor fetal nutrition and low birth weight, the issue of maternal and hence fetal overnutrition is of growing importance in the context of the current global obesity ‘epidemic’ (McMillen et al., 2008). By the early 2000s, the fetal origins hypothesis had become part of the child obesity lexicon, extending the understanding of obesity ‘back to the future’, and locating the origins and potentiality of obesity in the fetal environment.

Ebbeling and colleagues (Ebbeling et al., 2002: 475), in a landmark paper on the childhood obesity ‘crisis’, reported an:

intriguing hypothesis that prenatal overnutrition might affect lifelong risk of obesity. According to this hypothesis, maternal obesity increases transfer of nutrients across
the placenta, inducing permanent changes in appetite, neuroendocrine functioning, or energy metabolism... The implications of these findings are formidable: the obesity epidemic could accelerate through successive generations independent of further genetic or environmental factors.

The simplified ‘truth’ of the maternal origins hypothesis is that weight gain during pregnancy (or maternal obesity pre-pregnancy) can lead to fetal overnutrition and high birth-weight and contribute to childhood obesity independent of the family circumstance (La Coursiere et al., 2005).

Although many researchers investigating fetal origins in the scientific community speak of epidemiological uncertainty, caution, and degrees of imprecision (Moore and Davies, 2008; Susser and Levin, 1999; Wells, 2010), the media reporting of these ‘new scientific findings’ follows a simple storyline, and suggests that as women become too large, their fetuses also grow too large, and as a result ‘obesity is programmed in the womb’ (Paul, 2010). It is now frequently reported that: ‘women who are overweight or obese are 2 to 2.5 times more likely to have heavier babies... and larger babies create problems with delivery, and are more at risk of infection, diabetes, obesity and heart disease in later life’ (Shepherd, 2009). ‘Obese mums-to-be [are thus] urged to diet’ (Hall and Davis, 2009), as ‘the first nine months [can shape] the rest of your life’ (Paul, 2010). A reductive account of the fetal origins of disease is gold for scientific journalists, for obesity is both individualised and gendered, and characterised in the popular press as ‘a mother of a problem’ (Parker, 2009: 1).

A number of scholars have examined the media reporting of new scientific research and ‘have found that this type of media tends to lack critical coverage or comment by journalists... [and neglects] both the tentative nature of scientific inquiry and its political context’ (Parker, 2009: 4; see also Dyck, 1995). In spite of this, ‘scientific journalism’ relies on authoritative discourse, and locates its reporting in a context where ‘scientific knowledge continues to hold cultural authority as objective, rational and empirical’ (Parker, 2009: 2). Through this powerful legitimisation, scientific journalism becomes simultaneously a crucial source of scientific and public health information (Petersen et al., 2009; Boero, 2007; Saguy and Almeling, 2008) and ‘a key contributor to the shaping and definition of public health issues as social problems’ (Maher et al., 2010: 236). In relation to obesity, Monaghan et al. (2010) describe the media as ‘amplifiers/moralisers’ as they sensationalise, stereotype, and repeatedly focus on ‘dramatic’ or ‘moralising’ aspects of obesity.

Our study

In 2009 we examined the reporting of obesity over a three-month period (1 January to 31 March 2009) in three metropolitan Australian newspapers – The Advertiser, The Australian and The Sydney Morning Herald. The Sydney Morning Herald, owned by Fairfax Media, and The Australian, of News Limited, are both...
broadsheets. *The Australian*, the only national newspaper, has a broader nationwide audience than *The Sydney Morning Herald*, one of the main newspapers published in Sydney. Owned by Murdoch’s News Corporation, *The Advertiser* is a tabloid-format newspaper and has the widest circulation in Adelaide. These three newspapers were selected to represent Australia’s two dominant media outlets (Fairfax and Murdoch) and different readership and circulation. In order to collect data on visual images we opted against using text-based databases such as Factiva or LexisNexis and manually searched microfilm of the newspapers in our sample. We sourced 181 articles that included at least two of our search terms (obesity/obese AND pregnancy, parenting, child, eating, and diet), made multiple copies of each original (to allow for multiple analysis), and conducted a thematic analysis of text and visual images (see Bernard and Ryan, 2010). This involved identifying and describing both implicit and explicit themes within the data and critically exploring the relationships between these themes (for example, the recurrent links between childhood obesity and mothering). We also compared our results with two other Australian studies on media representations of maternal responsibility and obesity (Maher et al., 2010; Malik, 2007).

**Maternal blame**

In our media sample, obesity was frequently constructed as a parenting issue and was closely aligned with food consumption. When obesity was constructed in terms of parental responsibility, the onus was on the parent to help their child lose weight for the specific purpose of reducing overweight-associated health problems. As we (Zivkovic et al., 2010) and others (Boero, 2009; McNaughton, 2011; Maher et al., 2010) have highlighted, this ‘parent’ is consistently coded as ‘the mother’, ‘entrenching women’s roles as managers of children’s health and inequitably blaming them for childhood obesity’ (Maher et al., 2010: 236).

As Malik (2007) notes in her discourse analysis of how mothers of overweight and obese children are portrayed in the Australian media, mothers are often singled out as the culprit of childhood obesity, with headlines such as: ‘Fat mums set the trend for obese kids’ (Fox, 2005); ‘Fat kids? Yes, Mum’s the word’ (Cornes, 2006); and ‘A large legacy – Overweight children may not have to look too far to find the reason – it could all be mum’s fault’ (Steele, 1999). ‘Bad’ mothers are morally denigrated as overly permissive (‘Refrigerator mums’) or relying on junk food (‘McMums’), and often blamed for an epidemic in childhood obesity because of a perceived lack of education and lack of care for children. Even ‘30 years of feminist careerism’ (Malik, 2007: 13) is used to blame women being time poor, not making ‘home cooked’ meals, and working outside of the home.¹ Such reporting effectively uses what Armstrong refers to a ‘medical-moral authority’ (2003: 189), in that women who fail to act ‘maternally’ are held morally responsible and culpable for adverse health outcomes in their children.

