The Impact of Our Lifestyle on the Epigenome and Metabolism

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract:

Lifestyle factors, including physical activity and dietary intake of lipids and glucose, play a role in metabolism and influence susceptibility to diseases such as obesity and type 2 diabetes. Recent human investigations conducted by our research team have revealed that lifestyle factors such as exercise and altering lipid and glucose intake can induce changes in DNA methylation patterns in controlled laboratory settings and within human subjects. These epigenetic modifications may modulate the expression patterns of genes and proteins in human tissues and, as such, affect metabolism. Thus, epigenetic modifications may act as the link between different lifestyle factors, such as dietary intake, and the likelihood of developing metabolic diseases. However, we need to enhance our understanding of how lifestyle factors modulate DNA methylation. We, therefore, aim to explore the impact of various lifestyle factors, such as dietary factors and physical activity, on DNA methylation in human tissues and cells. Additionally, we want to investigate how these molecular changes influence metabolism and the risk of developing diseases. *In vitro* analyses in human pancreatic islets showed that elevated glucose and lipids (glucolipotoxicity) induced methylation and expression changes, contributing to impaired insulin secretion and increased apoptosis. Analyses in human offspring cord blood show that lifestyle intervention and gestational weight gain among pregnant women with obesity impact the DNA methylome of several genes which seem to be linked to offspring anthropometrics measures important for offspring health. Furthermore, our findings in human sperm indicate that a one-week sugar-rich diet does not significantly alter the sperm methylome. However, nominal evidence suggests potential DNA methylation modifications of specific CpG-sites linked to male fertility and imprinted genes after the sugar-rich diet. Overall, these findings support that DNA methylation may link lifestyle factors to health implications, including metabolic diseases.

Keywords: DNA methylation, Diet, Epigenetics, Lifestyle, Metabolism, Obesity, Physical Activity, WGBS

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Till min älskade familj
(och alla er som höll mig flytande)

Tacksamhet fyller mitt hjärta för er kärlek och ert stöd

”Make a habit of two things –
to help, or at least, to do no harm”
- Hippocrates

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Abstract

Lifestyle factors, including physical activity and dietary intake of lipids and glucose, play a role in metabolism and influence susceptibility to diseases such as obesity and type 2 diabetes. Recent human investigations conducted by our research team have revealed that lifestyle factors such as exercise and altering lipid and glucose intake can induce changes in DNA methylation patterns in controlled laboratory settings and within human subjects. These epigenetic modifications may modulate the expression patterns of genes and proteins in human tissues and, as such, affect metabolism. Thus, epigenetic modifications may act as the link between different lifestyle factors, such as dietary intake, and the likelihood of developing metabolic diseases. However, we need to enhance our understanding of how lifestyle factors modulate DNA methylation. We, therefore, aim to explore the impact of various lifestyle factors, such as dietary factors and physical activity, on DNA methylation in human tissues and cells. Additionally, we want to investigate how these molecular changes influence metabolism and the risk of developing diseases. *In vitro* analyses in human pancreatic islets showed that elevated glucose and lipids (glucolipotoxicity) induced methylation and expression changes, contributing to impaired insulin secretion and increased apoptosis. Analyses in human offspring cord blood show that lifestyle intervention and gestational weight gain among pregnant women with obesity impact the DNA methylome of several genes which seem to be linked to offspring anthropometrics measures important for offspring health. Furthermore, our findings in human sperm indicate that a one-week sugar-rich diet does not significantly alter the sperm methylome. However, nominal evidence suggests potential DNA methylation modifications of specific CpG-sites linked to male fertility and imprinted genes after the sugar-rich diet. Overall, these findings support that DNA methylation may link lifestyle factors to health implications, including metabolic diseases.

Popular Science Summary

Your body needs energy to function. For example, basal functions such as breathing and pumping your blood consume energy, as does moving around. We humans get this energy through the food and drink we consume. For the body to be able to use the food as energy, several chemical reactions take place where the food is converted to energy that our body can use; this is called metabolism.

Obesity is a global health problem that is only getting worse, and today, approximately 1 in 8 people lives with obesity. Obesity often arises because you eat too much food and engage in insufficient physical activity, resulting in a surplus of energy intake compared to energy expenditure. The surplus energy gets stored in your body as fat, leading to weight gain over time. Being overweight or obese can lead to many different health problems, such as heart disease, type 2 diabetes, and certain types of cancer. Obesity during pregnancy also increases the risk for complications during pregnancy and delivery and both short and long-term adverse health effects in the offspring.

DNA, the blueprint detailing the construction of the body, is present in identical copies in the cell nucleus of all the trillion cells constituting our body. Despite this uniformity, our bodies are comprised of more than 200 cell types, each with its distinct functions. This diversity in cell types is partially due to epigenetics, which allows for the construction of all the different tissues and organs in our body. Epigenetics includes several different mechanisms that regulate which parts of the DNA the cells can read and thus control which instructions the cell receives regarding its structure and function. Currently, we are unable to change our DNA sequence; it is something we inherited from our parents and which our children will inherit from us. However, we can change our epigenetics! It has been shown that when the body's circumstances change, for example, if we change what we eat or how we exercise, the instructions on how the cells should be built also change. Still, our knowledge of how and what is affected by our lifestyle is limited. We, therefore, wanted to explore how lifestyle factors, such as diet and physical activity, affect DNA methylation in our bodies. We also wanted to investigate how these potential DNA methylation changes affect our metabolism and the risk of disease.

This was investigated using four different studies. Study I investigated the effect of elevated levels of glucose and lipids (glucolipotoxicity), mimicking circulating levels of an individual with obesity and/or type 2 diabetes, on gene expression and DNA methylation patterns in human pancreatic islets. We found that glucolipotoxicity affected methylation and gene expression and that these changes seem to lead to altered insulin secretion and increased cell death. Study II was conducted on pregnant women with obesity, where 2/3 got a lifestyle intervention, including physical activity, with and without dietary advice. We then explored if there were any effects on the DNA methylation pattern in the offspring by analyzing

cord blood taken at delivery. Here, we found that the lifestyle intervention had an impact on the offspring's DNA methylation pattern in the cord blood, and several of these were linked to offspring muscle mass at birth and growth during the first three years of life. In Study III, we investigated if the amount of weight gain during pregnancy in women with obesity affects the offspring's DNA methylation pattern in cord blood. We found that the weight gain during pregnancy was, in fact, linked to changes in the DNA methylation pattern and that several of the altered methylation sites have been linked to asthma, BMI, and/or type 2 diabetes in other studies. We also found that several of the observed methylation changes were linked to muscle mass in the offspring at birth and their birthweight. In Study IV, we wanted to investigate the effect of a one-week-long diet intervention with excess sugar on the DNA methylation pattern in human sperm. Our findings indicate that a one-week sugar-rich diet does not influence the overall sperm methylome. However, there might be effects on methylation of individual sites linked to e.g., male fertility. In this study, we also provide a map of the global DNA methylome in human sperm. Overall, in this thesis, I show that DNA methylation can be affected by our lifestyle and possibly affect our metabolism, which can lead to various health consequences such as metabolic diseases.

In future studies, it would be highly interesting to include several different tissues from the same individuals and perform different biomedical analyses to better understand the link between lifestyle choices and the underlying mechanisms of diseases such as obesity and type 2 diabetes.

Populärvetenskaplig Sammanfattning

Din kropp behöver energi för att fungera. Basala funktioner så som andningen och att hålla kroppstemperaturen förbrukar energi, liksom att röra på sig. Vi människor får denna energi genom den mat och dryck vi konsumerar. Det sker flera kemiska reaktioner där det vi konsumerar omvandlas till energi som vår kropp kan använda; detta kallas ämnesomsättning eller metabolism.

Fetma är ett globalt hälsoproblem som bara blir värre, och idag lever cirka 1 av 8 människor med fetma. Fetma uppstår ofta för att man äter för mycket mat och rör på sig för lite, vilket leder till ett överskott i energiintag jämfört med energiförbrukning. Överskottet av energi lagras i din kropp som fett, vilket leder till viktökning över tid. Att vara överviktig eller ha fetma kan öka risken för många olika hälsoproblem, såsom hjärtsjukdom, typ 2-diabetes och vissa typer av cancer. Fetma under graviditeten ökar också risken för komplikationer under graviditet och förlossning samt både kort- och långsiktiga negativa hälsokonsekvenser hos avkomman.

DNA, ritningen som beskriver kroppens konstruktion, återfinns i exakta kopior i cellkärnan hos var och en av de biljoner celler som bygger upp vår kropp. Trots denna likformighet består våra kroppar av mer än 200 olika celltyper, var och en med sina unika funktioner. Denna mångfald av celltyper beror delvis på epigenetik, vilket möjliggör uppbyggnaden av alla olika vävnader och organ i vår kropp. Epigenetik inkluderar flera olika mekanismer som reglerar vilka delar av DNA:t som cellerna kan läsa och därmed styr vilka instruktioner cellen får angående sin struktur och funktion. I dagsläget kan vi inte ändra vår DNA-sekvens; den har vi ärvt från våra föräldrar och vi kommer föra vidare den till våra barn. Men vi kan ändra vår epigenetik! När kroppens omständigheter förändras, till exempel genom att vi ändrar vår kost eller vår fysiska aktivitet, ändras också cellernas instruktioner om deras uppbyggnad. Vår kunskap om hur och vad som påverkas av vår livsstil är dock begränsad. Därför ville vi undersöka hur livsstilsfaktorer, såsom kost och träning, påverkar epigenetiska markörer, så som DNA-metylering, i våra kroppar. Vi ville också undersöka hur dessa potentiella DNA-metylerings förändringar påverkar vår metabolism och risken för utvecklandet av sjukdom.

Detta undersöktes i fyra olika studier. I Studie I undersökte vi effekten av förhöjda nivåer av glukos och lipider (s.k., glukolipotoxicitet), som efterliknar cirkulerande nivåer hos en individ med fetma och/eller typ 2-diabetes, på genuttryck och DNA-metyleringsmönster i mänskliga pankreasöar. Vi fann att glukolipotoxicitet påverkade metyleringsmönstret och genuttrycket och att dessa förändringar verkar leda till förändrad insulinfrisättning och ökad celldöd. Studie II utfördes på gravida kvinnor med fetma, där 2/3 fick en livsstilsintervention med fysisk aktivitet, med eller utan kostråd. Vi utforskade sedan om det fanns några effekter på avkommas DNA-metyleringsmönster genom att analysera navelsträngsblod insamlad i

samband med förlossningen. Här fann vi att mammans livsstilsintervention hade en effekt på avkommans DNA-metyleringsmönster i navelsträngsblodet, och flera av dessa var kopplade till avkommans muskelmassa vid födseln och tillväxt under de första tre åren. I Studie III fann vi att viktökningen under graviditeten hos kvinnor med fetma påverkar avkommas DNA-metyleringsmönster i navelsträngsblod. Flera av dessa förändringar har tidigare kopplats till astma, BMI och/eller typ 2-diabetes. Vi hittade också att flera av metyleringsförändringarna var kopplade till mängden muskelmassa hos avkomman vid födseln och deras födelsevikt. I Studie IV ville vi undersöka effekten av en en-veckas dietintervention med hög andel socker på DNA-metyleringsmönstret i human sperma. Våra fynd tyder på att en en-veckas sockerrik diet inte påverkar det globala metyleringsmönstret i sperma. Det verkar dock finnas effekter på metyleringsmönstret på specifika ställen i vårt DNA som är kopplade till t.ex. manlig fertilitet. I denna studie kartlade vi även det globala DNA-metyleringsmönstret i human sperma. Sammantaget visar jag i denna avhandling att DNA-metylering kan påverkas av vår livsstil och möjligen påverka vår metabolism, vilket kan leda till olika hälsokonsekvenser såsom metabola sjukdomar.

I framtida studier skulle det vara mycket intressant att inkludera flera olika vävnader från samma individer och genomföra olika biomedicinska analyser för att bättre förstå sambandet mellan livsstilsval och de underliggande mekanismerna bakom sjukdomar såsom fetma och typ 2-diabetes.

List of Papers Included in the Thesis

Paper I

Hall E*, **Jönsson J***, Ofori JK, Volkov P, Perfilyev A, Dekker Nitert M, Eliasson L, Ling C, Bacos K. *Glucolipotoxicity Alters Insulin Secretion via Epigenetic Changes in Human Islets.* Diabetes. 2019 Oct;68(10):1965-1974. doi: 10.2337/db18-0900. Epub 2019 Aug 16. PMID: 31420409.

Paper II

Jönsson J*, Renault KM*, García-Calzón S, Perfilyev A, Estampador AC, Nørgaard K, Lind MV, Vaag A, Hjort L, Michaelsen KF, Carlsen EM, Franks PW, Ling C. *Lifestyle Intervention in Pregnant Women With Obesity Impacts Cord Blood DNA Methylation, Which Associates With Body Composition in the Offspring.* Diabetes. 2021 Apr;70(4):854-866. doi: 10.2337/db20-0487. Epub 2021 Jan 11. PMID: 33431374; PMCID: PMC7980200.

Paper III

Jönsson J, Renault KM, Perfilyev A, Vaag A, Carlsen EM, Nørgaard K, Franks PW, Ling C. *Gestational weight gain in pregnant women with obesity is associated with cord blood DNA methylation, which partially mediates offspring anthropometrics.* Clin Transl Med. 2023 Mar;13(3):e1215. doi: 10.1002/ctm2.1215. PMID: 36929108; PMCID: PMC10019770.

Paper IV

Jönsson J, Perfilyev A, Kugelberg U, Skog S, Ruhrmann S, Ofori J, Bacos K, Rönn T, Öst A, Ling C. *Impact of Excess Sugar on the Whole Genome DNA Methylation Pattern in Human Sperm.* Manuscript submitted, 2024 March 12.

*Equal contribution

Author's Contribution to the Papers

Paper I

In *Paper I*, Josefine was part of designing and executing a series of functional follow-up experiments in the laboratory setting; she conducted statistical tests, scrutinized and analyzed data, and compiled results; she wrote the majority of the first draft of the paper and was involved in revising and improving the paper before and after journal submission.

Paper II

In *Paper II*, Josefine was part of designing the analysis of the DNA methylation data and devised a plan encompassing various statistical methods to be used, potential cofounders to consider, and strategies to mitigate biases from cell-type composition and statistical inflation; she performed statistical analyses and scrutinized data, interpreted and compiled results, drafted the initial manuscript, crafted figures, and tables summarizing key findings, and was part of the submission and revision process and revised and improved the paper before and after journal submission.

Paper III

In *Paper III*, Josefine was part of designing the analysis of the DNA methylation data and devised a plan encompassing various statistical methods to be used, potential cofounders to consider, and strategies to mitigate biases from cell-type composition and statistical inflation; she performed statistical analyses and scrutinized data, interpreted and compiled results, drafted the initial manuscript, crafted figures, and tables summarizing key findings, and was the main contributor to the submission and revision process (responding to nine reviewers), revised and improved the paper before and after journal submission.

Paper IV

In *Paper IV*, a submitted manuscript, Josefine researched and devised the optimal method for DNA extraction and extracted the samples. She was the main person responsible for the contact with the company that performed the Whole Genome Bisulfite Sequencing (WGBS) analysis; she was part of designing the analysis of the WGBS data and devised a plan encompassing various statistical methods to be used; she performed statistical analyses and scrutinized data, interpreted and compiled results, drafted the initial manuscript, and crafted figures and tables summarizing key findings, revised and improved the paper before journal submission and contributed to the submission process.

List of Papers Not Included in the Thesis

Maguolo A, **Jönsson J**, Perfilyev A, Maziarz M, Vaag A, Carlsen EM, Nørgaard K, Franks PW, Renault KM*, Ling C*. *DNA methylation in cord blood partially mediates the effects of pre-pregnancy BMI on early childhood offspring BMI.* Under Review. Submitted 2024 Feb 6.

Rönn T, Perfilyev A, **Jönsson J**, Eriksson KF, Jørgensen SW, Brøns C, Gillberg L, Vaag A, Stener-Victorin E, Ling C. *Circulating triglycerides are associated with human adipose tissue DNA methylation of genes linked to metabolic disease.* Hum Mol Genet. 2023 May 18;32(11):1875-1887. doi: 10.1093/hmg/ddad024. PMID: 36752523; PMCID: PMC10196668.

Anand V, Li Y, Liu B, Ghalwash M, Koski E, Ng K, Dunne JL, **Jönsson J**, Winkler C, Knip M, Toppari J, Ilonen J, Killian MB, Frohnert BI, Lundgren M, Ziegler AG, Hagopian W, Veijola R, Rewers M; T1DI Study Group. *Islet Autoimmunity and HLA Markers of Presymptomatic and Clinical Type 1 Diabetes: Joint Analyses of Prospective Cohort Studies in Finland, Germany, Sweden, and the U.S.* Diabetes Care. 2021 Jun 23;44(10):2269–76. doi: 10.2337/dc20-1836. Epub ahead of print. PMID: 34162665; PMCID: PMC8929180.

Abbreviations

5mC	5-methylcytosine
BMI	Body Mass Index
bp	Base Pair
CpG	Cytosine-phosphate-Guanine
CVD	Cardiovascular Disease
DMR	Differently Methylated Region
DNA	Deoxyribonucleic Acid
DNMT	DNA methyltransferase
EWAS	Epigenome-Wide Association Study
FDR	False Discovery Rate
GA	Gestational Age
GDM	Gestational Diabetes Mellitus
GSEA	Gene Set Enrichment Analysis
GO	Gene Ontology
GWG	Gestational Weight Gain
HDL	High-Density Lipoprotein
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBW	Low Birthweight
mRNA	Messenger Ribonucleic Acid
NBW	Normal Birthweight
RNA	Ribonucleic Acid
SAM	S-adenosulmethionine
SAT	Subcutaneous Adipose Tissue
SD	Standard deviation
siRNA	Small Interfering Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
SPLAT	SPlinted Ligation Adapter Tagging
T2D	Type 2 Diabetes

TEE	Total Energy Expenditure
TET	Ten-Eleven Translocation
TF	Transcription Factor
TSS	Transcription Start Site
UTR	Untranslated Region
WGBS	Whole Genome Bisulfite Sequencing
WHO	World Health Organization

Preface

My interest in nutrition and its impact on our health stems from my childhood. Growing up with childhood obesity, I got an early insight into the importance of our diet through the healthcare system. Although the approach may not have been the best, it slowly started to create an interest and curiosity as to why I struggled with obesity and not my friends. After struggling with obesity throughout my growing up and young life, I sat down one day at my extra job and thought about what to do with my life. I had managed to lose some weight on several occasions, to be completely truthful, but it was a constant struggle to keep the weight off. Why was that? Internet searches and evening newspaper articles were not enough to answer my questions, and then it came to me like a lightning strike from above: why not turn this interest into a career? I get to learn "for real" and delve into some of the things I find most interesting - nutrition and health. That's how I found the training to be a nutritionist.