In contemporary ‘western’ societies mothers continue to be held culpable for making the wrong choices in regards to their fetuses’ wellbeing. Pregnant bodies
and the fetuses they contain are increasingly accessible to the medical and legal professions for inspection and intervention (Epstein, 1995: 140). This has resulted in a shift in pregnancy discourses from maternal health to a concern with fetal personhood (Ruddick, 2007). In this new discourse of fetal personhood, mothers can be ‘constructed as antagonistic towards their fetus, who becomes an object of collective concern, with its own public identity as the potential [healthy] citizen’ (Longhurst, 1999, cited in Fox et al., 2009: 62). Fetuses and children are portrayed as innocent victims in need of protection from irresponsible parents, and in some cases mothers have been prosecuted for neglect and abuse in raising obese children (Zivkovic et al., 2010).

Media headlines amplify this failure of duty of care in terms of women’s biological and social roles as mothers. Childhood obesity, it is claimed, ‘might start in the womb’ (Brown, 2009), and lies in the ‘improper’ nutrients supplied to fetuses by their mothers. In response to scientific reports in early 2009, Australian broadsheets had stories with headlines such as: ‘Obese mums-to-be urged to diet’ (Hall and Davis, 2009), ‘Weighty problems born of bad diet in pregnancy’ (Brown, 2009), ‘Overweight mums putting newborns at greater risks’ (Shepherd, 2009), ‘Breastfed children least likely to be abused by mothers’ (Taor, 2009) and ‘Child neglect linked to [breast] feeding’ (Medew, 2009).

The storyline to these headlines positions women as responsible for obesity and other chronic diseases in their children if they do not prepare their bodies for pregnancy, do not maintain their bodies during pregnancy, do not breastfeed, do not put the right choices in lunchboxes or make nutritious, home cooked meals (Fox et al., 2009; Malik, 2007). Levels of responsibility attributed to mothers in relation to obesity occur at key stages of a child’s development, travelling from the ‘placenta to breast, from breast to lunchbox, from lunchbox to the dinner table’ (Malik, 2007: 46). If women do not accept their ‘natural’ responsibilities as caregivers (both biologically and through social roles) they fall into what Blum (2007) calls a mother-valor/mother-blame binary. Mothers who fail to perform these key maternal activities are held ‘responsible for [poor] child outcomes and thus for the health of families, future citizens, and the nation’ (Blum, 2007: 202).

The permeable womb

Locating the source of high birth-weight and childhood obesity in the generative female body marks a long history of association between women’s bodies and that which is considered dangerous. Hailed as a monstrosity, Epstein (1995) and Ussher (2006) note that the female capacity for reproduction was considered an act of horror during the Enlightenment and, well before the scientific classifications of women’s bodily parts and functions in the 19th century, birth disabilities and malformations were seen to signify the desires and cravings of mothers. According to a widespread belief, it was the passions bound up with maternal appetites that posed the greatest threat to the assumed permeability of pregnant bodies. A pregnant woman’s appetite (including the ingestion of foods and drinks
and other sensory experiences such as fear and lust) was the explicit mechanism that transferred the effect from maternal environment to fetus (Kukla, 2005). Pregnancy was thus a dangerous process, for women’s appetites increased, which in turn increased the possibility of harmful passions and appetites corrupting the fetus.

This historical assigning of responsibility for defective births to mothers’ minds and bodies is, according to Epstein (1995: 155), indicative of a ‘legacy of blaming the mother for her children’s appearance and behaviour’, and it ‘serves to justify a wide range of strategies for containing women’s minds by containing women’s bodies’. In the 18th and 19th centuries, these measures included the restraining and hospitalisation of women in order to calm their minds, reduce their passions and decrease their chance of having a deformed infant.

While knowledge and practices surrounding pregnancy have significantly changed through time, Kukla (2005) suggests that preoccupations with pregnant bodies and the potentiality to harm the fetus still govern our imagination. In the news media, maternal obesity is constructed as harming the fetus, and it is the uncontrollable appetites of mothers-to-be that are blamed for the obesity epidemic: ‘Blame your mother if you’re overweight. Sounds Freudian and perhaps a bit mean, but a breakthrough study on obesity indicates that the path to becoming a podgy adult begins in the womb’ (Taranaki Daily News, July 2007, cited in Parker, 2009: 1).

**Intergenerational reproduction of obesity**

While several academics have highlighted the discourses of risk associated with such representations of maternal obesity (Keenan and Stapleton, 2010; McNaughton, 2011; Maher et al., 2010), we argue that the focus on intergenerational ‘passing on’ or transmission of fat from mothers to fetuses and babies constructs women and their reproductive capacities as potentially polluting. In line with social anthropologist Mary Douglas’ concept of ‘matter out of place’ (1966), pregnant bodies are already symbolically marked as dangerous because the flow of reproductive fluids represents a transgression of bodily boundaries. Pregnant bodies expand with fat, fetus, and fluid. Corporeal boundaries become confused and blurred ‘with the merging of two bodies’ (Johnson, 2010: 252) as the fetus distorts the category of subjectivity, feeding from the mother’s body through the placenta.

Fat too is ‘matter out of place’. In a world of plenty, it represents gluttony, it provokes disgust, contravenes the standards of ideal beauty, and is at the core of our dietary restrictions and understandings of bodily purity (Murray, 2005). The boundary between the body and the world is challenged and reconfigured by fat, which represents an invasion of the body by the world (Huff, 2001). This invasion means that the fat body (which translates to personhood) is discursively constructed as a failed body project, existing as a ‘deviant, perverse form of embodiment’ (Murray, 2005: 155). Women who are obese and pregnant are thus all the more visible, and
doubly grounded in biology (Unnithan-Kumar, 2011) as reproductive, and as fat. Consequently, fat, pregnant women are a threat to the fetus: ‘Researchers believe fat mothers pass their obesity to their children. Professor Ross Shepherd, from University of Queensland’s Nutrition Research Centre, said initial studies indicated maternal obesity was related to overweight infants’ (Steele, 1999: 3).

The contravention of order is not limited to the transgression of external and internal bodily boundaries: dangerous substances also course through the interiority of women’s bodies. Bell et al. (2009) argue that in public health discourses on Fetal Alcohol Spectrum Disorder (FASD), smoking when pregnant, and childhood overnutrition, the exposure of the fetus to alcohol, drugs, or to fat carries the risk of damaging the child’s health. ‘Modes of seepage’ permeate at the intersection of mother and fetus, a connection between placenta and umbilical cord, where matter can pass from one being to another in a process of feeding and excretion.

However, instead of exploring this placental process as a protective barrier that limits exposure of the fetus to harmful substances, the media (and some social scientists) completely ignore this clinical evidence and revert to a simplistic discourse in which pregnancy and the womb operate as a ‘performance of contagion… where the passage of fluid inside the pregnant body, backwards and forwards between the pregnant woman and the fetal entity, enacts the process of contagion’ (Maher, 2001: 201). Thus transmission of fat is no longer presented as a potential risk: instead intergenerational certainty of transmission is presented. Such logic couples appetite and emotions of guilt through successive generations of gendered blame: ‘Gulp…You are what your Grandma ate… Research by the Victor Chang Institute shows that what mothers and grandmothers ate during pregnancy affects the health of a particular generation through the genes that are passed on’ (The Sydney Morning Herald, 2006: 34).

**Pregnant bodies as smoking guns**

The ‘Mothers’ smoking gun’ (Stewart, 2009) article, cited at the beginning of this article, extends the potentiality of harm to the interiority of women’s bodies. Despite the lead-researcher (one of the authors of this article) emphasising to the journalist that life course and intergenerational health are ‘intertwined in very complex ways’, the representation of obese women as ‘smoking guns’ took precedence. This representation feeds into a simplistic discourse of genetics and deviance; the smoking gun is a popular media metaphor for understanding complex medical relationships where there appears strong circumstantial evidence for a causal relationship between an exposure and a disease process, but where the direct causal mechanism is obscure or unavailable for direct observation. A smoking gun represents a gun that has already ‘gone off’ and as such presents indisputable evidence of a crime that has been committed. In this case, the crime is to be overweight and pregnant, thus harming the unborn fetus. It is not the microscopic or invisible workings of genes that are the gun, but obese women’s bodies in pregnancy that are viewed as culpable.
The metaphor of (fat, pregnant) women as smoking guns is limited and inadequate, as the overnutrition hypothesis speaks to a bodily environment (the womb) that is very much mediated by the socio-economic environment in which the woman is situated. In reducing scientific understandings to genetic determinism, the interplay between bodies and their socio-cultural context is entirely overlooked. Within the fetal overnutrition hypothesis, there is no social circumstance without risk to the mother and the child. Every action involving a diet, food, and social practice is a compromise of interests, hazards, and likelihoods. Every action is constrained by a range of factors, whether social or biological. It is equally inadequate to attempt to explain the body of the mother as entirely reducible to either genetic or intra-individual motivational factors. The body of the mother is therefore a socio-biological accomplishment, the product of selection and survivorship pressures across generations that is in turn shaped by the social context within a lifetime.

The ways in which our bodily environment is mediated by diverse social factors is overlooked in the imagery of the smoking gun and in other print media claims that childhood obesity is triggered by ‘mothers who eat junk food during pregnancy’ (Science Daily, 2007) or that fat, pregnant women ‘condemn their children to a life of overeating and obesity’ (Connolly, 2008). In narrowing the frame of potential risks to the behaviour and biology of expectant women, the bodies of mothers are solely blamed for the misfeeding of their children, even inutero.

In this popularist discourse of the maternal lineage of fat transmission, men are absent from the production and reproduction of obesity across generations. It is assumed that the mother, and only the mother, influences the fetal environment and effects the transmission of obesity in her children. Recent studies, however, contest this position, finding the association between maternal and offspring BMI is comparable to that between paternal and offspring BMI, and concluding that intergenerational obesity involves both the father and the mother (Cole et al., 2008; Davey Smith et al., 2007; Hawkins et al., 2009; Kivimäki et al., 2007). And, among studies which find a stronger correlation between maternal and offspring BMI than that between paternal and offspring BMI, it does not necessarily follow that offspring BMI is an indicator of offspring fat mass during childhood (Lawlor et al., 2008). Lawlor et al. (2008: 491) caution that ‘developmental overnutrition related to greater maternal BMI is unlikely to have driven the recent obesity epidemic’.

One interpretation of these data is that while there are specific clinical conditions in pregnancy that can directly influence the growth trajectory of the fetus, and thereby the offspring, these factors can be largely swamped within an environment where the majority of the adult population is overweight or obese. Hence, most of the variation in child BMI may be attributable to social factors common to both the mother and father, and not the direct biological effect of maternally mediated fetal nutrition operating specifically in pregnancy. Again, while the mother may be holding a smoking gun, so too is the father.
Implications and conclusion

In this article we have shown how women’s reproductive bodies are entangled in historical, scientific, and media discourses that intimately link the behaviours and biology of mothers with harm to the fetus. The reporting on the ‘new science’ of the fetal overnutrition hypothesis now extends the gendered nature of infant feeding practices to the interiority (including molecular and genetic levels) of women’s bodies. Social environments have segued into intrauterine environments, in which fat, pregnant mothers can transmit obesity on to their children and future generations. Coupled with a political shift to individual responsibility and moralising discourses on obesity (Wright and Harwood, 2009), maternal obesity has become a powerful meta-discourse of blame.

Reductive understandings of obesity lead to reductive solutions. Blaming mothers for children’s excess weight in both biological and social terms narrows the cause of ‘the obesity problem’ and therefore the solution to the individual bodies of women. Clinical suggestions of pharmacological contraception, gastrointestinal surgery, and the frequent use of weighing stations, all proposed to reduce obesity in women (e.g. Kral, 2004), girls, and young women, have proliferated yet are misplaced. Alarmingly, it is not only the corpulent pregnant woman who is to blame for obesity. Kral (2004: 1544), a US surgeon writing in the prestigious journal *Paediatrics* in 2004, argues that all women, even ‘newborn girls’, have the potential to become ‘doubly damaging’, both polluted and polluting, since fat is passed on through the female body. Accordingly, the only way to curb the obesity epidemic is to ‘urgently’ target girls and young women: ‘[f]rom birth to menarche, behaviour modification in mothers and children should be the first choice’ in obesity prevention (Kral, 2004: 1544).