After many evening courses to qualify for science education (I was going to be an interior architect, I thought), I applied and then moved to Stockholm to start my bachelor's education. After a semester of (brutal) chemistry courses, I realized I did not want to be a nutritionist. Although I find population science (which nutrition education focuses on) very interesting, I was more interested in what happens inside the cells when we eat different things. So, I chose to change my focus and instead completed my bachelor's degree in molecular biology. During my education, albeit very briefly, we were introduced to epigenetics. Now another interest was sparked; here was perhaps the mechanism behind how external factors, such as diet, can affect our health. After completing my bachelor's education, I chose to move back to Skåne and do my master's in molecular biology with a specialization in molecular genetics and biotechnology at Lund University. When it was time for our master's project, I came across the Epigenetics and Diabetes group at Lund University, CRC. I was lucky enough to get the opportunity to do my project in this group. About a year after I finished my master's, I got the opportunity to do a Ph.D. in the same group, where I would investigate the effect of lifestyle, including diet, on epigenetics and health.

Although one may primarily think of acquiring specialized knowledge as the greatest learning experience during doctoral studies, some of the most significant learning experiences I will cherish from my Ph.D. education are the development of critical thinking, resilience, problem-solving skills, and adaptability. Research is far from a straight path, and setbacks and overcoming challenges are a big part of the journey, contributing to these skills. Additionally, the more I have learned, the more questions have arisen, revealing how much knowledge there is yet to be gained and leaving me with the humbling realization of how little we truly know.

This thesis was carried out within the Epigenetics and Diabetes Group, Faculty of Medicine, Department of Clinical Sciences, Malmö, Clinical Research Centre, Lund University.

Throughout this thesis, we wanted to investigate the influence of lifestyle factors, such as diet, on DNA methylation in human tissues and cells and assess their implications for human health. This was done using four studies, which differ, among other things, by which tissues and lifestyle factors have been analyzed.

Introduction

Metabolism

Metabolism encompasses all the enzyme-mediated chemical reactions taking place within each cell of a living organism providing energy for fundamental processes and for synthesizing and assembling new organic material. The two main processes of metabolism are anabolism and catabolism. Anabolism involves the synthesis of lipids, nucleic acids, and proteins, whereas catabolism involves the breakdown of, e.g., proteins, fats, and carbohydrates. The metabolism is tightly regulated through metabolic pathways, which are affected by the changes in metabolic demands due to internal and external conditions. For example. the body's physiological state is very different depending on whether it is in a prandial (before a meal/fasting) or postprandial (after a meal) state and coordination and fine-tuning of these processes are known as metabolic regulation (1). In animals, energy is obtained from nutrients in our diet, which can be grouped into two categories based on the amount required per day. Macronutrients include carbohydrates, proteins, and fat of which we require grams daily. Micronutrients include vitamins and minerals; we require milligrams or micrograms daily. The primary function of metabolism is to generate energy, for which the macronutrients are metabolized to produce adenosine triphosphate (ATP). ATP is the primary energy molecule used for cellular processes in the body. Carbohydrates are broken down into glucose and, finally, ATP through glycolysis and the citric acid cycle (1). Carbohydrates are the body’s main source of energy and dietary sources of carbohydrates are, e.g., fruits, vegetables, and grains. Proteins are made up of amino acids – the building blocks of tissues, hormones, enzymes, and antibodies. Some of the amino acids are essential, i.e., the body cannot produce them, and we need to acquire these through our diet. Proteins can be found in food sources such as meat, fish, eggs, legumes, and nuts. Proteins are metabolized into amino acids, which can be broken down into glucose or used as intermediates for the citric acid cycle to produce ATP (1). Fats, also known as lipids, are rich in energy and important for cell membranes' structure and function and absorption of fat-soluble vitamins. Fats can, in turn, be divided into saturated fats with a straight molecular structure and unsaturated fats, with a bent molecular structure due to one or more double chemical bonds. Dietary fats can be found in, e.g., dairy products, oils, fatty fish, and nuts. Fats are converted to ATP through hydrolysis into fatty acids and glycerol and beta-oxidation (1). Vitamins are organic components needed

in various metabolic processes, where they act as coenzymes or cofactors in enzymatic reactions (1). Vitamins are said to be either water-soluble (B and C) or fat-soluble (A, D, E, and K) and can be found in foods such as fruits, vegetables, whole grains, meat, and fish. Minerals are inorganic elements essential for various physiological functions, e.g., muscle contraction, bone formation, and nerve transmission (1). Minerals, such as calcium, iodine, iron, and magnesium, can be found in dietary sources such as dairy products, fish, nuts and seeds, and leafy greens.

Obesity

Obesity is a global health and economic burden that is estimated to only become more severe in the coming years (2). In 2022, approximately 878 million adults (≥20 years) and about 157 million children and adolescents (aged 5-19), that is to say, 1 in 8 were obese (3). Obesity is most commonly measured by the Body Mass Index (BMI) scale, but other methods, such as waist circumference or waist-to-hip ratio, are also used. BMI is defined as the body weight in kilograms (kg) divided by the square height in meters (kg/m^2) and is divided into four categories: underweight (≤18.5), healthy (18.5-24.9), overweight (25-29.9), and obese (≥30). Other thresholds are used for children, adolescents, and pregnant women. In children under five, the WHO Child Growth Standards are used as a reference, and if the child's weight-for-height is more than two standard deviations (SD) from the median, the child is said to be overweight. For childhood obesity, the cut-off is three SD. It is important to note that several obesity-related risk factors seem to depend more on fat distribution than excess weight, where visceral fat accumulation is considered to be metabolically worse than subcutaneous fat accumulation (4).

Although studies have shown that a genetic predisposition is estimated to account for about 40% to 70% of obesity cases (5), obesity is often caused by lifestyle factors such as diet with an energy surplus. The energy surplus is often due to nutritional overconsumption and low physical activity levels – leading to metabolic dysregulation (6). In fact, lifestyle has been shown to be important in preventing obesity (7) and its comorbidities (8).

Complications of Obesity

Obesity is associated with widespread metabolic alterations that affect circulating levels of various metabolites, affecting almost every organ system in the body, and can lead to a variety of complications (**Figure 1**). Adipose tissue is an endocrine organ in itself that regulates whole-body metabolism by producing several hormones (adipokines, e.g., leptin, TNF-α, and adiponectin) (9). Obesity leads to

an imbalance in the adipokines and, thus, metabolic effects. Obesity is highly associated with type 2 diabetes (T2D), with incidence rates going in parallel. This may partly be due to several of the adipokines, such as adiponectin and TNF-α, affecting insulin sensitivity (9). Insulin is a hormone produced by the beta-cells of the pancreas and plays a central role in coordinating glucose metabolism by enabling or enhancing the uptake of blood glucose by tissues and organs such as muscle, adipose tissue, and liver. When levels of circulating glucose are high, such as after a meal, insulin is released into the bloodstream. The elevated levels of insulin lead to the inhibition of glucose production in the liver and promote storage in the form of glycogen as well as the synthesis of fatty acids and the inhibition of their release (5). In the muscle, the glucose is either used directly as energy or stored as glycogen. In the adipose tissue, glucose is converted into triglycerides for storage, while insulin also inhibits lipolysis.

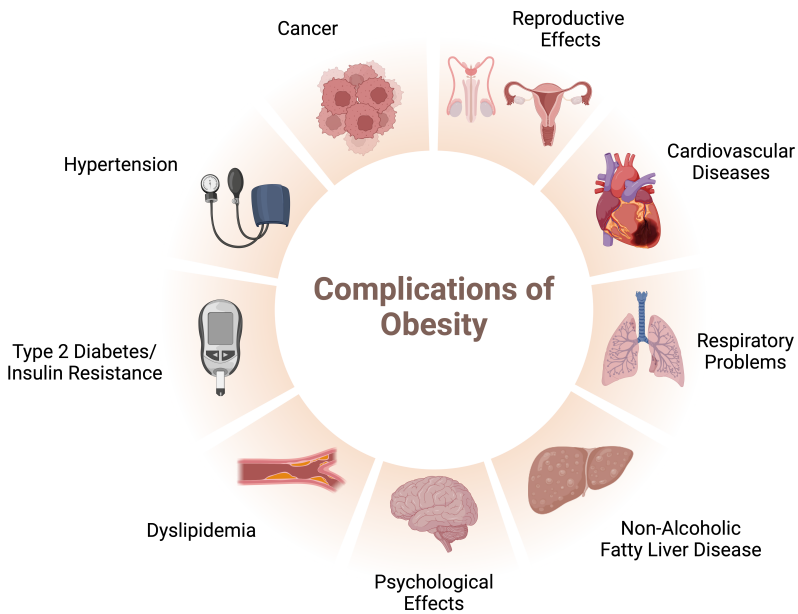

Figure 1. Common complications of obesity. Created with BioRender.com

Insulin resistance occurs when tissues and organs become less responsive to insulin, leading to inadequate glucose uptake and elevated blood glucose levels (5). Ectopic lipid accumulation has been linked to insulin resistance in muscle and liver (10). Ectopic lipid accumulation is the abnormal deposition of lipids in tissues other than adipose tissue, such as the liver and muscle, disrupting normal signaling pathways and cellular metabolism (10). In the muscle, excess lipids lead to impaired insulin signaling and glucose uptake (10). Ectopic lipid accumulation in the liver impairs

insulin signaling, in part due to the activation of processes such as the unfolded protein response and endoplasmic reticulum stress (10). Insulin resistance has also been suggested to impair the uptake of and the ability to metabolize triglycerides, leading to elevated triglyceride levels in the blood (5) and an increased risk of cardiovascular diseases (CVD). Obesity also affects lipid metabolism in a more direct manner through lipolytic adipokines, which increases lipolysis and leads to the breakdown of triglycerides and elevated circulating free fatty acids (also known as non-esterified fatty acids) (5). In fact, obesity is characterized by dyslipidemia with increased levels of free fatty acids, triglycerides, very low-density lipoproteins, and HDL-cholesterol (11), increasing the risk of, e.g., CVD (12), Non-Alcoholic Fatty Liver Disease (13), and insulin resistance (14).

Obesity and Pregnancy

Maternal obesity, most often defined as a pre-pregnancy BMI of ≥30kg/m^2, poses a risk to both mother and offspring. One of the most common obesity-related pregnancy complications is gestational diabetes mellitus (GDM), where the risk of developing GDM increases 3.5-fold in women with obesity and 8.5-fold in women with severe obesity (15). Adverse outcomes for the mother also include a higher frequency of cesarean deliveries, preeclampsia (16), and stillbirths (17). Adverse outcomes in the offspring include macrosomia (18), congenital anomalies (19), cardiovascular morbidity (20), and childhood overweight and obesity (21). Maternal obesity can be seen as a double burden as women with obesity tend to exceed the gestational weight gain (GWG) recommendations more often than normal-weight women (22). Both burdens increase the risk of similar complications. Excessive GWG is, for instance, associated with obesity due to a higher risk of maternal postpartum excess weight retention, cesarean delivery (23), GDM (24) and macrosomia (23), increased BMI in childhood, and an increased risk of obesity in adulthood (25). It is considered excessive GWG when the weight gain exceeds the Institute of Medicine recommendations based on pre-pregnancy BMI categories (**Table 1**).

Table 1. Institute of Medicines recommendations for Total Weight Gain During Pregnancy

Pre-pregnancy BMI	Total Weight Gain, Range in kg
Underweight (<18.5kg/m^2)	12.5–18
Normal weight (18.5-24.9kg/m^2)	11.5–16
Overweight (25.0-20.9 kg/m^2)	7–11.5
Obese (≥30.0 kg/m^2)	5–9

It has been shown that lifestyle interventions with physical activity can be beneficial in pregnant women with obesity, with reduced GWG (26) and high-sensitivity C-reactive protein: an inflammation marker (27). Additionally, the intake of soft drinks, sweets, and snacks was a strong predictor of GWG (28). Together, this

suggests that advice regarding increased physical activity and reducing the intake of soft drinks, sweets, and snacks in pregnant women with obesity is highly relevant for limiting GWG.

Epigenetics and DNA Methylation

Epigenetics is the study of changes in cellular phenotype that do not involve alterations to the DNA sequence. The term "epigenetics" comes from the Greek prefix "epi", meaning "on top off", and genetics, reflecting something taking place "outside off" or "on" the genetic sequence. Epigenetic mechanisms involve modifications of DNA or histone proteins as well as RNA-mediated processes (**Figure 2**) (29). DNA is wrapped around a core of histone proteins, making up the nucleosome, a fundamental unit of chromatin. Histone modifications are post-translational modifications to the histone proteins, such as acetylation, phosphorylation, ubiquitination, and methylation. These modifications alter the structure of the nucleosomes altering the compaction and accessibility of the DNA to regulatory proteins and consequently affecting gene expression (30). RNA-mediated processes, often referred to as RNA interference, include non-coding RNAs, such as microRNAs and long non-coding RNAs. These RNA molecules do not code for proteins but aid in gene regulation at the transcriptional or post-transcriptional level by interfering with mRNA (the protein-coding molecule) stability or translation (30). DNA methylation is the process by which methyl groups (-CH3) are enzymatically added to or removed from the 5-carbon position of cytosine residues in the DNA. This most often occurs at CpG dinucleotides – so-called CpG-sites of which there are approximately 30 million in the human genome (31). These sites are not randomly distributed in the genome, rather, 1-2% are clustered in regions and known as CpG islands (31). A CpG island is defined as a >200 bp region of DNA with a C+G content of >50% and an observed CpG/expected CpG of >0.6 (32). CpG islands are often found in the promoters of protein-coding genes, where the methylation status may affect the accessibility of DNA to transcription factors and, hence, influence gene expression (31).

DNA methylation is a vital mechanism in many biological processes, such as X-chromosome inactivation, transposable element silencing, tissue-specific gene expression, cell fate determination, and genomic imprinting. DNA methyltransferase enzymes (DNMTs) establish and maintain the DNA methylation patterns during DNA replication. DNMT1 mainly maintains the DNA methylation pattern during DNA replication. It has the ability to recognize hemimethylated DNA and methylate the newly synthesized strand, ensuring the same DNA methylation pattern in the daughter cells. DNMT3A and DNMT3B are the *de novo* methyltransferases and can create new DNA methylation patterns (33). They are,

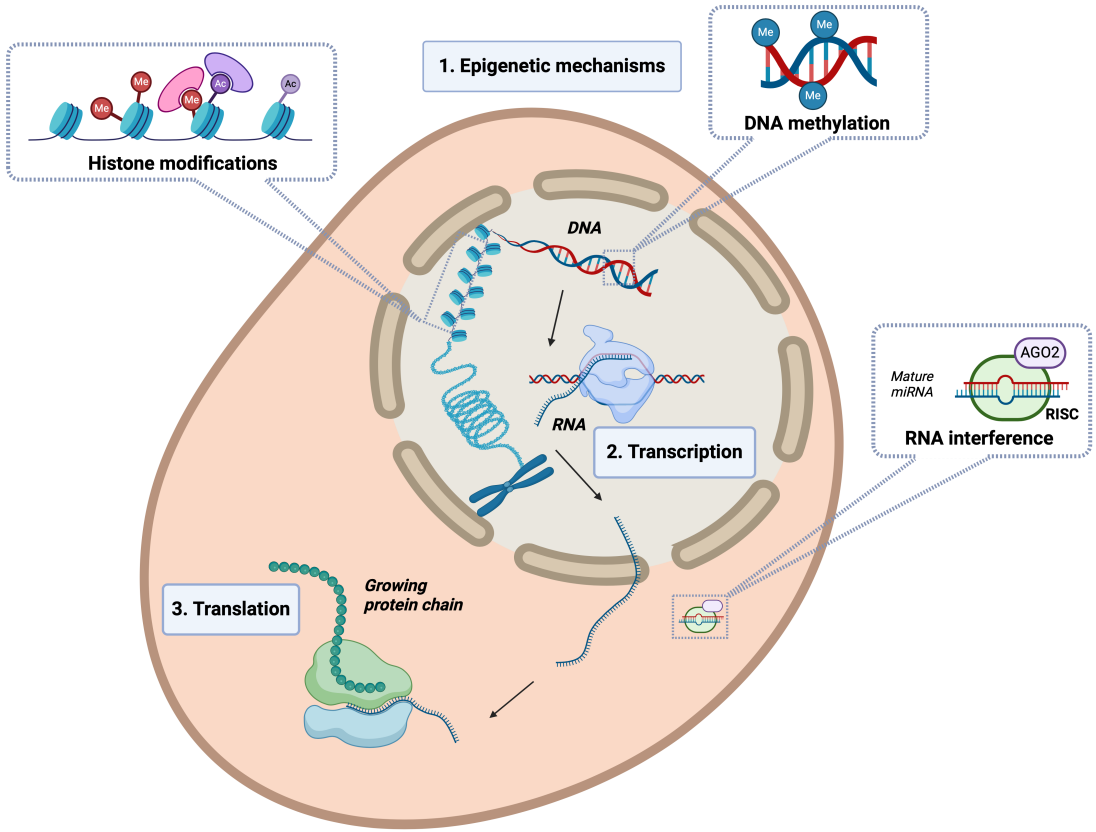

Figure 2. Epigenetic mechanisms regulate transcription and translation. Histone marks regulate chromatin structure. DNA methylation regulates gene expression by affecting the binding of, e.g., the transcription machinery, activators, or repressors. RNA interference regulates gene expression via the degradation of mRNA. Created with BioRender.com

therefore, fundamental in biological processes such as early embryonic development and gene regulation. DNMT3L is a paralog of DNMT3A and DNMT3B and is catalytically inactive. However, it interacts with and modulates the methyltransferase activity of DNMT3A and DNMT3B, making it an important DNA methylation regulatory protein in germ cells and genomic imprinting processes (34). The DNMTs catalyze the addition of a methyl group from S-adenosulmethionine (SAM) to the cytosine in DNA, forming 5-methylcytosine (5mC). DNA demethylation, on the other hand, involves Ten-Eleven Translocation (TET) enzymes, which, with multiple oxidation steps, lead to the removal of the methyl group (29). Passive demethylation also takes place and is a process where methyl groups are diluted with each round of DNA replication and cell division, leading to decreased methylation (29).

Epigenetics and Lifestyle Factors

The Cambridge Dictionary defines 'lifestyle' as "someone's way of living" or "the things that a person or particular group of people usually do". When considering lifestyle factors, diet and physical activity are most likely the first to come to mind, but stress, smoking, and alcohol consumption are also examples.

Tobacco smoking and alcohol consumption have for instance shown to affect DNA methylation (35).

Diet is an important lifestyle factor in epigenetics partly, due to altering the availability of substrate necessary for enzymatic reactions leading to epigenetic modifications. Folate (vitamin B9) and vitamin B12, for instance, have important roles in one-carbon metabolism, which combines the folate cycle and the methionine cycle and produces SAM (30). SAM acts as a methyl donor, enabling the addition of a methyl group e.g., during DNA methylation. Good dietary sources of folate include leafy greens, citrus fruits, and legumes. Sources of vitamin B12 include animal-derived foods, such as meat, dairy products, and eggs, but can also be found in a wide range of fortified foods, such as plant-based milk alternatives. Several studies in rodents have demonstrated the importance of folate in DNA methylation, with strong associations between folate metabolism and fetal growth and development. Diets rich in folate lead to hypomethylation of placental DNA with the opposite effect with diets low in folate, and protein-restricted diets lead to altered DNA methylation patterns in the adult offspring liver – reversible by folate supplementation (36). In humans, the DNA methylation study of the Dutch Hunger Winter is probably one of the most well-known studies in epigenetics, showing that intrauterine exposure to famine can cause epigenetic changes that persist throughout life (37). However, it is not only famine that affects DNA methylation; studies of overfeeding have shown that overfeeding affects the DNA methylation pattern in

human subcutaneous adipose tissue (SAT) (38) and skeletal muscle (39). Additionally, fat quality (saturated fatty acids versus polyunsaturated fatty acids) showcases distinct DNA methylation patterns in adipose tissue of normal-weight participants (40), suggesting that also the source and quality of foods matter. Studies of low birth weight (LBW, i.e., impaired fetal growth) versus normal birth weight (NBW) participants have also found alterations in DNA methylation; Gillberg et al. found that DNA methylation of genes previously associated with T2D was different in SAT of LBW versus NBW men (38) and that these groups show fasting-induced DNA methylation changes (41, 42). This supports the notion of intrauterine programming, and that the intrauterine environment plays a key role in the development of metabolic disease. Several studies in animal models and humans show that paternal diet is linked to offspring health, which is suggested to be due to epigenetic inheritance (43-45), implying that the father's lifestyle also significantly impacts the health of the offspring.

Physical activity has been found to affect DNA methylation in a wide range of tissues (46). Physical activity has, e.g., been linked to hypomethylation of LINE-1 elements in peripheral blood lymphocytes, which, in turn, has been found to lower the incidence and mortality of certain CVDs (35). Additionally, Rönn et al. showed that a six-month exercise intervention in men with previously low levels of physical activity resulted in genome-wide changes in adipose tissue DNA methylation (47) with effects on gene expression (48). Similarly, effects of exercise on DNA methylation in skeletal muscle have been found (49, 50). Exercise also seems to alter acetyl and methyl group availability and the activity of enzymes involved in methylation and demethylation (51). Studies in rodents show different effects of physical activity in the fathers and health effects in the offspring depending on the fathers' body type. A study in lean mice showed that the fathers who had been exercise-trained had altered sperm methylation patterns compared to sedentary controls and that the offspring of the exercise-trained fathers were programmed for low energy expenditure and more susceptible to the adverse effects of high-fat diets (52). An exercise study in obese mice, however, shows beneficial effects on the offspring with reduced risks of obesity and metabolic impairments (53).

Tissues

In the four studies that comprise this thesis, we use three different human tissues: pancreatic islets, cord blood, and sperm.

Pancreatic islets are micro-organs located within the pancreas. In humans, the pancreas is located behind the stomach, and the pancreatic islets are scattered throughout the pancreas. The pancreatic islets comprise several hormone-secreting cells vital for blood glucose homeostasis (54). The alpha, beta, delta, pancreatic

polypeptide (PP), and epsilon cells make up the pancreatic islet. The alpha cells secrete glucagon, a hormone that stimulates the release of stored glucose from the liver. The beta cells secrete insulin, which promotes glucose uptake into cells, glucose storage in muscle and the liver, and inhibits glucose production in the liver (54). Additionally, insulin promotes lipogenesis through the uptake of glucose into the adipocytes (5). Consequently, excess glucose gets converted to fatty acids and stored as triglycerides in the adipocytes (5). The delta cells secrete somatostatin, a hormone that helps regulate the overall balance of blood glucose levels by inhibiting insulin and glucagon release. The PP cells secrete PP, a hormone that regulates the secretion of digestive enzymes from the pancreas and, as such, helps optimize the digestive process. PP also has effects on gastrointestinal motility and appetite regulation (54). The epsilon cell produce ghrelin, a hunger-stimulating hormone playing a vital role in appetite control (55). In a healthy individual, the beta cells respond to elevated levels of blood glucose, such as after a meal, by secreting insulin into the circulation. Insulin facilitates glucose uptake and/or storage in the liver, muscle, and adipose tissue, normalizing blood glucose levels. In T2D, insulin secretion from the beta cells is impaired, and the insulin-sensitive tissues are often unable to respond to insulin (56). Consequently, this results in elevated levels of circulating fatty acids and glucose, which *in vitro* studies have shown to cause adverse effects on insulin secretion (57-59), which may partially be due to epigenetic mechanisms (60, 61).

Cord blood, also known as umbilical cord blood, and adult peripheral blood have several physiological differences. For instance, cord blood contains a small proportion of nucleated red blood cells (nRBCs), which are immature red blood cells with a cell nucleus. During the maturation process of the red blood cells in the bone marrow, the nuclei are typically lost before entering the bloodstream. Thus, adult blood normally does not contain nRBCs. Cord blood also contains a larger proportion of immunologically naïve hematopoietic stem cells than adult blood. Furthermore, the leukocytes are more immunologically immature (62).

Sperm cells are male haploid gametes containing 23 chromosomes, contributing to half of the embryo genome. The process by which sperm cells (spermatozoa) are produced is called spermatogenesis and involves a series of sequential steps. The first step involves the undifferentiated germ cells (spermatogonia), which undergo mitotic division, producing more spermatogonia and primary spermatocytes. The primary spermatocytes then undergo meiosis, resulting in haploid spermatids. The spermatids then undergo extensive morphological changes, which involve the formation of the head, a midpiece, and a tail, before they are called mature sperm cells (63). The nucleus is found in the sperm's head, where the DNA is highly condensed by protamines, ensuring the protection and integrity of the DNA. However, the chromosomal elements essential for embryonic development are histone-bound and thus more accessible. Furthermore, the chromatin is organized

into loop domains anchored to the nuclear matrix. This organizes the chromatin, which aids in regulating DNA replication and gene expression (64).

Rationale

Homo sapiens – the modern man – arose around 300,000 years ago in Africa (65) and humans of today, our genetics are adapted to our ancestral environment. However, the introduction of agriculture and domestication of animals happened recently on an evolutionary time scale, only about 10,000 years ago (66). This has brought about significant changes in our diet and lifestyle, and our genomes have, as such, not had the time to adapt to our modern lifestyle (66). In more recent years, much due to advancing technology after the Industrial Revolution, this acceleration continued. Today, the growing availability of ultra-processed foods with high levels of sugars, saturated fats, refined carbohydrates, and sodium has led to another change in our diets, which now contain calorie-rich foods high in fats and sugars (67). Additionally, sedentary jobs, expanded transportation options, and urbanization have made us more physically inactive, adding to the burden (67). It is believed that these, in combination, are the reasons for the steep rises in health-related problems, such as obesity, type 2 diabetes, and cardiovascular disease, worldwide.

It is crucial to deepen our understanding of what happens in the body as a reaction to different foods and lifestyles. This knowledge can inform better policies and allow individuals to make healthier life choices. In this thesis we, therefore, wanted to explore the effect of various lifestyle factors on DNA methylation in human tissues and cells and assess their implications on human health.

Aims

The overall aim was to investigate the influence of various lifestyle factors, such as diet, on DNA methylation in human tissues and cells and assess their implications on metabolism and disease risk.

Specific Aims

Study I	To investigate the effect of glucolipotoxic treatment on genome-wide mRNA expression and DNA methylation patterns in human pancreatic islets. Furthermore, to evaluate if glucolipotoxicity induced epigenetic changes underlie islet defects.
Study II	To explore the effects of a lifestyle intervention including physical activity, with and without dietary advice, in pregnant women with obesity on DNA methylation changes in offspring cord blood. Moreover, analyze whether specific epigenetic cord blood marks associate with offspring body composition at birth and growth during the first 36 months of life.
Study III	To analyze whether gestational weight gain in pregnant women with obesity is associated with offspring DNA methylation in cord blood. Additionally, to examine if specific cord blood epigenetic marks in the offspring associate with body composition in the newborn and birthweight.
Study IV	To characterize the human sperm DNA methylome and relate it to gene expression and study the effect of a diet intervention, including added sugar, on whole genome DNA methylation in sperm.

Material and Methods

This section offers an overview of the methods used in this thesis while allowing for an in-depth exploration of their technical aspects. For a detailed description of the conduction of methods, please refer to the respective article.

The four studies included in this thesis can be regarded as aligning with the progression of knowledge acquired throughout the doctoral studies, either by advancing laboratory or statistical methods or by increasing independence and responsibility.

Study Design and Overview

The first study analyzed the effect of excess metabolic substrates—glucose and palmitate, i.e., glucolipotoxicity—in human pancreatic islets. Studies number two and three explored the effect of lifestyle factors in pregnant women with obesity on the offspring. In the fourth study, we characterized the sperm DNA methylome and analyzed the effect of added sugar on sperm whole genome DNA methylation. The studies are summarized in **Table 2**.

The following sections are presented for each study/cohort.

Table 2. Overview of the four studies included in this thesis	
Study I	
Aim	To investigate the effects of glucolipotoxic treatment on genome-wide mRNA expression and DNA methylation patterns in human pancreatic islets. Furthermore, to evaluate if glucolipotoxicity induced epigenetic changes underlie islet defects
Study design	Within-subject experimental study
Setting	Human pancreatic islets from 13 donors, treated under control and glucolipotoxic conditions
Measurments/Methods	Human Islets: Glucolipotoxic treatment, DNA methylation 450k array, Affymetrics mRNA expression array, Glucose-stimulated insulin secretion (GSIS), Apoptosis. Functional follow-up experiments in cell-lines: Knock-down of genes of interest, Luciferase assay, qPCR, GSIS, Cell viability i.e., cell number, assesment of apoptosis, and ATP levels.
Statistics	Gene set enrichment analysis, Paired t-tests, Kruskal-Wallis One-Way ANOVA, Wilcoxon matched-pairs signed-rank test
Study II	
Aim	To explore the effects of a lifestyle intervention including physical activity, with and without dietary advice, in pregnant women with obesity on DNA methylation changes in offspring cord blood and if specific epigenetic cord blood marks associate with offspring body composition at birth and growth during the first 36 months of life
Study design	Randomized Control Trial
Setting	Umbilical cord blood from 208 newborns
Measurments/Methods	DNA methylation 450k array, Lifestyle intervetion, Lean mass, and Growth
Statistics	Linear regressions, Linear Mixed Models (LMMs), Spearman correlations, Student's t-test, Mann-Whitney U test, Chi-squared tests, Causal Mediation Analyses, FDR, Bonferoni correction
Study III	
Aim	To analyze whether gestational weight gain in pregnant women with obesity is associated with offspring DNA methylation in cord blood. Additionally, to examine if specific cord blood epigenetic marks in the offspring associate with body composition in the newborn and birthweight
Study design	Randomized Control Trial
Setting	Umbilical cord blood from 208 newborns
Measurments/Methods	DNA methylation 450k array, Gestational weight gain, Lean mass, and Birthweight
Statistics	Linear regressions, Causal Mediation Analyses, FDR, Bonferoni correction
Study IV	
Aim	To characterize the human sperm DNA methylome and relate it to gene expression and study the effect of a diet intervention, including added sugar, on whole genome DNA methylation in sperm
Study design	Cross over study
Setting	Ejaculates at three timepoints from 15 participants
Measurments/Methods	Whole genome bisulfite sequencing, DMR calling, Individual CpG methylation analyses, Characterization of the human sperm methylome
Statistics	Generalized Least Squares (GLS) with a nested autoregressive correlated error structure, Wilcoxon signed rank tests with continuity correction, Friedman rank sum test, Dunns' post hoc test

Study Design

Experimental Study (Study I)

Metabolic Substrate Analyses in Human Islets (Study I)

Pancreatic islets from human donors were acquired from the Nordic Network for Islet Transplantation at Uppsala University, Uppsala, Sweden, through the Human Tissue Laboratory at Lund University Diabetes Centre. The islets were prepared by collagenase digestion and density gradient purification (68). Here, we investigate the effect of glucolipotoxic treatment in human islets by measuring mRNA expression, insulin secretion, and apoptosis. DNA methylation was measured to evaluate if glucolipotoxicity-induced epigenetic changes underlie the potential islet defects (**Figure 3**).

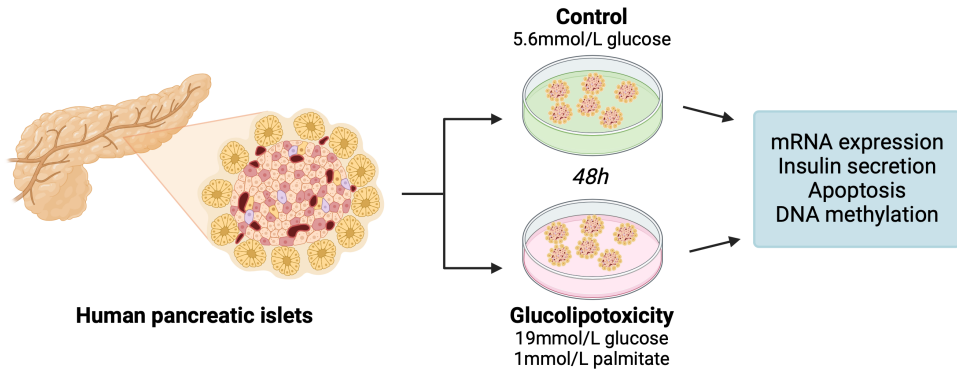

Figure 3. Study design of the glucolipotoxicity study in human pancreatic islets. Created with BioRender.com

DNA methylation and mRNA expression were analyzed from 13 donors. Eight donors were included in both the DNA methylation and mRNA array analyses, while five donors were unique for each array (**Table 3**).

Table 3. Characteristics of pancreatic islet donors

Analysis	Donors (*n*)	Sex (F/M)	Age (years)	BMI (kg/m2)	HbA1c (%)
DNA methylation	13	5/8	48.6 ± 16.4	26.4 ± 4.5	5.4 ± 0.7*
mRNA expression	13	6/7	53.5 ± 14.3	25.5 ± 4.3	5.6 ± 0.9**
Insulin Secretion	9	5/4	58.9 ± 9.2	24.3 ± 2.6	5.5 ± 0.2
Apoptosis	4†	0/4	59.3 ± 6.2	25.8 ± 3.4	6.3 ± 0.8***

*Data are presented as mean ± SD. F, female; M, male. †One donor had T2D. *Data missing for three donors. **Data missing for five donors. ***Data missing for two donors.*

Intervention Studies (Study II-IV)

The Treatment of Obese Pregnant women (TOP) study (Study II & III)

The TOP-study is a randomized controlled trial initially conducted to evaluate the effect of lifestyle intervention on GWG in 425 pregnant women with obesity, by comparing three groups I) PA, physical activity intervention (n = 142); II) PA+D, physical activity and dietary intervention (n = 142); and III) C, a control group receiving standard care (n = 141). Due to participant attrition by miscarriages, voluntarily withdrawing, and moving from the region, 389 women completed the study. On the mothers, we have anthropometric, questionnaire, and blood test data; in the newborns, we have DNA methylation data from cord blood, anthropometric and body composition data; follow-up data in the form of anthropometric data is available for a subset of the offspring at 9, 18, and 36 months (**Figure 4A**). Whole cord blood samples, used for DNA methylation analyses, were available for 232 offspring and for these analyses, the two lifestyle intervention groups (PA and PA+D) were merged, and after quality control (QC), 208 samples remained (**Figure 4B**, and explained in more detail in papers II and III) (**Table 4**).