While Kral’s view is extreme, McNaughton (2011: 1) argues that ‘core assumptions at the heart of obesity science have been taken up uncritically in medical arenas focused on conception, pregnancy, and reproduction and that this is providing new opportunities for the surveillance, regulation, and disciplining of ‘threatening (fat) female bodies’. We would agree that the Foucauldian gaze is firmly on the interiority of women’s bodies, but we do not take the fetal origins field as a homogenous one. Some scientists working in this field do critique the narrow framing of obesity causality (Warin et al., 2011), arguing that this draws attention away from the very real structural inequalities in health care, education, and employment that are often felt hardest by women and minorities (Boero, 2009: 118). As Kukla (2008) notes, public discourse tends to focus on individual responsibility and displays of will-power (or failure of these), rather than the structural conditions that enable or undermine people’s ability to make choices over the long term.

Longhurst (1999) noted that pregnancy is a biological process but exists within socio-cultural, economic, and political realms and is both spatially and temporally located. If we focus on the relationships between the social and the biological, and the ways in which they fundamentally interact with each other, then we have a much more powerful framework to understand gendered bodies and the social
determinants of health. Barker’s work potentially offers a way to reduce social inequalities in health by giving attention to the living conditions, health, and nutrition of young women, pregnant mothers, and their children. Fetal origins, for example, provide a compelling framework to understand the high rates of obesity amongst women in disadvantaged populations. In a discussion of the obesity amongst the Pima Indians of Arizona, Wells argues that ‘obesity arrived [most notably for the Pima women] in combination with poverty and US government rations of sugar and flour’ (2010: 296). Poor maternal diets, gender bias through preferential feeding of male children, and fetal exposure to maternal work during pregnancy means that obesity is structurally embedded in socio-cultural contexts. Addressing women’s livelihoods will bring particular benefits to their health, whilst further passing these on to subsequent generations.

Our research focuses on the ways in which the politics of mothering and individualised (gendered) responsibility is implicated in obesity debates and policy (Moore and Davies, 2008; Zivkovic et al., 2010). The media is central to this politics as it is part of a neoliberal paradigm that aims to individualise responsibility and individualise the biocultural. In its shallow approach, it reaches into women’s bodies to locate obesity as simply a matter of good or bad mothering. Our research aims to invert the gaze to phenomena outside the body – to focus on bodily action, biological endowment, evolutionary history, and the organisation of society (and not just one of these). This approach means challenging the communication of knowledge that is strongly influenced by the media’s power to characterise women’s bodies (in both their reproductive capacities and social roles) in ways that make it seem ‘natural’ to blame them for obesity transmission across generations.

**Funding**

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

**Note**

1. For example, in January 2010 London buses and billboards were awash with the slogan ‘Career women make bad mothers’. Following public outcry (predominately from working mothers), the Outdoor Advertising Association (who ran the ad campaign in an attempt to promote the effectiveness of billboard advertising) removed them.

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**Megan Warin** is a social anthropologist and Senior Lecturer in the Discipline of Gender, Work and Social Inquiry at the University of Adelaide. Her current research interests span theories of embodiment, intersections of class and gender in experiences of obesity, public understanding of obesity science, and desire and denial in eating disorders.

**Tanya Zivkovic** is a social anthropologist whose research explores the body and cultural trajectories of the life course. Tanya’s research interests include work on death, relics, and reincarnation among Tibetan Buddhist lamas, ‘the child’, gender and obesity, and more recently Tibetan healing and biomedicine. Tanya is a Research Fellow in the Discipline of Gender, Work and Social Inquiry, University of Adelaide, and has recently completed a residency in Ascona, Switzerland as a 2011 Fellow of the Centro Incontri Umani.

**Vivienne Moore** is a Professor and (with Michael Davies) is Co-Director of the Life Course and Intergenerational Health Research Group at the University of Adelaide, South Australia. Her research investigates the social and behavioural influences on women’s and children’s health within a life-course perspective, and gender inequalities in health.

**Michael Davies** is an Associate Professor, ARC Future Fellow and Co-Director of the Research Centre of Early Origins of Health and Disease at the University of Adelaide. His research in reproductive epidemiology investigates early life factors that impact on the health of Australians within and across generations. He is also researching decision-making, safety, and effectiveness of assisted reproductive technologies.
From the womb to the tomb: obesity and maternal responsibility

Darlene McNaughton*

School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University, P.O. Box 6811, Cairns, QLD 4870, Australia

(Received 17 May 2010; final version received 9 September 2010)

In recent decades overnutrition and obesity have been presented as a looming threat to the health and wellbeing of children and infants, most notably in western industrialised societies. However, this threat is not simply limited to ‘children’ who are ‘over fed’ by their ‘parents’. Increasingly, maternal overweight and obesity are said to inhibit conception, cause recurrent miscarriage, pose a serious threat to the development and health of the foetus and have long-term implications for the future wellbeing of the child. Parental responsibility looms large in these discourses, in which women in particular are held responsible for the future (fat free) health of their offspring from the womb to the tomb. In this article, it is argued that core assumptions at the heart of obesity science have been taken up uncritically in medical arenas focused on conception, pregnancy and reproduction and that this is providing new opportunities for the surveillance, regulation and disciplining of ‘threatening’ (fat) female bodies. It is shown that although all women of a reproductive age are being brought under the gaze of this deeply punitive medico-moral discourse, it is the bodies, lives and bedrooms of marginalised women that are singled out as posing the greatest ‘risk’ to their offspring and then targeted for even greater degrees of health/State intervention and surveillance.

Keywords: children; health behaviour; public policy

Introduction

An increasing body of evidence suggests that obesity does indeed beget obesity: children of obese parents have a stronger tendency towards obesity…the vicious spiral of obesity is rapidly spiralling upwards as this tendency is passed from parent to child (Reece 2008, p. 24).