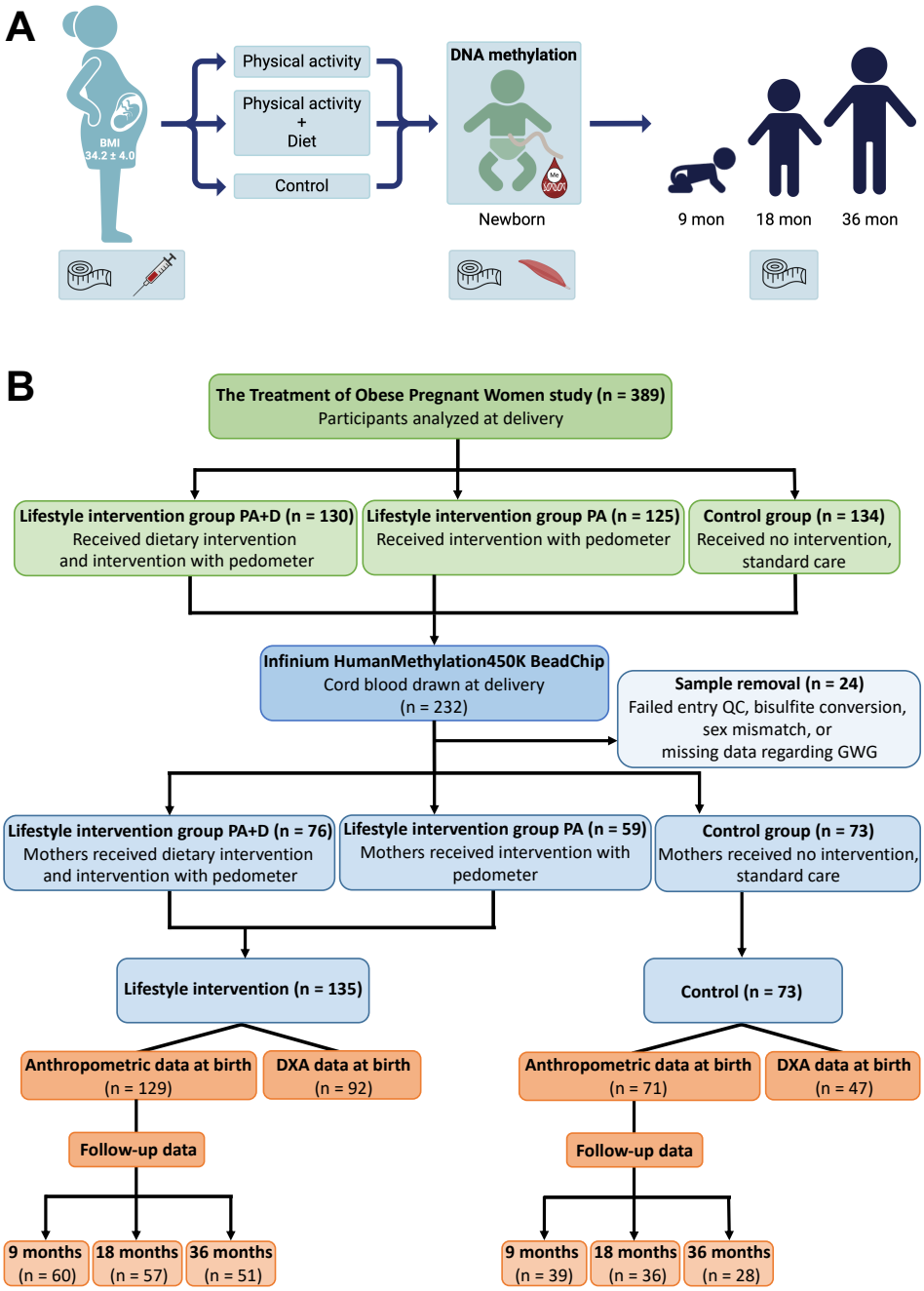

Figure 4. A) Study design of the methylation cohort of the TOP-study. Anthropometric data and blood work are available for the mothers, anthropometric and body composition data for the newborns, and anthropometric follow-up data for the offspring at 9, 18, and 36 months. Created with BioRender.com **B)** Flowchart and available data for analyses in Paper II & III (69).

Table 4. Parental and offspring baseline characteristics according to the lifestyle intervention and control groups in the methylation cohort

	Lifestyle intervention	Control	*P*-value
Maternal characteristics	*n* = 135	*n* = 73	
Age at enrollment (years)*	30.90 (4.30)	31.40 (4.74)	0.440
Prepregnancy BMI (kg/m^2)*	34.19 (4.00)	34.36 (3.98)	0.763
Education level, *n* (%)†			0.970
Grammar school, 10y	15 (11.1)	6 (8.2)	
Secondary school, 12y	16 (11.9)	9 (12.3)	
Vocational training school	13 (9.6)	6 (8.2)	
Further education 1-2y	26 (19.3)	12 (16.4)	
Tertiary education 3-4y (Bachelor level)	46 (34.1)	29 (39.7)	
Advanced (postgraduate)	18 (13.3)	10 (13.7)	
NA	1 (0.7)	1 (1.4)	
Smoking during pregnancy (yes/no), *n* (%)†	10/125 (7.4/92.6)	3/70 (4.1/95.9)	0.524
Parity (single/multi), *n* (%) †	75/60 (55.6/44.4)	39/34 (53.4/46.6)	0.882
Energy intake at enrollment (kJ)‡§	8 019 (2 784)	7 540 (3 246)	0.829
Paternal characteristics	*n* = 115	*n* = 65	
BMI (kg/m^2) at enrollment*	27.39 (4.51)	27.01 (4.52)	0.585
Offspring characteristics	*n* = 135	*n* = 73	
Sex, *n* (%) (F/M)†	66/69 (48.9/51.1)	34/39 (46.6/53.4)	0.862
Gestational age (weeks)*	40.17 (1.23)	40.01 (1.31)	0.393
Birthweight (g)*	3 724 (482)	3 677 (513)	0.515
Weight (kg), 9 months*‖	9.61 (1.03)	9.38 (1.15)	0.299
Weight (kg), 18 months*¶	11.86 (1.18)	11.26 (10.27)	0.014
Weight (kg), 36 months*#	15.30 (1.86)	14.71 (12.97)	0.141
Length (cm), birth***	52.50 (2.17)	52.48 (2.24)	0.958
Length (cm), 9 months*‖	73.14 (2.31)	72.99 (1.97)	0.740
Length (cm), 18 months*††	82.75 (2.87)	82.55 (2.48)	0.724
Height (cm), 36 months*‡‡	96.42 (4.21)	95.95 (3.07)	0.599
Breastfeeding, exclusively (weeks)*§§	10.98 (9.41)	8.38 (10.07)	0.163
Breastfeeding, partially (weeks)*§§	16.30 (11.05)	14.88 (10.71)	0.501

*Enrollment, weeks 11-14; NA, not available. *Mean (SD), two-sided Student t test. †Frequencies, χ^2 test. ‡Median (interquartile range), two-sided Mann-Whitney U test. §Lifestyle intervention, n=133; control, n=68. ‖Lifestyle intervention, n=60; control, n=39. ¶Lifestyle intervention, n=58; control, n=36. #Lifestyle intervention, n=51; control, n=29. **Lifestyle intervention, n=129; control, n=71. ††Lifestyle intervention, n=57; control, n=36. ‡‡Lifestyle intervention, n=51; control, n=28. §§Lifestyle intervention, n=77; control, n=42.*

Added Sugar, Sperm (Study IV)

In this crossover study, 15 voluntary men aged 20-27 and a BMI below 30kg/m^2, performed a 2-step diet intervention during a 2-week period (**Table 5**). The first week of the diet intervention consisted of a standardized healthy diet based on the Nordic Nutrition Recommendations, with an energy intake corresponding to the study participants' calculated total energy expenditure (TEE). During week two, the diet involved the same standardized healthy diet, with the addition of sweets and sweetened drinks corresponding to 50% of their estimated TEE, resulting in an energy intake of 150% of their estimated TEE (**Figure 5**).

Table 5. Characteristics of participants in the Added Sugar cohort (n=15)

	Mean ± SD (range)		
	Baseline	Healthy Diet	Added Sugar
Age (years)	23.27 ± 2.28 (21.50 – 25.00)	23.27 ± 2.28 (21.50 – 25.00)	23.27 ± 2.28 (21.50 – 25.00)
Height (m)	1.84 ± 0.08 (1.77 – 1.88)	1.84 ± 0.08 (1.77 – 1.88)	1.84 ± 0.08 (1.77 – 1.88)
Weight (kg)	75.83 ± 10.23 (68.23 – 81.80)	75.25 ± 9.86 (68.54 – 80.83)	76.70 ± 9.92 (70.39 – 83.13)
BMI (kg/m^2)	22.48 ± 2.57 (20.31 – 24.37)	22.31 ± 2.39 (20.17 – 24.31)	22.73 ± 2.32 (20.92 – 24.42)
Sperm concentration (x10^6/ml)	32.40 ± 25.25 (11.00 – 44.00)	32.20 ± 20.18 (22.00 – 44.00)	28.87 ± 18.27 (14.00 – 39.50)
Progressive motile sperm (%)	47.93 ± 14.36 (33.50 – 60.50)	53.00 ± 13.04 (49.00 – 61.00)	55.93 ± 8.63 (52.50 – 63.00)
Total motile sperm (%)	53.00 ± 14.10 (40.00 – 65.00)	58.00 ± 13.11 (54.50 – 66.00)	60.87 ± 8.07 (57.00 – 67.50)
Fat mass (kg)	11.53 ± 4.08 (9.11 – 12.84)	11.71 ± 3.76 (9.22 – 12.92)	11.86 ± 4.12 (9.77 – 13.31)
Fat free mass (kg)	64.30 ± 8.06 (59.93 – 70.02)	63.55 ± 7.61 (58.89 – 68.80)	64.85 ± 7.99 (61.20 – 71.33)
Plasma cholesterol (mmol/L)	4.59 ± 1.15 (3.80 – 5.20)	4.38 ± 1.38 (3.70 – 4.65)	4.46 ± 1.31 (3.85 – 4.70)
Serum triglycerides (mmol/L)	1.20 ± 0.81 (0.84 – 1.30)	1.08 ± 0.63 (0.66 – 1.10)	2.13 ± 1.28 (1.40 – 2.55)
Plasma HDL cholesterol (mmol/L)	1.55 ± 0.42 (1.25 – 1.75)	1.49 ± 0.41 (1.20 – 1.60)	1.36 ± 0.38 (1.10 – 1.55)
Serum LDL cholesterol (mmol/L)	2.48 ± 0.87 (1.80 – 2.80)	2.38 ± 1.18 (1.85 – 2.35)	1.90 ± 0.54 (1.45 – 2.15)
Plasma non-HDL cholesterol (mmol/L)	3.02 ± 1.17 (2.20 – 3.30)	2.87 ± 1.44 (2.25 – 2.85)	3.08 ± 1.40 (2.50 – 3.20)
Fasting plasma glucose (mmol/L)	5.13 ± 0.33 (4.90 – 5.30)	5.09 ± 0.29 (4.90 – 5.20)	5.15 ± 0.38 (4.90 – 5.35)

BMI, body mass index; HDL, High-density lipoprotein; kg, kilogram; LDL, low-density lipoprotein; m, meter; SD, standard deviation.

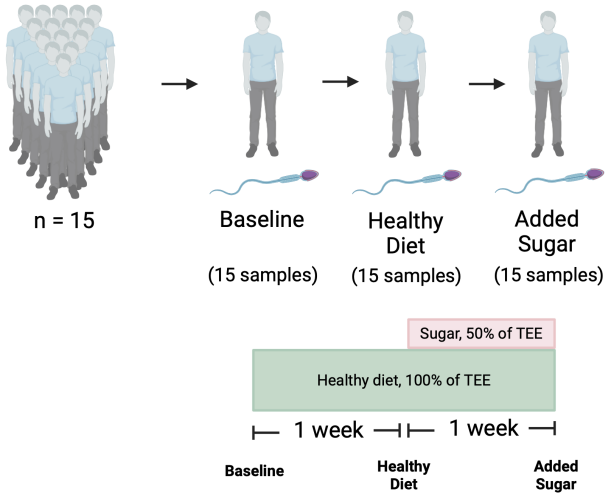

Figure 5. Study design of the Added Sugar cohort in human sperm. TEE, total energy expenditure. Created with BioRender.com

DNA Methylation

In the first three studies of this thesis, DNA methylation was measured using the Infinium® HumanMethylation450 BeadChip. In the fourth study, we used Whole-Genome Bisulfite Sequencing to analyze DNA methylation.

Bisulfite Conversion (Study I-IV)

Bisulfite conversion is a necessary first step in analyzing DNA methylation. It results in the unmethylated cytosines (C) being chemically converted to uracil (U) while methylated cytosines (5mC) are unaffected. The first step of bisulfite conversion is to produce single-stranded DNA by chemical or temperature denaturation. The denatured DNA is subsequently treated with sodium bisulfite ($NaHSO_3$) enabling the deamination of the unmethylated cytosine through the release of ammonia, leading to the conversion to uracil (**Figure 6A**). Methylated cytosines remain as cytosines during bisulfite conversion and the amplification process, while unmethylated cytosines are converted into uracil, and during the amplification process, the uracil is converted to thymine (**Figure 6B**). Incomplete bisulfite conversion may lead to misinterpretation of the DNA methylation status, it is hence important to consider the bisulfite conversion rate and have built-in controls to assess this.

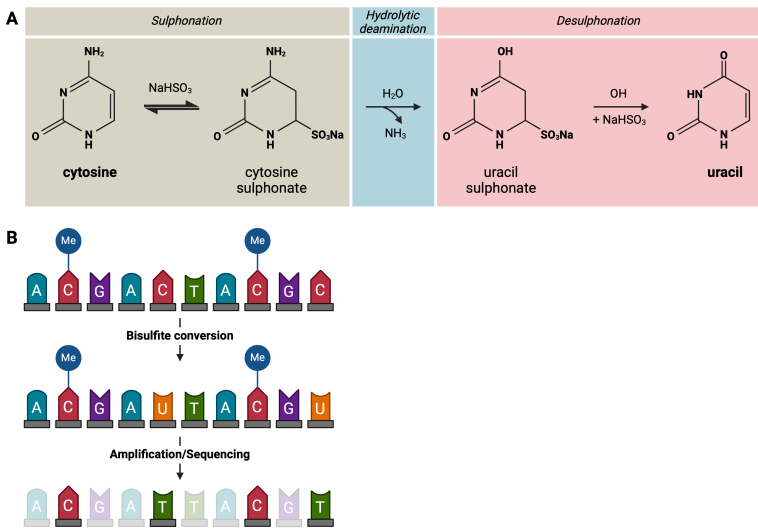

Figure 6. A) Scheme of the chemical reaction of bisulfite conversion, where cytosine is converted to uracil in DNA after bisulfite treatment. **B)** During bisulfite conversion and the subsequent amplification process, methylated cytosines remain as cytosines. Meanwhile, unmethylated cytosines are converted to uracil during bisulfite treatment, and during the amplification process, uracil is converted to thymine. Created with BioRender.com

Infinium® HumanMethylation450 BeadChip (Study I-III)

The Infinium® Assays are used for studying CpG methylation using bisulfite-converted genomic DNA. The Infinium® HumanMethylation450 BeadChip entered the market in 2011 and is a high-throughput DNA methylation platform providing a genome-wide coverage of more than 485,000 CpG-sites. It covers 99% of all RefSeq genes with a global average of 17.2 probes per gene region, and 96% of all CpG islands with an average of 5.6 probes per CpG island (70). Probes per gene range between 1 to 1,299. It has a broad coverage distributed across gene regions (**Figure 7A**), CpG island regions (**Figure 7B**), and non-CpG cites. Methylation profiles have been found to correlate well with the predecessor HumanMethylation27 array and with whole-genome bisulfite sequencing data (70).

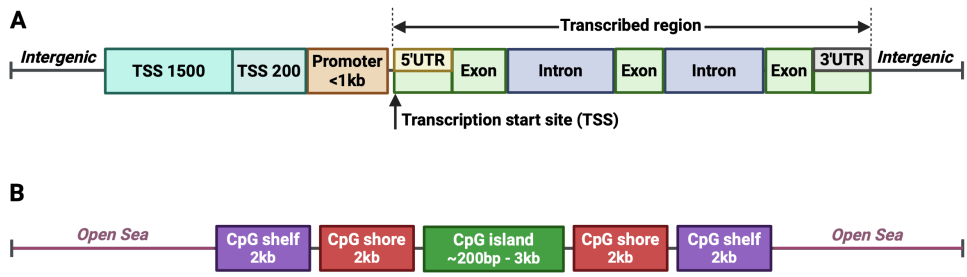

Figure 7. A) CpG-sites are mapped to gene regions based on functional genome distribution, where the promoter is defined as the region between the TSS and 1 kb upstream, and to **B)** CpG island regions based on CpG content. Shore: the flanking region of CpG islands, i.e., covering 0-2,000 bp distant from the CpG island; Shelf, regions flanking island shores, 2,000-4,000 bp distant from the CpG island. TSS, Transcription Start Site. Created with BioRender.com

The HumanMethylation450k array involves two types of 50-base long probes: Infinium I and Infinium II. In the Infinium I assay, a paired probe approach is used for each CpG-site, the 3' terminus of the probe is designed to recognize either the methylated state (M probe) or the unmethylated state (U probe) (**Figure 8A**). For the Infinium II assay, only one probe corresponds to each CpG-site, and the methylation state is detected by single-base extension where cytosines are incorporated in the methylated state and thymine in the unmethylated state (**Figure 6B** and **8B**) (70).

Both Infinium assays use a color-coded fluorescence detection system that allows discrimination between methylated and unmethylated states. After DNA bisulfite conversion, the DNA is amplified and hybridized to the BeadChip, and the methylation level is determined by the fluorescence intensity (70).

Although the Infinium Methylation assays are useful tools for measuring DNA methylation, there are some known problems. Multiple Infinium I and II probes have been found to cross-hybridize with non-intended genomic regions by 47-50 base

matches, especially on the X- and Y-chromosomes. Furthermore, several probes have been found to overlap with single nucleotide polymorphisms (SNPs) (71). Additionally, technical variability and batch effect are other problems to be aware of. These issues could lead to misinterpretation of data and have hence been considered in the studies where the HumanMethylation450k array has been used.

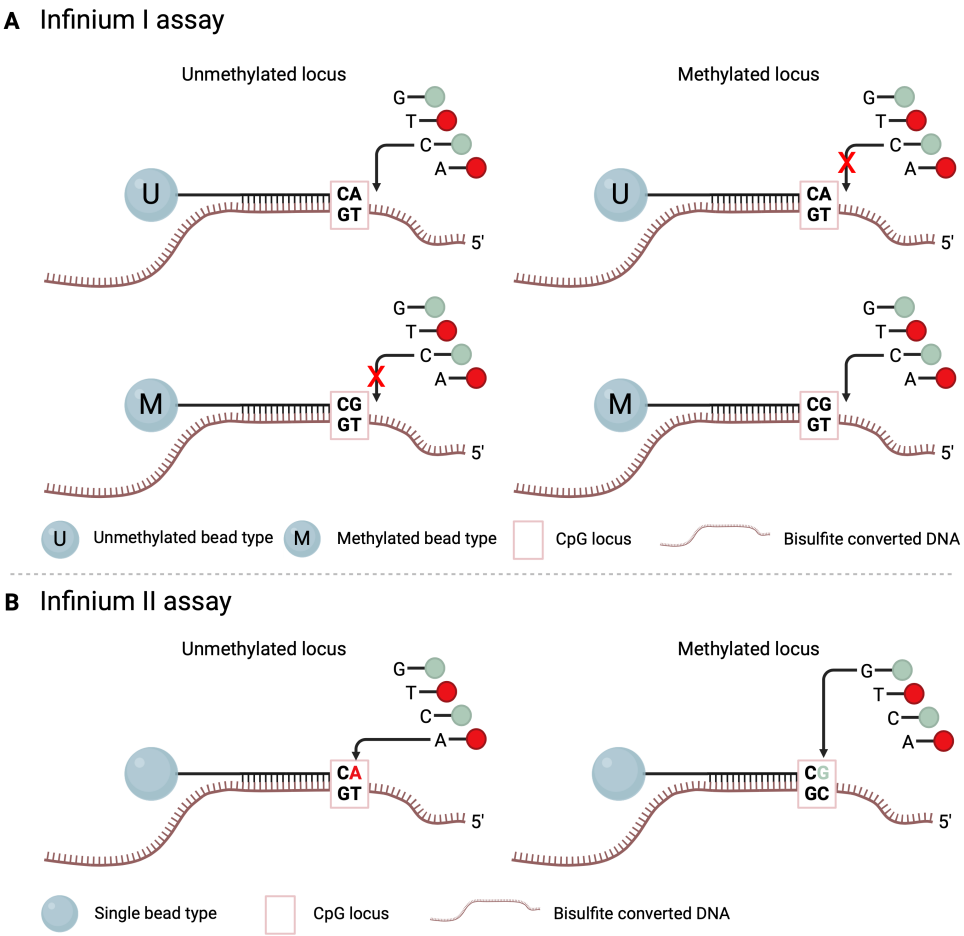

Figure 8. The Illumina Infinium® HumanMethylation450 BeadChip utilizes a dual-assay design with both Infinium I and Infinium II assays. **A)** In the Infinium I assay, two bead types are employed per CpG locus, representing the methylated and unmethylated states. **B)** The Infinium II design utilizes a single bead type, determining the methylated state at the single base extension step following hybridization. Created with BioRender.com

Whole Genome Bisulfite Sequencing (Study IV)

Although array-based methods for studying DNA methylation are robust, there is a great difference in genomic coverage, where the latest Infinium Methylation array on the market, the EPIC version 2, covers about 3% of the genome. Whole-genome bisulfite sequencing (WGBS) on the other hand, is a technique for analyzing DNA methylation patterns at a single-base resolution across the entire genome (**Figure 9**).

In this thesis, WGBS was performed using the SPlinted Ligation Adapter Tagging (SPLAT) protocol (72) for library preparation. This protocol combines bisulfite treatment of DNA with next-generation sequencing and allows for low quantities of DNA input.

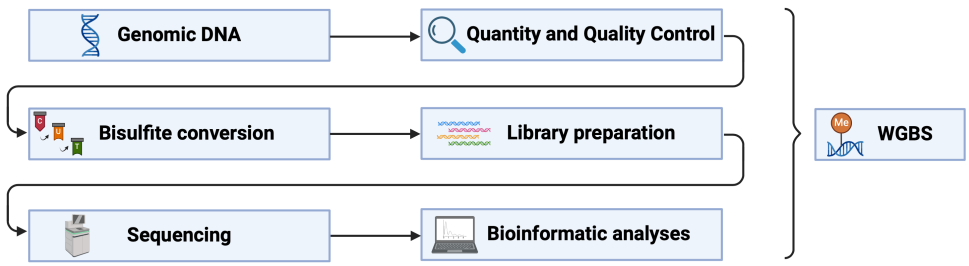

Figure 9. Workflow of the whole genome bisulfite sequencing analysis. Created with BioRender.com

Gene Expression

Affymetrix GeneChip™ Human Gene 1.0 ST Array (Study I)

The Affymetrix GeneChip™ Human Gene 1.0 ST Array is an array-based method for gene expression profiling, providing comprehensive measurements of protein-coding and long non-coding RNA transcripts. It covers 28,869 genes with 764,885 probes, which are distributed across the full length of the gene, allowing for whole-transcript coverage (73).

Publicly available RNA sequencing data (Study IV)

Publicly available human sperm RNA-sequencing (RNA-seq) data for 12 subjects (74) was used to explore the relationship between DNA methylation and gene expression levels. The publicly available RNA-seq data was downloaded from the NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra, Accession:

PRJNA573604). Genes were annotated to hg38, and a total of 33,408 genes were identified. The expression levels were split into four factions, with 20,791 genes being classified as non-expressed genes (<2 mean normalized counts) and the remaining expressed genes with a mean normalized count of ≥2 were categorized into low- (n=4,151), medium- (n=4,171), and high-expressed (n=4,295) genes.

Additional Methods

Pathway Analyses (Study I-III)

Pathway analyses encompass the systematic study of biological pathways, allowing for a biological context and interpretation of, for example, DNA methylation and gene expression data. DNA methylation or gene expression changes of genes enriched in certain pathways can provide insight into potential underlying biological mechanisms and how these contribute to phenotypic outcomes.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a resource providing information on biological pathways and interactions of genes and molecules such as proteins and metabolites (75). The Gene Ontology (GO) database is another resource for annotating genes and their products based on their molecular function, involvement in biological processes, and cellular components (76).

Gene Set Enrichment Analysis (GSEA) is one method for analyzing the enrichment of genes in certain pathways of differently expressed genes. This method uses a priori-defined gene sets from databases such as KEGG or GO terms. GSEA ranks the genes and provides a GSEA enrichment score for each gene set with a nominal *P*-value based on permutations (77).

Using methods such as gene ontology analysis on DNA methylation data from array-based methods has been shown to create a great bias due to the differing number of probes per gene (78). That is to say, a gene with many probes is more likely to be identified as differently methylated. Another source of bias arises as several of the probes are annotated to more than one gene, deviating from the assumption of independent measurement. To account for these biases, we implemented the *gometh* function of the missMethyl R package (79) in studies II and III.

Small interfering RNA transfection (Study I)

Small interfering RNA (siRNA) transfection is a widely used method in targeted gene silencing (knock-down) experiments. siRNAs, which will target the mRNA sequence of choice, need to be designed if not readily available. The siRNA is then

transfected into cells, in our case we used Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA), a lipid-based transfection reagent. The siRNA is incorporated into the RNA-induced silencing complex (RISC, depicted in **Figure 2**) which targets the mRNA with a matching sequence, leading to its degradation. The siRNA's efficiency was evaluated by varying volumes and incubation times and a negative control was used for baseline comparisons.

Luciferase Assay (Study I)

We used a luciferase assay, a biochemical assay, for studying gene expression. Luciferases catalyze the oxidation of luciferin, leading to the emission of light, and their enzymatic activity is measured by luminescence (**Figure 10A**). The Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) was used in these experiments. It implements a dual reporter system with a "control" reporter (internal control) and an "experimental" reporter. Normalizing the experimental reporter to the activity of the internal control minimizes experimental variability potentially caused by differences in cell viability or transfection efficiency.

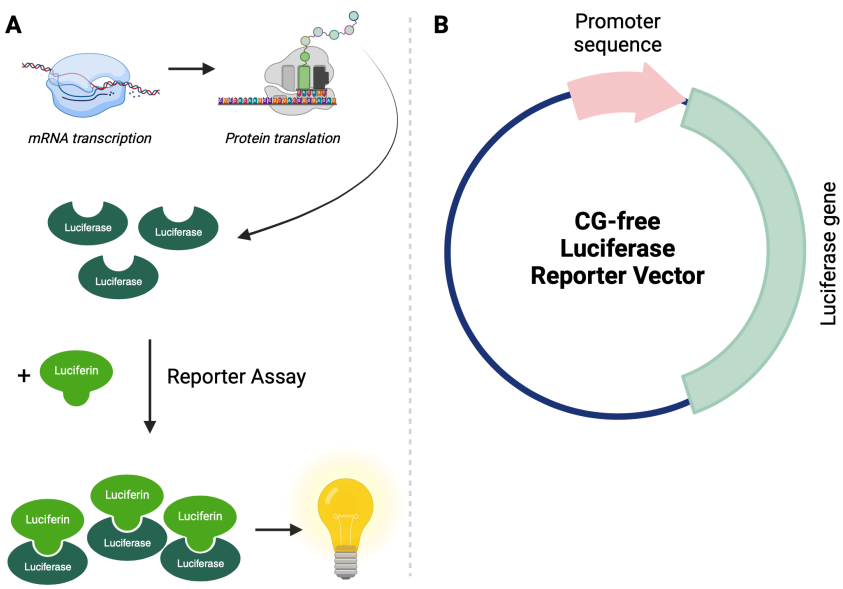

Figure 10. Simplified scheme of the Luciferase Assay. **A)** Expression of the luciferase gene following translation to the luciferase enzyme and the addition of luciferin (the substrate of luciferase) lead to light emission, which allows for quantification of gene expression. **B)** A simplified depiction of the CG-free Luciferase Reporter vector. Created with BioRender.com

In Study I, we implemented this method for studying the effect of DNA methylation on gene expression of genes of interest. Promoter sequences corresponding to 1,500bp upstream of the transcription start site (TSS) were synthesized and inserted into a CG-free vector containing the luciferase gene (pCpGL-basic, GeneScript USA Inc., Piscataway, NJ, USA) (**Figure 10B**). The impact of DNA methylation was evaluated using three different methyltransferases, SssI, HhaI, and HpaII (New England Biolabs, Frankfurt, Germany) known to methylate cytosines in distinct patterns. Methyltransferase SssI methylates all CpG-sites and leads to a highly methylated construct. In contrast, HhaI methylates the internal cytosine residue within a GCGC sequence, and HpaII methylates the internal cytosine within a CCGG sequence, leading to point-methylated constructs. Depending on the promoter sequence, the number of methylated CpG-sites will differ, allowing for evaluation of promoter methylation's impact on gene expression.

Apoptosis Assessment (Study I)

The Apo-ONE® Homogeneous Caspase-3/7 Assay is a simple and highly sensitive method for analyzing programmed cell death (apoptosis). It encompasses activity measurements of caspase-3 and caspase-7, two key enzymes involved in the apoptotic pathway. This method provides insight into cell viability and cytotoxicity. In Study I, this allowed for the evaluation of the impact of the glucolipotoxic treatment in the human islet and also, in the functional follow-up experiments, the effect of altered gene expression (knock-down experiments).

Statistical Analyses

In Studies I and IV, R software and GraphPad were used for statistical analyses; for Study II-III, R was used for all statistical analyses (80, 81).

Paired t-tests were used to identify differences in DNA methylation and mRNA expression and to analyze functional experiments in human islets (Study I). To analyze results from experiments performed in beta cell lines, 832/13 INS-1 cells (rat beta cell line) (82) and EndoC-βH1 cells (human fetal pancreatic beta cell line) (83), Kruskal-Wallis One-Way ANOVA or Wilcoxon matched-pairs signed-rank test were used (Study I).

Linear regression models were used to analyze the DNA methylation pattern in Study II-III. Several versions, with varying covariates, were run as sensitivity analyses to evaluate the robustness of the results. Linear regressions were also run to assess whether DNA methylation is associated with offspring measurements. Linear Mixed Models for repeated measurements were used to evaluate the association between DNA methylation and offspring growth. Longitudinal data on

growth expressed as BMI z-scores (weight relative to height and adjusted for age and sex of the child) were used in these calculations (Study II). Spearman correlations, a nonparametric measure of rank correlation, were used to check for correlations between two variables (Study II-III). Descriptive analysis of clinical variables was done using different statistical methods, depending on the data tested. Student's t-test (parametric) or the Mann-Whitney U test (nonparametric) were used for continuous data, and frequencies were analyzed using chi-squared tests (Study I-IV). In Studies II and III, causal mediation analyses were performed to investigate whether DNA methylation at individual CpG-sites was part of a pathway through which lifestyle intervention (Study II) or GWG (Study III) exerts its effects on offspring measurements. The mediation analysis breaks down the total effect of exposure on the outcome into two components: the indirect effect, which acts via the mediator of interest, and the direct effect, which acts either directly or through a mediator other than the one that is under study (**Figure 11**). A nonparametric causal mediation analysis and the R 'mediation' package with default settings were used for these analyses (84). The effect is estimated for each association between the treatment, i.e., lifestyle intervention or GWG, and the outcome(s), i.e., offspring measurement(s), with the discovered lifestyle intervention or GWG-associated DNA methylation sites as the mediator (**Figure 11**).

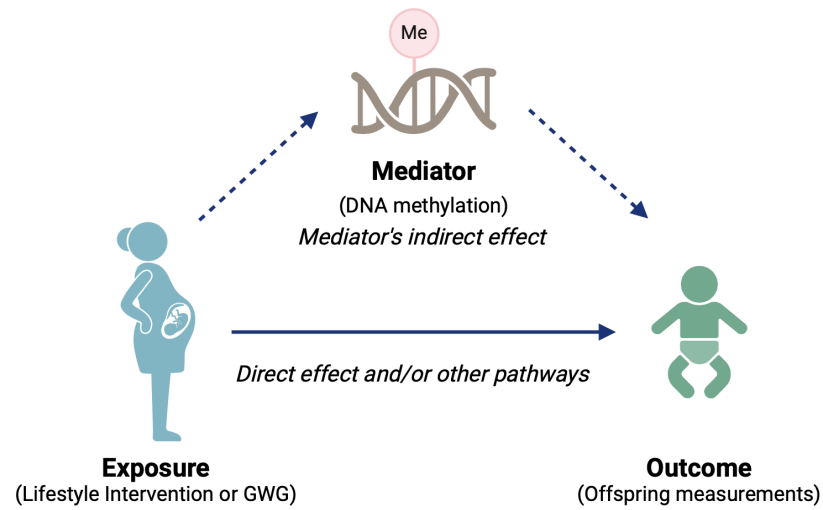

Figure 11. The scheme tested by the causal mediation analysis and the potential mechanisms linking the exposure to the outcome. Created with BioRender.com

Study IV used the R package 'dmrseq' (85) to identify diet-associated differentially methylated regions (DMRs). This package utilizes a method that fits a linear regression model by using generalized least squares (GLS) with a nested autoregressive correlated error structure. The GLS regression allows for correlation among the residuals and hence accounts for the complex correlation patterns present in DNA methylation data by including the CpG-sites' genomic positions. Autoregressive correlation means that the correlation between CpG-sites decreases as their distance from each other increases. Wilcoxon signed rank tests with continuity correction were used when analyzing individual CpG-sites from the WGBS data. Continuity correction was applied to account for the small sample size. The Friedman rank sum test was used to explore the relationship between DNA methylation and gene expression levels. The non-parametric Friedman rank sum test is an extension of the Wilcoxon signed-rank test, allowing for analysis of repeat measurements of more than two groups/conditions. The Dunns' post hoc test was then applied to identify which groups were significantly different.

To correct for multiple testing, we applied either Benjamini-Hochberg (86), a statistical method used to control for the false discovery rate (FDR), or both Benjamini-Hochberg (86) and Bonferroni. Bonferroni, a correction method controlling for the family-wise error rate, is an effective method for reducing the likelihood of Type I errors (false positives) but may also increase the risk of Type II errors (false negatives). In epigenome-wide association studies (EWAS) it is said to be too conservative due to the known correlation patterns at nearby sites in DNA methylation data and the nonvariability of sites present on the array, leading to many false negatives. An alternative method, widely used in EWAS, is the Benjamini-Hochberg procedure (86); however, it may produce some false-positive results.

Results

Study I

Glucolipotoxicity Alters Insulin Secretion via Epigenetic Changes in Human Islets

In this study, we investigated the effect of glucolipotoxic treatment in human islets by measuring insulin secretion and apoptosis. We then investigated potential molecular mechanisms contributing to the phenotypes observed by measuring mRNA expression. Finally, DNA methylation was measured to evaluate whether the gene expression changes caused by glucolipotoxicity were prompted by epigenetic changes (**Figure 3**).

The human islets treated under glucolipotoxic conditions for 48 hours were found to have impaired glucose-stimulated insulin secretion (GSIS), with similar results in the human EndoC-βH1 beta-cell line (**Figure 12A-B**). Increased apoptosis was also seen in the glucolipotox-treated islets compared to islets under control conditions (**Figure 12C**).

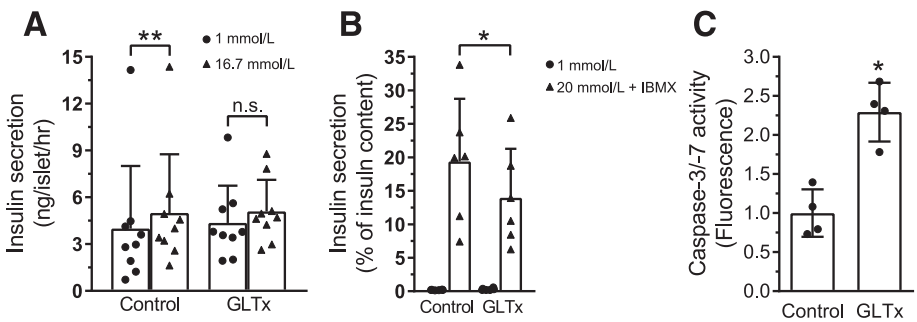

Figure 12. Impact of glucolipotoxic treatment on **A)** glucose-stimulated insulin secretion in human islets (n=9) ***P*<0.01, paired *t*-test and **B)** glucose-stimulated insulin secretion in the human EndoC-βH1 beta-cell line (n=6, with two technical replications per condition) **P*<0.05, Wilcoxon signed rank test, and **C)** apoptosis (n=4), measured as the combined activity of Caspase-3/-7, **P*<0.05 paired *t*-test. Data are shown as mean ± SD. GLTx, glucolipotoxicity. (87)

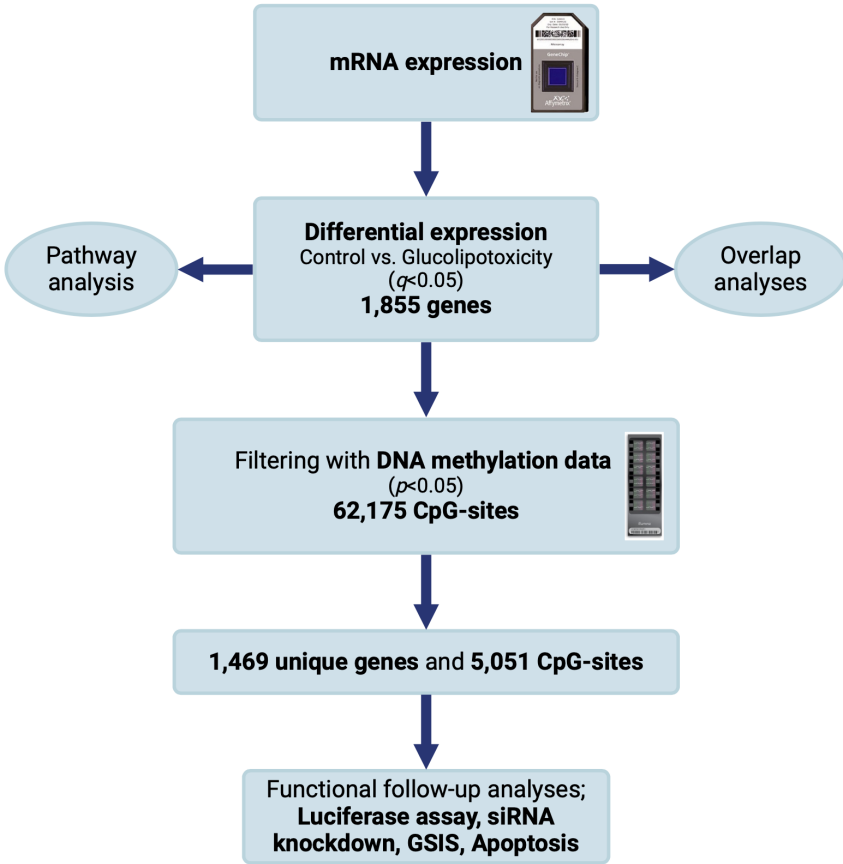

Figure 13. Workflow of the gene expression and DNA methylation analyses of islets treated under glucolipotoxic conditions. Created with BioRender.com

Using microarrays, we found 1,855 genes with differential expression when comparing control- and glucolipotox-treated islets (q<0.05; FDR<5%, **Figure 13**).