We must look at the womb to understand what is producing today’s obesity (Leibowitz quoted in Taylor 2009, p. 1).

As many commentators have noted, the culture of public health policy, practice and research has shifted in recent decades and increasingly focuses on the regulation of private behaviour, rather than public projects and infrastructure (Petersen 1996, 1997,
Petersen and Bunton 1997, Petersen and Lupton 1997). During this period, obesity and overweight have become a central focus of the politics of private regulation (Petersen and Lupton 1997, p. 9). In the context of an alleged global obesity epidemic, fatness is increasingly understood as dangerous and debilitating – an unhealthy state of being that places the individual at much greater risk of a growing list of ailments ranging from diabetes to cancer. Since the mid-1990s, the central trope of most obesity discourse, public health messaging, and media commentary is ‘be alarmed’: because we are getting fatter at a disturbing rate, being overweight or obese has serious health consequences and everyone is at risk.

Within this discourse, fatness and overnutrition have also been consistently presented as a looming threat to the health and wellbeing of children, most notably in western industrialised societies (Austin 1999, Campos 2004, Gard and Wright 2005, Campos et al. 2006, Murray 2008). Public health campaigns unfailingly emphasise the short- and long-term risks of fatness in children, the scale of the issue, the changeable nature of behaviours purported to produce fatness and the role of parents in inflicting their ‘unhealthy’ habits on their innocent offspring. In some quarters, there are increasing calls for legislation to control or criminalise those who ‘abuse’ their children through ‘over feeding’ or expose them to unhealthy dietary behaviours (Bell et al. 2009). Although fathers and more rarely same sex partners are implicated in these statements, gender stereotypes about responsibility for feeding children are very much at play and invariably, these inadequate or irresponsible parents are cast as mothers and as overweight or obese.

However, the alleged threat posed by fatness or overnutrition is not simply limited to children who are ‘over fed’ by their parents (read parent). Concerns regarding maternal obesity during pregnancy, foetal obesity, and infant feeding are becoming more commonplace in scholarly research and popular commentary. Increasingly, maternal fatness is said to inhibit conception, cause recurrent miscarriage, pose a serious threat to the development and health of the foetus and have long-term implications for the future wellbeing of the child. Parental responsibility also looms large in these discourses, in which women in particular are held responsible for the future (fat free) health of their offspring from the womb to the tomb.

In this article, it is argued that core assumptions at the heart of obesity science regarding the scale of the obesity problem, the nature of the risk, and where responsibility for health should fall, have been taken up uncritically in medical arenas focused on conception, pregnancy, and reproduction. This in turn is providing new and disturbing opportunities for the surveillance, regulation, and disciplining of ‘threatening’ (fat) female bodies while at the same time perpetuating a number of taken for granted medico-moral assumptions about individuals and the causes of fatness.

Framing obesity as risky

As many commentators have shown, discussions of health risk also serve as part of the increasing surveillance functions of modern medicine, which shifts the medical gaze from the individual to the population at large and encourages individuals to adopt increasing vigilance over their own bodies and behaviours (Armstrong 1995, Lupton 1995). Presenting obesity as a serious health ‘threat’ of epidemic proportions is, among other things, an exercise in power, disciplining and surveillance
in which ‘new forms of governance are creating problematic social conditions [i.e. unsatisfactory parenting, mothering] in which the state can ‘reasonably’ intervene’ (Evans et al. 2008, p. 11). Fatness is a highly visible and deeply stigmatised physical characteristic that cannot be hidden in the same way as smoking or drinking and this makes it open to considerable surveillance and judgement (Saguy and Riley 2005, p. 913), particularly in the context of a medical encounter.

Within the discourses examined here, epidemiology and biomedical research are the primary source of expertise and knowledge regarding obesity and are characterised by a belief in law-like mathematical regularities in the population (Hacking 1990, Lupton 1995, Gard and Wright 2005). National and international obesity statistics are consistently held up in this literature as ‘evidence’ that an increasing BMI equals greater risk from a number of diseases, when, in fact, any data correlating obesity prevalence with disease incidence is, at best, ‘an indication of a possible link between body size and health for a population’ rather than an undeniable truth (Gard and Wright 2005, pp. 101–102). Despite the certainty expressed in these discourses and in public health circles that ‘fat is a killer’, we do not actually know exactly how dangerous it is to be overweight as many critics have shown1 (Austin 1999, Campos 2004, Gard and Wright 2005, Campos et al. 2006, Murray 2008). The ideology of fatness as unhealthy and as an entirely controllable and avoidable risk to life long (i.e. fat free) health is also ubiquitous in the body of literature under examination. Here, ‘healthiness’ acts a metaphor for self-control, self-denial and willpower and as a moral discourse (Crawford 1994, p. 1352). Indeed, when overeating and inactivity are constructed as avoidable, ‘fat bodies are read as evidence of both preventable illness and moral failings’ (Saguy and Riley 2005, p. 885).

Despite the inconclusive state of the evidence on obesity and its impact on ‘health’, public health campaigns, media reports and the medical literature urge parents to vigilantly monitor their own and their children’s weight (see Campos 2008 for a discussion). As I have previously argued (Bell et al. 2009), mothers are particularly singled out in obesity discourse as responsible for the body size and weight of their offspring. In contemporary biomedical and public health discourse, a mother’s work patterns (Anderson et al. 2003, Zhu 2007) and feeding practices (Rising and Lifshitz 2005) have both been deemed a source of ‘risk’ for childhood obesity – an argument that serves to reinforce traditional gender roles and stereotypes.2 As Lee (2008, p. 468) has noted, mothering in modernity is understood as ‘both the private responsibility of individual mothers, and also a matter of public scrutiny and intervention, with mothering practices defined as ‘good’ or ‘bad’ in expert and policy discourses’. Of course, ideals about what constitutes a ‘good’ or ‘bad’ mother are deeply cultural, shaped by larger structural forces and underwritten by a range of classist, racist and sexist assumptions. They are also linked to discourses of risk, where in a ‘good’ mother resists or avoids any action or activity that might be potentially ‘unhealthy’ for the child (Lee 2008, p. 468).

Breastfeeding (doomed if you do, doomed if you don’t)

While maternal feeding practices more generally have received considerable notice in the obesity literature, breastfeeding has been a particular focus of attention. Breastfeeding is a practice that has received substantial support from primary care and public health sectors in recent decades. Given its raised status as the most appropriate way to feed one’s child it can be crucial to a woman’s identity as a ‘good’
mother (Schmeid and Lupton 2001), and women who choose not to breastfeed, or find they are unable to do so, face considerable challenges in maintaining a positive ‘maternal’ identity (Lee 2008).

Coinciding with a recent national report suggesting that ‘90% of UK born children are being formula fed after 6 months of age’ (Lee 2008, p. 469), research into the ‘protection’ breastfeeding might confer against obesity is becoming increasingly common, with media reports on these studies including headlines such as ‘Mums encouraged to breast feed in public to fight childhood obesity’(Ely Standard 2008). However, evidence of the protective effects of breastfeeding against overweight and obesity is far from convincing. For example, one study found that:

The prevalence of obesity was significantly lower in breastfed children, and the association persisted after adjustment for socio-economic status, birthweight, and sex. The adjusted odds ratio for obesity (BMI > 98th percentile) was 0.70 (95% CI 0.61–0.80). Our results suggest that breastfeeding is associated with a reduction in childhood obesity risk (Armstrong and Reilly 2002, p. 2003).

Although results such as these are statistically weak and rather inconclusive (Beyerlein et al. 2008), similar surveys of women and their infants are being undertaken at a furious rate, with most concluding that overweight and obese women should be specifically targeted for breastfeeding (Buyken et al. 2008). Yet, some researchers are arguing that ‘the increased glucose and insulin levels in the breast milk of mothers with diabetes may actually increase the risk of subsequent obesity in childhood’ and that ‘rather than being causally related to later protection against obesity, the presence of breast-feeding may actually be a marker for other factors related to leanness’ (Toschke et al. 2002, pp. 765–766; see also Braegger 2003).

Many of these epidemiological studies collect data on the fat status of women through the documentation of their BMI before, during and after pregnancy, on how many women breastfeed and for how long (Armstrong and Reilly 2002, Arenz et al. 2004). Some of these studies suggest that overweight or obese women, notably women of colour, are not breastfeeding as much as their thin counterparts (Liu et al. 2009, p. 175). According to one study, obese women were less likely to initiate breastfeeding and ‘women who were obese before pregnancy fed for 2 weeks less than their normal-weight counterparts’ (Li et al. 2003, p. 931). These results have the capacity to further stigmatise fat women, especially as they make no effort to examine why the participants did or did not breastfeed (e.g. returning to work, shame, lactation problems, etc.) or to explore the broader socio-economic, cultural or political contexts that might have been at play.

As Petersen and Lupton (1997) have noted, the positioning of women as producing ill health in their children has long been a central element of public health initiatives and biomedicine more broadly. In the future, fat post-partum women who cannot breast feed, who struggle to but find it too painful, choose not to in the first instance or are advised against it because they are diabetic are going to have to work even harder than their thin compatriots to keep their identity as a good mother secure.

A search for origins

More recently, research into the origins of obesity has extended its gaze to pregnant women, whose eating habits are suspected of influencing the weight and health of
their future offspring (Catalano and Ehrenberg 2006, Wu and Suzuki 2006, Rodriguez et al. 2008). Underwriting this literature is a view that fat women will be fat mothers and have fat babies: ‘Nutrition in the womb is central for foetal development ... a mother's pre-pregnancy or early pregnancy birth weight is a likely determinate of the birth weight of her child and that infant birth weight is a likely predictor for adolescent and adult weight’ (Smith et al. 2008, p. 178).

One paper, entitled Maternal and child obesity: the causal link, encapsulates some of the key assertions regarding maternal weight and future child health and the ‘cycle of obesity’ (Oken 2009). The authors write:

High maternal weight entering pregnancy increases risk for obesity and cardiometabolic complications among offspring ... higher maternal gestational weight gain is associated with higher weight and consequent risk for obesity and elevated blood pressure among children ... and that while these ‘associations’ are partly mediated by shared genes and behaviour, the abundance of human evidence, supported by extensive ... animal studies, suggests that intrauterine exposure to an obese intrauterine environment programs offspring obesity risk by influencing appetite, metabolism and activity levels (Oken 2009, p. 361).

For many such commentators, the source of obesity is the womb: the intrauterine environment. For Oken (2009, p. 362) and others, the only way to slow the obesity epidemic and improve the lives and life expectancy of future generations is to ‘interrupt this cycle of obesity’ by intervening throughout a woman’s reproductive life: before she conceives, while she is pregnant and in the years after she has given birth (Gunderson 2009). Although several of these same commentators acknowledge that many of the findings are contradictory, based on animal studies or too weak to show any clear relationship between maternal overweight, foetal or infant obesity and long-term health effects, most call for greater levels of intervention and surveillance of overweight women (Guillaume 1999). Alongside this research are related discussions regarding the possible existence of an ‘obesity gene’ which, like the ‘gay gene’, is attached to commentaries about prenatal genetic diagnosis being used for obesity testing (see LeBesco 2009 for a trenchant critique).

With striking consistency, the literature emerging from reproductive medicine begins with the premise (stated or unstated) that we are in the midst of worldwide obesity epidemic. This, it is asserted, is resulting in more women being overweight or obese prior to, during and after pregnancy, especially women of colour, poor women and those from minority groups (see, e.g. Phelan 2009). The uncritical acceptance of a childhood obesity epidemic leads many to imagine the avalanche of fat women of a reproductive age that is come and the impact this will have on health care systems. Acceptance of the threat of obesity and the risks it is purported to pose, operates not only as a justification for the research itself, but also as a call for urgency in the generation of knowledge and for the development of immediate and earlier interventions into women’s lives (pre-pregnancy) on the part of experts. The discourse constructs itself.