The GSEA showed 64 significant KEGG pathways being upregulated, including pathways involved in exocytosis and protein export (pathways important for, e.g., insulin secretion), and key metabolic pathways in islet cell function being downregulated in glucolipotox-treated islets (q<0.05, **Figure 14**). In the overlap analyses of T2D candidate genes (using the genome-wide association studies (GWAS) catalog), the donor's HbA_{1c} levels, glucotoxic- (60), and lipotoxic-treatment (61), we found 35, 187, 6, and 972 genes overlapping, respectively. We analyzed differential DNA methylation using the Illumina Infinium® HumanMethylation450 BeadChip in control versus glucolipotox-treated islets. Here

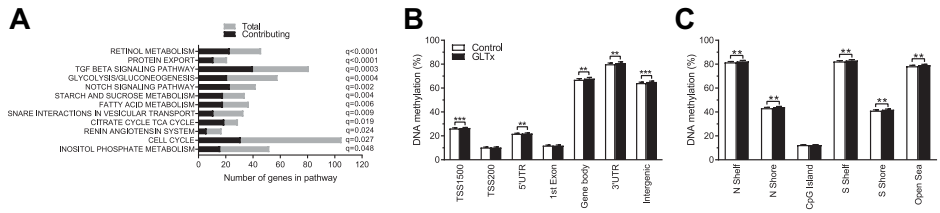

Figure 14. A) A selection of KEGG pathways, several with important functions in pancreatic islets, from the GSEA analysis of the mRNA expression data (q<0.05). The black bars indicate the number of contributing genes and the gray bars indicate the total number of genes in the pathway. **B-C)** DNA methylation of different **B)** genomic and **C)** CpG island regions in control and glucolipotox-treated pancreatic islets (13 donors). ***q*<0.01, ****q*<0.001, paired *t*-test. (87)

we found that several gene and CpG-island regions (**Figure 7**) had slightly higher methylation in the glucolipotox-treated islets (**Figure 14A-B**).

In the analyses of individual CpG-sites, 62,175 CpG-sites (**Figure 13**), annotated to 16,320 genes, were identified as differently methylated ($P<0.05$) with the majority showing increased methylation. The mRNA expression and DNA methylation data were then overlapped to identify genes with altered expression and methylation. A total of 5,051 CpG-sites showed altered DNA methylation in 1,469 genes of differently expressed genes (**Figure 13**), including some known T2D-associated genes such as *BCL11A*, *CDKN2B*, and *TCF7L2*. Functional follow-up experiments were then performed to further support our findings. Here four genes showcasing differential expression and DNA methylation were chosen: *CDK1*, *FICD*, *TPX2*, and *TYMS* (i.e., genes of interest, **Figure 13**). Luciferase assays were performed in a rat beta-cell line (832/13 INS-1) (82) where a 1.5kb fragment upstream of the TSS of the *CDK1* gene, inserted in a CpG-free firefly luciferase reporter vector, were used to analyze the effect of DNA methylation on gene expression. Methylation by SssI and HhaI, the methyltransferases that methylate most cytosines, showed strong effects on transcription from the *CDK1* promoter, while methylation by HpaII showed no effect (**Figure 15A**). The effect of the genes of interest on insulin secretion was assessed by knockdown experiments using siRNA in EndoC-βH1 cells. GSIS was increased after the knockdown of *FICS* and *TPX2*, where *TPX2*-knockdown also led to a slight increase in basal secretion (**Figure 15B-C**).

CDK1-knockdown led to a slight decrease in insulin content (negative control siRNA 458.3 ± 78.7 vs. *CDK1* siRNA 389.4 ± 64.8 mU/mg protein; $P = 0.03$), while knockdown of the other genes did not show an effect on insulin content (data not shown). No effect of the glucolipotox-treatment was seen on cell number or apoptosis after the knockdown of any of the four genes of interest in the EndoC-βH1 cells (data not shown).

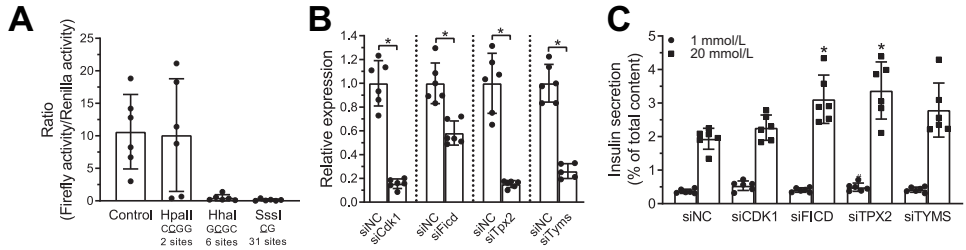

Figure 15. Functional follow-up experiments. **A)** A luciferase assay showing inhibition of the transcriptional activity of the *CDK1* promoter after methylation of the promoter with SssI or HhaI, whereas there was no effect after methylation with HpaII (n = 6). *P* = 0.001, Kruskal-Wallis one-way ANOVA. **B)** The relative expression measured by quantitative PCR analysis of siRNA-mediated *CDK1*, *FICD*, *TPX2*, and *TYMS* knockdown in EndoC-βH1 cells. Data are presented as the mean ± SD (n = 6). **P*<0.05, Wilcoxon matched-pairs signed rank test. **C)** GSIS in EndoC-βH1 cells after siRNA-mediated knockdown of genes of interest. Data are presented as the mean ± SD (n = 6, with two or three technical replicates for each condition). **P*<0.05 vs. negative control siRNA (siNC) at 20 mmol/L, Wilcoxon matched-pairs signed rank test. (87)

Study II

Lifestyle Intervention in Pregnant Women With Obesity Impacts Cord Blood DNA Methylation, Which Associates With Body Composition in the Offspring

This study aimed to assess whether a lifestyle intervention, including physical activity with and without dietary advice, in pregnant women with obesity affected the DNA methylome in their offspring's cord blood. To do this, we used offspring cord blood from the randomized control trial, the TOP-study. The baseline characteristics of the mothers, paternal BMI, and offspring at birth are presented in **Table 4**. There was no difference in energy intake at enrollment, but there was a trend towards a lower energy intake in the intervention group compared to controls at weeks 36-38. Wearing a pedometer was part of the lifestyle intervention, and during week 17 of pregnancy, the daily step counts were 8,623 ± 2,615 (no available step counts for the control group). The offspring were similar at birth regarding gestational age (GA), weight, length, and breastfeeding (**Table 4**).

Cord blood DNA methylation was examined at individual sites to determine if there were any differences between the lifestyle intervention and control groups. We found 379 CpG-sites (q<0.05, **Figure 16A**), annotated to 370 unique genes, to differ between the groups after adjusting for known confounding factors and using the Houseman reference-free method (88) to adjust for cell-type composition and controlling for FDR using the Benjamini-Hochberg method (86).

To evaluate the robustness of our findings, we did a sensitivity analysis in which we **I)** used a reference-based method to adjust for cell-type composition (62), **II)** adjusted for the first five principal components of the residuals, and **III)** added fewer covariates to the models or adjusted for smoking, **IV)** adjusted for maternal age, maternal BMI, smoking, GA, and offspring sex. In **I)** we found 376 of the 379 CpG-sites to be nominally associated with the lifestyle intervention (P=5.12x10^{-7}–3x10^{-2}), **II)** 377 of the 379 CpG-sites were found to be nominally associated with the lifestyle intervention (P=3.08x10^{-9}–4.7x10^{-2}), where a post hoc Benjamini-Hochberg analysis (86) on the 379 sites showed that all of the 377 had FDR<5%, **III)** all CpG-sites were nominally associated with lifestyle intervention after adjustment with fewer covariates and after adjustment for smoking (P<0.05), and **IV)** where 377 of the 379 sites were nominally associated with the lifestyle intervention (P=1.3x10^{-8}–2.4x10^{-4}). Additionally, we checked if GWG was associated with any of the 379 CpG-sites, of which none were (q<0.05).

Using GO Term Mapper for the 370 unique genes with differently methylated CpG-sites we found that about 60% of the genes have a role in metabolic processes (89). Additionally, combining GO and REVIGO analyses, 15 biological pathways were

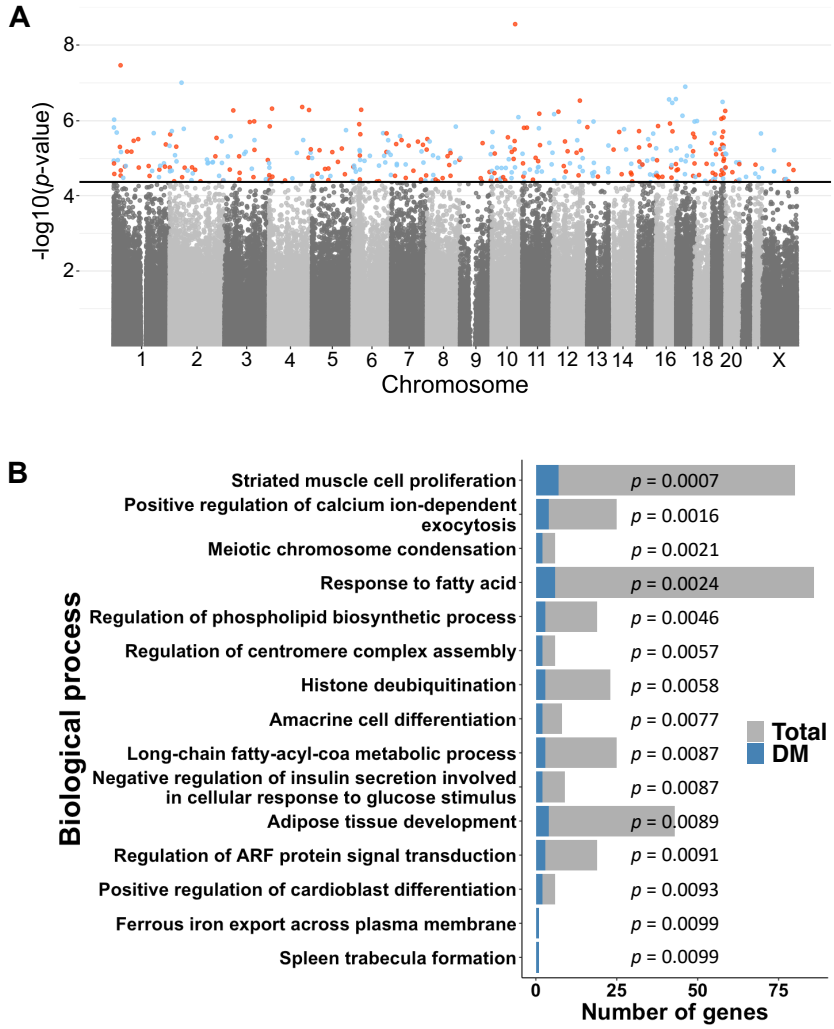

Figure 16. A) A Manhattan plot, representing the distribution of methylation sites across the genome. The black line shows the FDR threshold for multiple testing (P<4.17 x 10^{-5}) where surpassing hypermethylated sites are red and hypomethylated sites are blue in the lifestyle intervention group vs. the control group. **B)** Pathways with P<0.05 from a GO analysis focusing on the biological processes and where redundant GO terms were removed using REVIGO. Gray bars, the total number of genes in the pathway; blue bars, the number of differently methylated (DM) genes in lifestyle intervention vs. control subjects. (69)

found with a $P<0.01$, some of which are important in metabolic regulation and in metabolic diseases, such as response to fatty acids, negative regulation of insulin secretion involved in cellular response to glucose stimulus, and adipose tissue development (**Figure 16B**)(79, 90).

We investigated the relationship between genetic variation and DNA methylation by utilizing the methylation quantitative trait loci (mQTL) database. We identified 110 out of the 379 CpG-sites to be associated with SNPs, so-called mQTLs. Notably, the CpG-site cg21753618 is among the three sites that withstood the Bonferroni correction. Among these mQTLs, 18 SNPs have been linked to diseases in GWAS. Furthermore, previously published EWAS have linked 56 of these mQTLs to traits such as T2D, obesity, sperm viability, and maternal stress. Additionally, we explored whether any of the 370 genes for which we found differential methylation, had been associated with diseases/traits of interest. Here we found, three genes associated with adiposity (*MAP2K5*, *MEIS1*, and *IPO9*), four with obesity (*MAP2K5*, *PCDH9*, *SCNN1A*, and *TCF4*), four with T2D (*ACSL1*, *HMGA2*, *RPSAP52*, and *SLC9B2*), and seven genes (*TENM4*, *HMGA2*, *MAP3K10*, *RB1*, *KLHL29*, *LRIG1*, and *PMFBP1*) have SNPs associated with birthweight.

We were interested in finding out if the lifestyle intervention affected offspring characteristics, at birth and during the first three years of life. We chose to study lean mass in the offspring, as this cohort comprises very valuable data from Dual-energy X-ray Absorptiometry (DXA) scans of the newborns (within 48h of birth). Here we found that offspring born to mothers randomized to the lifestyle intervention group were born with more abdominal lean mass (**Figure 17A-B, Table 6**). Additionally, a trend toward more overall lean mass was seen for the offspring of mothers in the lifestyle intervention group (**Figure 17C-D, Table 6**). As seen in **Table 4,** the birthweight did not differ between the groups, indicating that the distinction between the groups is due to altered body composition.

Furthermore, we found that children of mothers in the lifestyle intervention group were larger in size at 9, 18, and 36 months although only significantly so at 18 months of age (**Figure 17E**, **Table 4** and **6**). This led us to the question of whether the found lifestyle intervention-associated CpG-sites (379 sites) were associated with the offspring characteristics of interest. Here we found that 25 out of the 379 CpG-sites were associated with lean mass ($q<0.05$), with a majority being hypermethylated in the lifestyle intervention group and positively associated with greater lean mass. BMI z-scores were used as a proxy for measuring growth, and as we were interested in exploring if the 379 lifestyle intervention-associated CpG-sites were associated with offspring growth over the first three years of life we used LMMs. Methylation of 22 out of the 379 CpG-sites was associated with BMI z-scores ($P<0.05$). Some of these 22 CpG-sites were annotated to genes of interest in metabolism and metabolic diseases, e.g., *ACSL1* harboring an SNP associated with T2D and *TCF4* with an SNP associated with obesity.

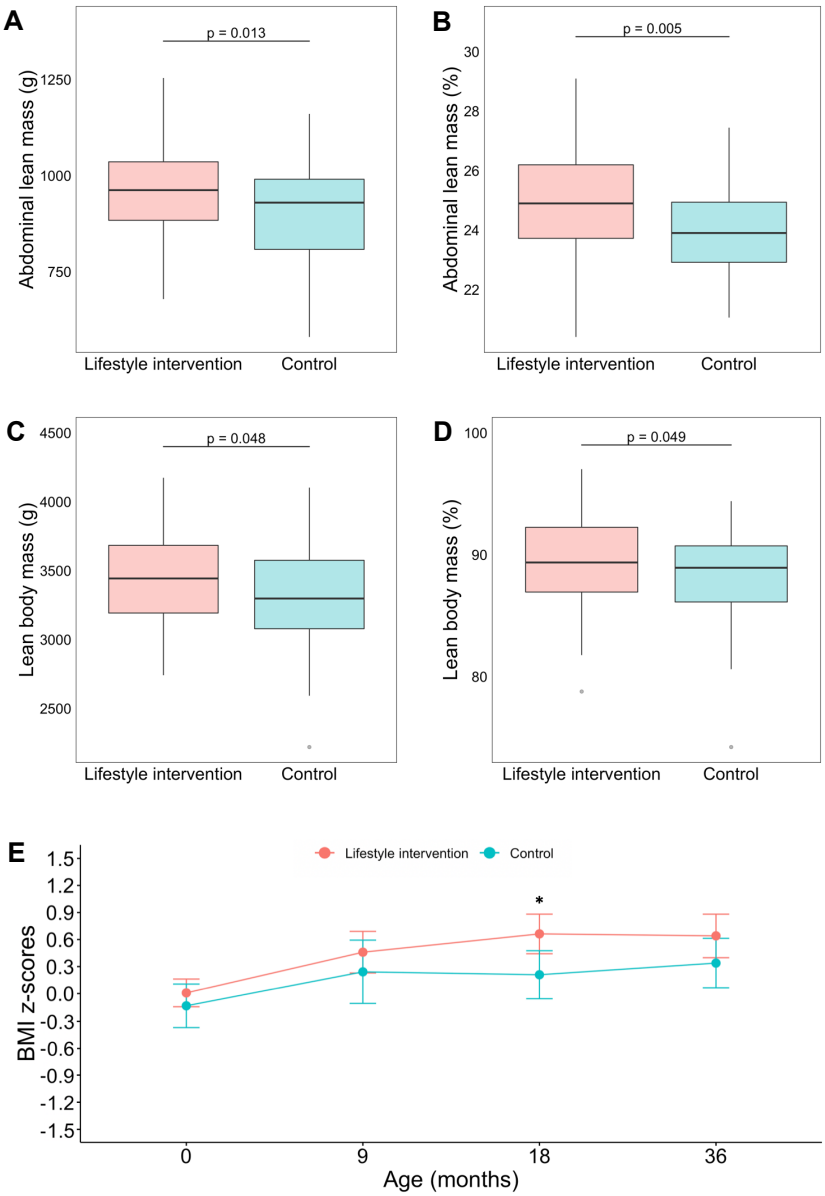

Figure 17. Boxplots showing **A)** abdominal lean mass (g), **B)** abdominal lean mass (%), **C)** total lean mass (g), and **D)** total lean mass (%) in the lifestyle intervention and control groups at birth, medians (IQR). The *P*-values are based on linear regression models adjusted for maternal education level, maternal smoking during pregnancy (yes/no), GWG (kg), pre-pregnancy BMI (kg/m2), parity (single/multi), GA (weeks) and offspring sex. **E)** Offspring BMI z-scores at four time points, birth, 9, 18, and 36 months. **P*<0.05 based on linear models adjusted for maternal education level, maternal smoking during pregnancy (yes/no), GWG (in kilograms), prepregnancy BMI, parity (single/multi), breastfeeding partially and exclusively, and BMI z-score at birth.