**Fat women produce fat (unhealthy) foetuses and infants**

In fat-averse societies, pregnancy is one of the few times women (particularly middle class women) are encouraged to eat freely (‘to eat for two’) and gain weight legitimately without the guilt and stigma traditionally attached to an increase in body size. Not anymore. For the nature of a woman’s dietary and exercise habits
and the conditions these are thought to create in her womb are coming under greater scrutiny. This focus on the womb is encapsulated in the recent work of Barker and colleagues who suggest that ‘a woman provides her unborn baby with a ‘nutritional forecast’ that guides metabolic development’ and that ‘it is experiences before birth, primarily, that are held to have a permanent legacy’ with regards to the development of obesity and overweight (Barker 2004 cited in Moore and Davies 2005, pp. 341–342).

Although many of these studies commonly assert that a great deal is still not known about the causes of foetal or infant overweight or obesity, the female body is increasingly the site of new research into these questions:

...inadequate or excessive energy intake is not optimal for the developing fetus. Against a history of inconsistent results, several recent studies suggest that in Western settings the balance of macronutrients in a woman’s diet can influence newborn size. Effects appear to be modest, but this relationship may not encapsulate the full significance for health of the child, as there is emerging evidence of associations with long-term metabolic functioning that are independent of birth size (Moore and Davies 2005, p. 341).

Other commentators are less moderate in their assertions, claiming that ‘paediatric obesity has reached critical proportions’ and is contributing to the worldwide obesity epidemic (Lieb et al. 2009). Other epidemiological studies argue that ‘individuals who were small at birth have an increased risk of type II diabetes and cardiovascular disease in adulthood’ and overweight and obesity (Grivetti 1998 cited in Moore and Davies 2005, p. 341). The internal contradictions in this research are notable.

Also significant is that although underweight and overweight are both constructed as a risk for mothers and babies, it is overweight and obese mothers who currently receive the greatest focus in this literature. Although there was historically much interest in underweight mothers and underweight babies, women who are considered underweight or ‘normal’ weight are often excluded from contemporary studies examining the impacts of weight on mothers and offspring, which commonly focus entirely on women identified as overweight or obese in terms of their BMI (<20) (see, e.g. Callaway et al. 2006). This limits the possibility of drawing a broader impression of the effects of weight and diet on foetal, infant or child health and evidences the powerful influence of ideas regarding the threat of obesity in these research areas.

Indirect links between maternal obesity and health effects in her future offspring are also being made via a focus on gestational diabetes. It has long been recognised that some women develop non-insulin dependent diabetes mellitus (type II) during pregnancy and that for many the condition dissipates after they give birth. However, links are now being made between gestational diabetes and obesity and type II diabetes in offspring. For example, in a commentary from the Journal of the Australian Medical Association entitled ‘Maternal diabetes and obesity may have lifelong impact on health of offspring’ we find the following:

Many obstetricians have traditionally struggled to help diabetic women maintain good blood glucose control during pregnancy. Then, once the infant was born, everyone would give a sigh of relief... We used to think, at least the baby’s out and it’s safe. Well, that baby is not safe. We have set up this child for adverse health downstream – certainly in childhood, and perhaps as an adult... It’s a vicious cycle where an obese insulin-resistant woman has an obese fetus who becomes an obese neonate, who
becomes an obese child, who is at greater risk to develop type 2 diabetes. We’ve always assumed that if you would just get up and get a gym membership and not drive to McDonald’s, you would be able to avoid these problems... And maybe to some extent that’s true, but it might also depend on your intrauterine nutrition (Hampton 2004, p. 789).

In this literature, obesity is sometimes referred to as a disease, for example: ‘In addition to being a serious disease in its own right, obesity has also added fuel to a multitude of other diseases and can be socially contagious’ (Fumento cited in Saguy and Riley 2005, p. 892; see also Reece 2008). It is also identified as a cause of diseases like diabetes mellitus, rather than as a symptom (Gard and Wright 2005, p. 95). However, overweight and obesity, like thinness, are not diseases, or diagnosable illnesses (Gard and Wright 2005, p. 25). Framing fat as an avoidable disease and a disease causing agent assists in characterising fat women of child bearing age as irresponsible and dangerous to themselves, to their offspring and to society. They are bad citizens and bad mothers.

Maternal obesity and the unproductive and deadly womb

Women of childbearing age are also a growing object of study into the implications of overweight and obesity on conception, miscarriage, birth defects and foetal and infant development. This research has been appearing in much greater quantities in a range of academic and practitioner-oriented journals from the fields of paediatrics, obstetrics, gynaecology, fertility, reproduction and midwifery.

Maternal fatness is now a central focus in studies on conception, where it is commonly hypothesised that overweight and obesity inhibit conception, including assisted conception with IVF and have a role to play in infertility (Norian et al. 2005, Rajasingham et al. 2009). Again, it is poor minority women of colour who feature most strongly statistically:

Obesity negatively affected CP [clinical pregnancy] in all races studied; however, obese Black and White women had a lower percentage of CPs [clinical pregnancy]. Although both Blacks and Hispanics had a higher incidence of obesity, obesity imposed the greatest negative impact on IVF CP [clinical pregnancy] success in Blacks compared to other races (Norian et al. 2005, p. 249).

To address this, some practitioners are calling for greater use of gastric banding to reduce a woman’s weight before she tries to conceive, but the risks are considerable, given the high mortality rates associated with this procedure. Coustan notes that one study has suggested that ‘gastric banding was more effective than lifestyle intervention in inducing remission of type 2 diabetes in an obese patient’ (2008, p. 2552). He goes on to suggest that

Theoretically, such interventions may reduce the risk of adverse pregnancy outcomes associated with diabetes, hypertension and obesity. However, gastric bypass carries risks including nutritional deficiencies because of decreased nutrient intake as well as decreased fat absorption (Coustan 2008, p. 2552).

Maternal obesity has also become the focus of studies into the causes of stillbirths and in some research is being identified as a significant cause, alongside other factors such as age and IVF, for which there are more compelling data (Lo 2008). In recent media coverage of an Australian study into maternal obesity and still births, it was reported that ‘There could be an epidemic of stillbirths in Australia in the next few
years if the nation’s obesity rate continues to soar and more women aged over 35 have children’ (Flenady, 2008). In an interview with the study’s author, it was asserted that:

40% of the 2000 Australian stillbirths a year are preventable if a woman loses any excessive weight, has children earlier and gives up smoking... That’s 800 babies a year which could be saved if we were able to remove these three modifiable factors (Flenady 2008).

In contrast to these alarming and alarmist claims, a meta analysis of studies on the topic concluded that ‘maternal obesity is associated with an increased risk of stillbirth, although the mechanisms to explain this are not clear’ (Chu et al. 2007, p. 223).

Who gets the intervention?

In the discourses on maternal obesity, fat women are scapegoated as irresponsible mothers/parents and citizens who set a poor example, and put their food addictions and bad habits ahead of the health of their offspring and their very capacity to reproduce society. Framing maternal obesity and overweight as the result of risky behaviour also suggests a need for intervention – usually in the form of education and increased surveillance. It also implies and potentially reinforces the view that fat people are stupid or ignorant (Saguy and Riley 2005, p. 886). For example, Dr Xavier Pi-Sunyer, who runs a weight loss clinic and is on the board of Weight Watchers (USA), writes:

Why does the average American woman gain weight with each pregnancy and end up [after] four kids, fifty pounds heavier? Its because nobody alerts her to the fact that this may happen and it many not be good for her to end up fifteen to twenty years later fifty pounds heavier (cited in Saguy and Riley 2005, p. 886).

It is argued here that within these discourses on maternal and child obesity is a shared and increasing concern regarding the ‘problems’ and ‘threats’ posed by the individualised behaviours of women whose actions are constructed as dangerous to the interests of their children, families, communities and nations. In these discourses and in obesity science more broadly, an unhealthy lifestyle is evidenced by higher than average weight, which in turn is read as evidence of a lack of self-control and of personal and civic (because of public health costs) irresponsibility (Saguy and Riley 2005, p. 887). These understandings are embedded in much of the discourse examined here with little or no reflection on the deeply cultural and classist assumptions that underwrite them.

As Petersen and Lupton (1997) note, the enforcement of state-imposed regulations tends to be exercised upon the most stigmatised and powerless groups. In particular, it is women of colour, single mothers and women living in poverty who are most often identified as posing the greatest risk to their offspring and targeted for intervention and surveillance – further stigmatising those who are already marginalised and powerless (Bell et al. 2009). In this literature, there is little recognition of the potential harms that arise from increased state interventions into the lives of these women, let alone consideration of the structural and contextual factors that create risks to health in the first place such as poverty, racism, disenfranchisement, poor housing, etc. This neo-liberal emphasis on individual responsibility is popular because it emphasises personal control over illness rather than requiring major...
changes in industrial practices, in the economy, or in the government (Saguy and Riley 2005, p. 887).

**Conclusion**

As many commentators have shown, the true impact of fatness on health is not known and obesity science is permeated with ambiguity and contradiction (Gard and Wright 2005). In this article, I have argued that certain assumptions regarding the inherent dangers of fatness, on conception, pregnancy foetuses, infants and children need to be critically examined. These include but are not limited to assumptions regarding the scale of the obesity epidemic, the nature of the risk and where responsibility for health should fall. These logics are far from neutral or objective (Austin 1999, Campos 2004, Gard and Wright 2005, Campos et al. 2006, Murray 2008). They also affirm certain moral, neo-liberal ideas and values while at the same time rendering invisible the political economy that produces ill health in the first place (poverty, racism, classism, sexism, etc.) (Crawford 1994).

These logics and the medico-moral assumptions that underpin them underwrite the design of studies, the examination of results and in claims about the risks of exposing foetuses, infants and children to food substances and lifestyles said to have the potential to negatively affect their health in the short- and long-term. Furthermore, in a search for the ‘origins’ of obesity, researchers are moving beyond the usual ‘suspect’ environments of the kitchen table, corporations and genetics as the causes of fatness, and turning their gaze to the female body and the womb.

Within these discourses it is asserted and assumed that women who exert self-control and maintain a healthy weight throughout their life are more likely to produce children of a ‘normal weight’ who, according to the core assumptions of obesity science, will be healthier in the long-term. By contrast, those who do not discipline themselves in these ways or do so unsuccessfully are not only less capable of reproducing, but their unhealthy lifestyles, behaviours and state of being can cause the untimely death of their unborn child, or doom those that do survive to a life of overweight, ill health and a shortened life span.

Even more disturbingly, the gaze of this deeply punitive medico-moral discourse is being expanded to encompass all women of childbearing age because of suspicions that their body weight and eating habits before pregnancy influence the future survival and health of their offspring. However, as demonstrated above, it is the bodies, lives and bedrooms of marginalised women that are singled out for even greater degrees of health/State intervention and surveillance and are seen to pose the greatest risk to their future and current offspring.

**Notes**

1. It is not clear that simple dietary intake causes many or even most cases of overweight or obesity (Gard and Wright 2005) and yet there is little awareness of these critiques in this literature. In a review of international studies, Rolland-Cachera and Bellisle (2002) found little evidence to suggest that overweight and obese children consume more calories than other children – with the exception of children experiencing the ‘highest indices of obesity’, where a correlation was found between body weight and the amount of protein consumed.
2. According to one highly publicized study (Zhu 2007), it is the mother’s actions adopted in response to time constraints and her struggle to fulfil her dual roles as caregiver and economic provider have partly ‘caused’ growing rates of childhood obesity. In light of such discourses it is unsurprising that research suggests that women are increasingly hostile to weight gain during pregnancy and in the years immediately after giving birth (Herring et al. 2008, Laraia 2009).

3. This speaks to Bell’s (2011, forthcoming) point that the threshold for public health intervention is a socio-political phenomenon and ‘scientific’ standards for action are particularly low when children (or foetuses) are seen to be involved.

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