Table 6. Differences estimated from linear regression models of offspring lean mass and BMI *z*-scores, Lifestyle intervention (n=92) versus control (n=47) groups.

Phenotype	Estimated differences (95% CI)	*P*-value
Lean mass (g), birth	126.55 (-4.52; 257.62)*	0.058*
Lean mass (%), birth	1.36 (20.05; 2.77)*	0.059*
Abdominal lean mass (g), birth	59.09 (10.53; 107.65)*	0.017*
Abdominal lean mass (%), birth	0.88 (0.24; 1.53)*	0.008*
BMI *z*-score, birth[1]	0.15 (20.14; 0.43)†	0.352†
BMI *z*-score, 9 months[2]	0.31 (20.14; 0.76)‡	0.315‡
BMI *z*-score, 18 months[3]	0.54 (0.14; 0.93)§	0.006§
BMI *z*-score, 36 months[4]	0.30 (20.13; 0.74)§	0.169§

*[1]Lifestyle intervention, n=129; control, n=71. [2]Lifestyle intervention, n=60; control, n=39. [3]Lifestyle intervention, n=57;control, n=36. [4]Lifestyle intervention, n=51; control, n=28. *Adjusted for maternal education level, maternal smoking during pregnancy (yes/no), GWG (in kilograms), prepregnancy BMI, parity (single/multi), GA (in weeks), and offspring sex. †Adjusted for maternal education level, maternal smoking during pregnancy (yes/no), GWG (in kilograms), prepregnancy BMI, parity (single/multi), and GA (in weeks). ‡Adjusted for maternal education level, maternal smoking during pregnancy (yes/no), GWG (in kilograms), prepregnancy BMI, parity (single/multi), GA (in weeks), breastfeeding partially and exclusively, and BMI z-score at birth. §Adjusted for maternal education level, maternal smoking during pregnancy (yes/no), GWG (in kilograms), prepregnancy BMI, parity (single/multi), breastfeeding partially and exclusively, and BMI z-score at birth.*

To analyze the potential causal relationship between DNA methylation and offspring characteristics, we performed causal mediation analyses. This allows us to investigate whether DNA methylation is part of a pathway through which the lifestyle intervention exerts its effect on offspring lean mass (**Figure 11**). We found that 17 of the 25 lean mass-associated CpG-sites partially mediate the effect of the lifestyle intervention on offspring lean mass at birth (q<0.05, **Table 7**).

Table 7. Causal mediation analysis on the significant associations between the lifestyle intervention and lean mass related methylation (CpG) sites as mediators and lean mass (%) as outcome (ACME *q*-value<0.05, FDR<5%)

CpG site	Gene	ACME estimate of mediator CpG (95% CI)	ACME *q*-value	ADE estimate (95% CI)	Total effect (95% CI)	Proportion mediated by CpG (95% CI)
cg07405330	*MOBP*	0.84 (0.35; 1.39)	<0.001	0.52 (-0.84; 1.89)	1.35 (-0.09; 2.74)	0.62 (-0.73; 3.58)
cg06480224	*KIAA2012*	0.78 (0.19; 1.51)	0.013	0.57 (-0.97; 1.98)	1.35 (-0.09; 2.74)	0.58 (-1.11; 4.27)
cg11612786	*AC079135.1; GBX2*	0.64 (0.13; 1.38)	0.013	0.71 (-0.66; 2.03)	1.35 (-0.09; 2.74)	0.47 (-0.58; 2.78)
cg20982052		0.53 (0.14; 1.11)	0.013	0.82 (-0.68; 2.18)	1.35 (-0.09; 2.74)	0.39 (-0.62; 2.63)
cg00154557	*DSE*	0.50 (0.08; 1.08)	0.021	0.86 (-0.64; 2.26)	1.35 (-0.09; 2.74)	0.37 (-0.58; 2.42)
cg13002044	*TMEM178B*	0.70 (0.14; 1.42)	0.021	0.65 (-0.73; 1.98)	1.35 (-0.09; 2.74)	0.52 (-0.85; 2.87)
cg18088415	*LSM2*	0.70 (0.19; 1.41)	0.021	0.65 (-0.77; 2.07)	1.35 (-0.09; 2.74)	0.52 (-0.73; 3.42)
cg11594420	*TEX101*	0.53 (0.10; 1.18)	0.025	0.82 (-0.59; 2.15)	1.35 (-0.09; 2.74)	0.39 (-0.45; 2.84)
cg04678315		0.48 (0.08; 1.02)	0.028	0.87 (-0.56; 2.20)	1.35 (-0.09; 2.74)	0.36 (-0.64; 2.05)
cg08144675		0.70 (0.10; 1.42)	0.032	0.66 (-0.85; 2.20)	1.35 (-0.09; 2.74)	0.52 (-0.98; 3.35)
cg22454673	*HERC2*	0.63 (0.16; 1.29)	0.032	0.73 (-0.71; 2.18)	1.35 (-0.09; 2.74)	0.46 (-0.77; 3.10)
cg04058675	*HUWE1*	0.45 (0.08; 1.03)	0.033	0.90 (-0.46; 2.22)	1.35 (-0.09; 2.74)	0.34 (-0.48; 2.09)
cg03190725	*RP3-468B3.2*	0.54 (0.10; 1.10)	0.035	0.81 (-0.67; 2.19)	1.35 (-0.09; 2.74)	0.40 (-0.65; 2.58)
cg06799721	*TARS*	0.52 (0.05; 1.27)	0.036	0.84 (-0.54; 2.07)	1.35 (-0.09; 2.74)	0.38 (-0.30; 2.00)
cg15157974	*DISC1*	0.43 (0.04; 0.96)	0.040	0.92 (-0.50; 2.21)	1.35 (-0.09; 2.74)	0.32 (-0.24; 2.10)
cg26142132	*AAT*	0.45 (0.06; 0.96)	0.041	0.90 (-0.57; 2.40)	1.35 (-0.09; 2.74)	0.34 (-0.44; 2.26)
cg00354884	*ABR*	0.53 (0.03; 1.18)	0.047	0.83 (-0.62; 2.17)	1.35 (-0.09; 2.74)	0.39 (-0.45; 2.32)

Models adjusted for GWG (in kilograms), maternal BMI, GA (in weeks) and offspring sex. Based on 139 participants, Lifestyle intervention (n = 92), control (n = 47). ACME, average causal mediator effect; ADE, average direct effect

Study III

Gestational weight gain in pregnant women with obesity is associated with cord blood DNA methylation, which partially mediates offspring anthropometrics

This study aimed to investigate whether GWG in women with obesity, spanning a broad range of GWG (-5.0-34.1kg), is associated with offspring cord blood DNA methylation. To do this, we analyzed DNA methylation using the Illumina Infinium® HumanMethylation450 BeadChip from 232 newborns enrolled in the TOP study (**Figure 18A**). The association was tested using four linear regression models (**Figure 18B),** which differed in included covariates and methods for adjusting for cell-type composition. This was done to estimate the covariates' influence on the association. Our main model, Model 1) was adjusted for maternal age (years), pre-pregnancy BMI (kg/m^2), lifestyle intervention (yes/no), offspring sex, and GA (days). Adjustment for cell composition was done using the reference-free method from Houseman et al. (88). Model 2) was an unadjusted model, and Model 3) was adjusted for the same covariate as Model 1 but without correcting for cell-type composition. Model 4) included the same covariates as Model 1 and correction for cell-type composition was done using a reference-based method (62). Using our main model, Model 1, we found 441 CpG-sites to associate with GWG (FDR<5%), annotated to 352 genes (**Figure 18**). Six sites surpassed the Bonferroni cut-off: cg01704198 in the gene body of *CLASP2;* cg19152518 in the 1st Exon and 5' untranslated region of *DENND5B*; cg19697475, in the promotor region of *HCN1*; cg22950754 in the promotor region of *PDRG1*; cg10383019, in the gene body of *TUB* – encoding a member of the Tubby family of bipartite transcription factors which is suggested to affect hypothalamic regulation of body weight; and cg13303461 in the promoter region of *UBE2L6* (**Figure 18C**). Additionally, GWG was nominally associated with DNA methylation at 410, 413, and all of the 441 sites in Model 2 with $P = 3.72 \times 10^{-8} – 4.91 \times 10^{-2}$, Model 3 with $P = 2.08 \times 10^{-8} – 4.92 \times 10^{-2}$, and Model 4 with $P = 1.59 \times 10^{-7} – 3.70 \times 10^{-2}$, respectively (**Figure 18B**). Together, these data indicate that the association between GWG and DNA methylation was not drastically impacted by these covariates. Further validation of the results was done by randomly splitting the cohort into a discovery (n=125) and validation cohort (n=83)(60:40), where 99% and 74% of the sites were replicated, respectively. More importantly, 74% of the sites in the discovery cohort were replicated in the validation cohort.

Next, we investigated the genetic influence of the 441 GWG-associated CpG sites in cord blood. Using the mQTL database, we identified 4911 SNPs linked to DNA

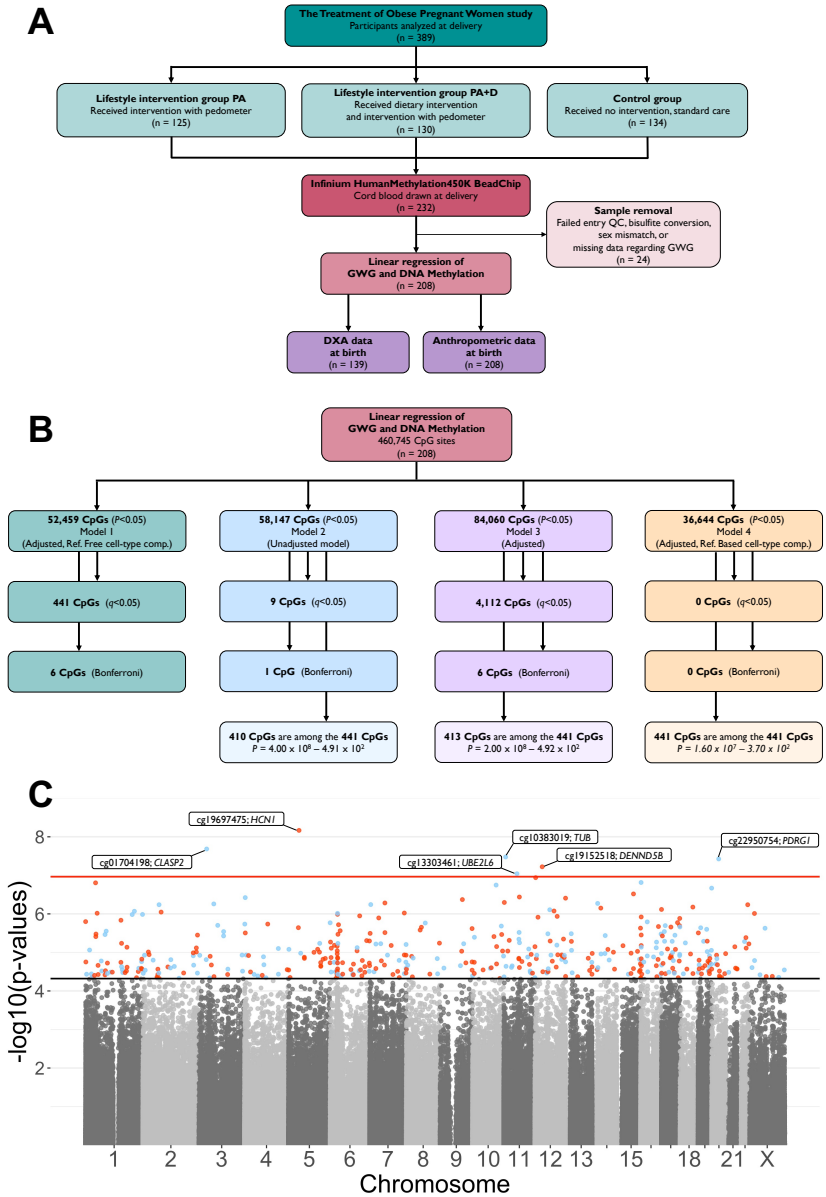

Figure 18. A) Flow diagram of the GWG and DNA methylation cohort of the TOP-study. QC, quality control; DXA, Dual-energy X-ray absorptiometry. **B)** Linear regression models of GWG and DNA methylation in cord blood. **C)** A Manhattan plot, representing the distribution of methylation sites across the genome, for the association between GWG and DNA methylation in cord blood from the offspring, (Model 1). The bottom (black) line indicates the FDR-adjusted *P*-value threshold (FDR<5%) and the top (red) line indicates the Bonferroni threshold (1.085199 x 10^{-7}, i.e., 0.05/460,745). Methylation sites that surpassed the FDR threshold are highlighted in color; red is hypermethylated and blue is hypomethylated sites. Hyper-/hypomethylation is based on beta coefficients from Model 2. (91)

methylation at 111 CpG-sites—mQTLs (92). Of these, a CpG-site—cg19152518, annotated to the *DENND5B* gene involved in vesicle-mediated transport— was one of the six CpG-sites that withstood the Bonferroni correction. Additionally, 39 SNPs among the 111 mQTLs were associated with disease traits such as asthma (e.g., *SIK2* and *WDR36*) and waist-to-hip ratio adjusted for BMI (*ATP6V0A2*) in GWAS (93). Furthermore, DNA methylation at 61 of the 111 mQTLs was linked to various traits, including asthma (e.g., cg21689291 annotated to *TMEM106A* and cg22227621 annotated to *ALG14*), birthweight (e.g., cg22441770 annotated to *CRTC2,* and cg24796852 annotated to *GMFG*), BMI (cg12338137 annotated to *TNS1*), and T2D (cg05411199 annotated to *PXDC1*), in published EWAS (94).

We proceeded to investigate whether GWG is also associated with offspring lean mass at birth (n=139) and birthweight (n=208). A negative correlation between GWG and offspring lean mass at birth was found (**Figure 19A**), a linear regression model adjusted for lifestyle intervention (yes/no), maternal smoking during pregnancy (yes/no), GA (days), and offspring sex, confirmed these results and estimated a decrease of lean mass at birth by 0.23 ± 0.05 percentage points (95% CI: -0.33; -0.13) with every kilogram of GWG. A positive correlation between GWG and birthweight was found (**Figure 19B**), also confirmed by an adjusted linear regression model (covariates: GA (days) and parity (single/multiple)), showing estimates of 21.1 ± 5.0g (95% CI: 11.3; 30.9) increase in birthweight for every unit of GWG. Following these analyses, we tested if cord blood methylation of the found 441 CpG-sites from Model 1 was associated with offspring lean mass at birth and birthweight, adjusting models as mentioned above. We found 62 CpG-sites associating with offspring lean mass and 21 CpG-sites associating with offspring birthweight (q<0.05). We explored these CpG-sites further and investigated whether the influence of GWG on the offspring's lean mass and/or birthweight was partially mediated through cord blood DNA methylation at any of the sites associated with lean mass (62 sites) and/or birthweight (21 sites). Using nonparametric causal mediation analyses, we found that DNA methylation partially mediates the effect of GWG on offspring lean mass and birthweight at 21 and 17 CpG-sites, respectively (FDR<5%, **Figure 19C-E**).

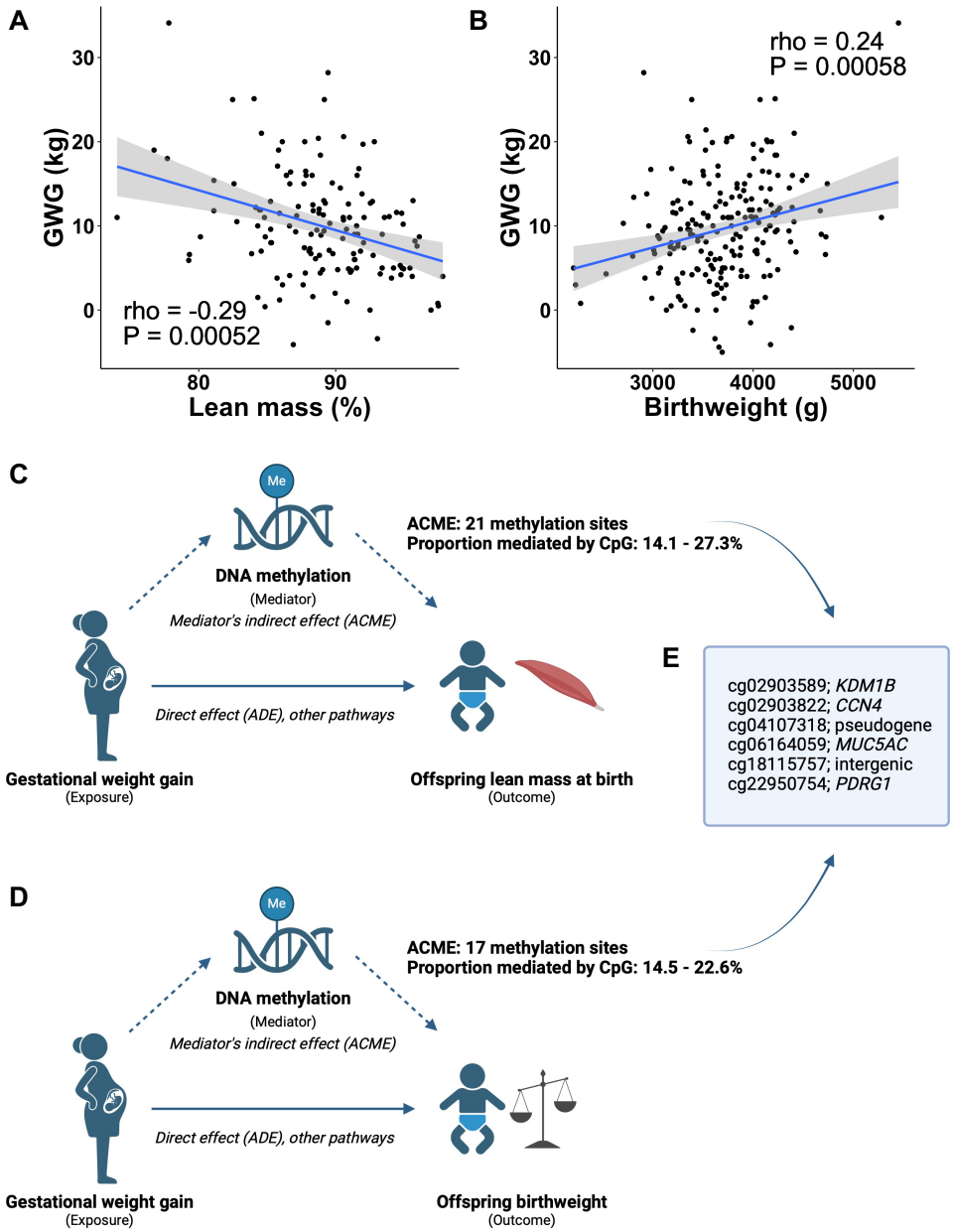

Figure 19. A) Spearman correlation plots of GWG (kg) and offspring lean mass at birth (%) and **B)** offspring birthweight (g). **C-D)** The scheme tested by the causal mediation analysis and the potential mechanisms linking GWG and **C)** offspring lean mass at birth and **D)** offspring birthweight. **E)** Show the six sites suggested to partially mediate the effect of GWG on both offspring anthropometric measurements (91). C-E) Created with BioRender.com

Study IV

Impact of Added Sugar on the Whole Genome DNA Methylation Pattern in Human Sperm

The aim of this study was to investigate the impact of dietary intervention, with and without added sugar, and to profile the DNA methylome in human sperm using WGBS.

DNA methylation at base-pair resolution was obtained in human sperm of 15 men using WGBS (**Figure 5**). We profiled genome-wide DNA methylation at 26.9 million CpG-sites in the human sperm with an average sequencing depth of 10x.

The effect of diet on the DNA methylome was investigated using the R package dmrseq (85). The following criteria were set for DMR calling: a minimum sequencing depth (CpG coverage) of 10x for individual CpG-sites across all samples, a minimum DMR length of three CpGs, a cut-off of 0.05 (indicating ≥5% difference in methylation between groups), and 10 permutations. DMR calling was performed for the three different diets, Baseline versus Healthy, Baseline versus Added Sugar, and Healthy versus Added Sugar, on approximately 2.9 million, 2.1 million, and 3.7 million CpG-sites, with an average sequencing depth of 11.7x, 12.5x, and 11.7x, respectively (**Figure 20**). We found seven DMRs in total, four DMRs for Baseline versus Healthy, two for Baseline versus Added Sugar, and one for Healthy versus Added Sugar (**Figure 20**), all of which are annotated to intergenic regions and with a DMR length of three CpG-sites.

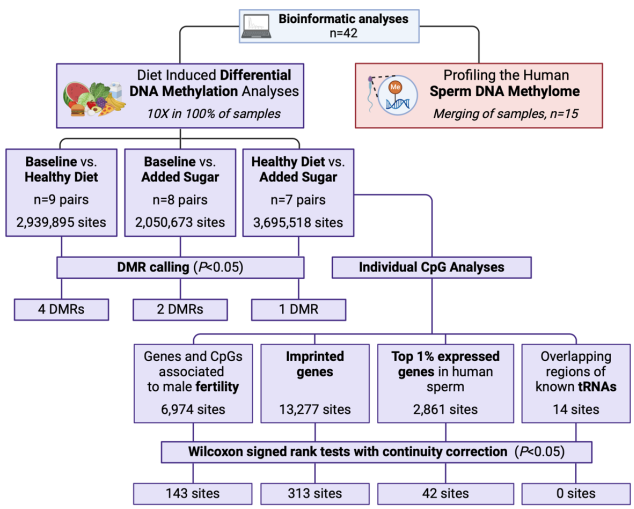

Figure 20. Flow chart of bioinformatic and statistical analyses of WGBS data in human sperm of 15 men. Created with BioRender.com

We further tested if the Healthy Diet versus Added Sugar impacted DNA methylation of individual CpG-sites. For these analyses we required CpG coverage of ≥10 reads across all samples, resulting in 7 pairs and approximately 3.7 million CpG-sites. We chose to investigate I) genes and CpG-sites with known function in male fertility based on the review by Åsenius et al. (95) of which 6,974 CpG-sites overlapped with our data, II) CpG-sites annotated to imprinted genes where 13,277 CpG-sites overlapped, III) CpG-sites annotated to the top 1% (159) of expressed protein-coding genes in human sperm, based on RNA sequencing (RNA-seq) data in human sperm from Corral-Vazquez et al. (74) with 2,861 CpG-sites overlapping, and IV) CpG-sites at exact genomic coordinates as known tRNAs, of which 14 sites were overlapping (**Figure 20**). Based on nominal *P*-values ($P<0.05$), DNA methylation was affected by the Added Sugar diet in I) 143 CpG-sites annotated to, e.g., *ACP1*, *AHRR*, *GNAS*, *HDAC4*, *PAX8*, *PTPRN2*, and *SNURF*, II) 313 CpG-sites annotated to, e.g., *GLIS3*,, *MAGI2*, *PEG3*, *PLAGL1*, and *SNURF*, III) 42 CpG-sites annotated to, e.g., *CHD2* and *PEG3*, and IV) none of the 14 CpG-sites with the same genomic position as known tRNAs (**Figure 21**). On the whole, we found DNA methylation of 486 unique CpG-sites to be nominally affected by the Added Sugar diet, of which numerous went in the same direction in all analyzed samples (**Figure 21**). However, these findings did not withstand correction for multiple tests.

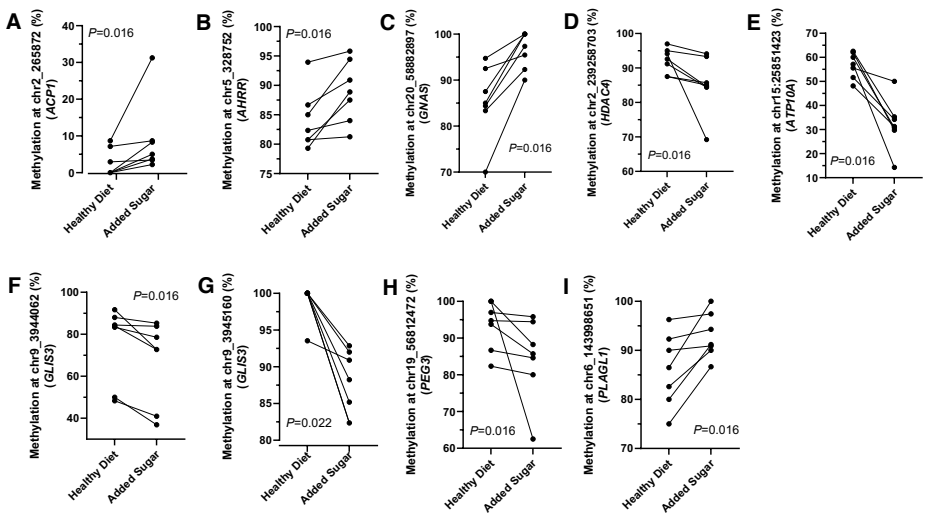

Figure 21. Diet-induced DNA methylation differences in individual CpG-sites based on nominal *P*-values ($P<0.05$), with inclusion criteria requiring ≥10 reads for CpG-sites and samples, and consistent directionality of DNA methylation changes across all sperm samples when comparing the Healthy Diet versus Added Sugar. DNA methylation of CpG-sites annotated to **A)** *ACP1*, **B)** *AHRR*, **C)** *GNAS* exhibited increased methylation, while **D)** *HDAC4*, **E)** *ATP10A*, **F-G)** *GLIS3*, **H)** *PEG3* showed decreased methylation, and **I)** *PLAGL1* displayed increased methylation after the Added Sugar diet compared to the Healthy Diet, (n=7 for all panels except F, n=6). Wilcoxon signed rank tests with continuity correction was used for these analyses.

Next, the impact of the three diets on the "global" methylome in human sperm was investigated. To do this, we calculated the 1st, 2nd, and 3rd quartiles per diet (Baseline, Healthy Diet, and Added Sugar) based on the WGBS data of 22,780,770 CpG-sites (the number of overlapping CpG-sites for all three diets) with a mean sample coverage of ≥10x (n=41). **Figure 22A** shows the 1st quartile representing the quarter of CpG-sites with the lowest methylation level, the 2nd quartile representing the median methylation level, and the 3rd quartile representing the quarter of CpG-sites with the highest methylation level. No evident impact of the diet intervention on the "global" methylome in human sperm could be seen, and no difference in the "global" DNA methylation pattern for the three different diets in the 15 men was detected (**Figure 22A**). Furthermore, no significant effect was found for the variance in the "global" degree of methylation in the 1st and 2nd quartiles (P=0.33 and P=0.81, respectively). Due to a small sample variance, the 3rd quartile could not be statistically tested (methylation values were 100% for 39 out of 41 samples, **Figure 22A**).

The profiling of the human sperm DNA methylome was carried out using merged methylation data from the three diets of the 15 men included in the study. This was done since we did not see a distinct difference between the diets and to increase the sequencing depth. The merging resulted in an average CpG coverage of 28.7x (**Figure 22B**).

When plotting the overall distribution of DNA methylation in human sperm, using dmrseq (85), the distribution was found to be bimodal. The first peak was around 0%, demonstrating CpG-sites with low levels of methylation, and the second peak was at about 100%, revealing that most CpG-sites are fully methylated, the average degree of methylation was 77.05% (**Figure 23A**). This is in concordance with what has been seen previously in pancreatic islets, although the average methylation in the human sperm seems to be slightly higher (96). The distribution and degree of DNA methylation vary with different genomic regions; in human pancreatic islets, for instance, the promoter regions have been found to have the lowest degree of methylation, and the gene body has the highest (96). Density plots visualizing the overall distributions of the methylation levels through different genomic regions in human sperm were generated using dmrseq (85)(**Figure 23A-H**). The introns and 3'UTRs were found to have the highest DNA methylation degree, with an average of around 82% (**Figure 23F-G**), and the lowest levels were seen in the 5'UTRs, with an average methylation degree of 13.7% (**Figure 23D**).

Thereafter, using publicly available RNA-seq data (n=12)(74), we investigated the relationship between gene expression and the DNA methylome of the 15 men included in this study. We identified 33,408 genes in the RNA-seq data, of which the majority (62%) were classified into non-expressed genes (<2 mean normalized counts). The remaining 12,617 genes were determined to be expressed genes and divided into three bins classified as low- (n=4,151), medium- (n=4,171), and high-expressed (n=4,295) genes. Associations between the degree of DNA methylation

in the different gene regions and the four gene expression levels were found using the Friedman rank sum test (**Figure 23I**). Based on Dunns' post hoc test, we, for example, found non-expressed genes in human sperm to have lower DNA methylation levels in exon regions in comparison to the expressed genes (**Figure 23I**).

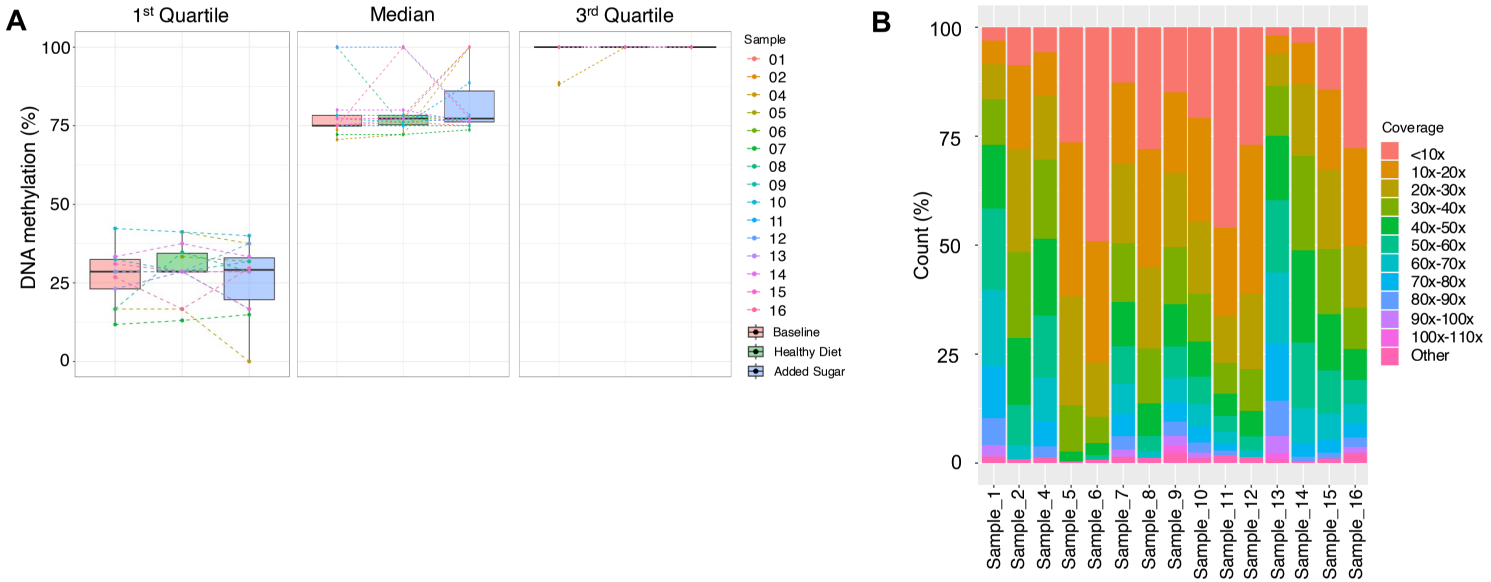

Figure 22. A) Boxplot visualizing the variability between individuals and diets based on the global degree of methylation. The 1st quartile represents the quarter of CpG-sites with the lowest methylation level, the 2nd quartile represents the median methylation level, and the 3rd quartile represents the quarter of CpG-sites with the highest methylation level. **B)** The coverage proportion of whole genome bisulfite sequencing data in sperm, determined for each individual after merging data from the three diets.

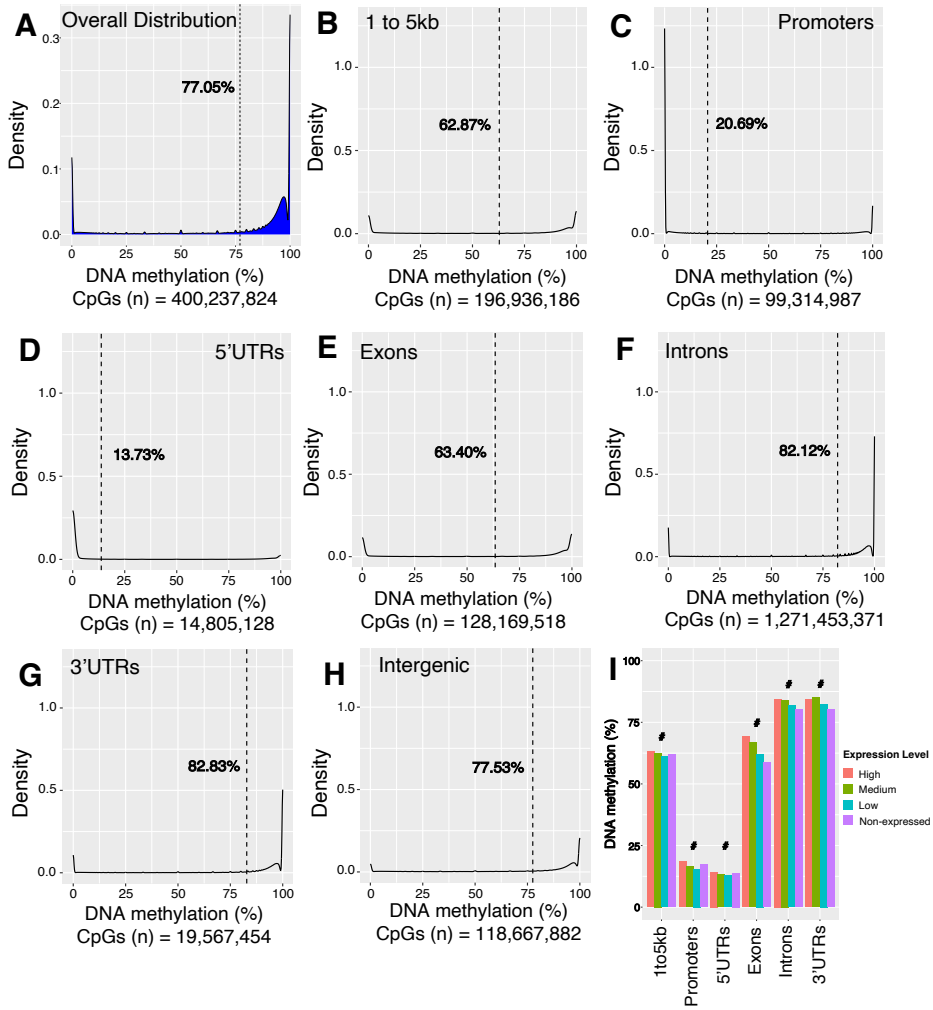

Figure 23. A) The overall distibution of DNA mentylation in human sperm (n=15) visualized in a density plot. Peaks at around 0% and around 100% methylation. The mean methylation degree was 77.05%. **B)** Average DNA methylation levels, using the merged WGBS data, in different gene regions of non-expressed genes and high-, medium-, and low-expressed protein-coding genes. #<0.05, as analyzed by Friedman rank sum tests, P=9.3x10^{-10} for 1-5kb, P=1.6x10^{-9} for promoters, *P*=9.3x10^{-10} for 5'UTR, *P*=9.3x10^{-10} for exons, *P*=9.3x10^{-10} for introns, and *P*=2.6x10^{-9} for 3'UTR, as analyzed by a post hoc Dunn's multiple comparison test. Density plots showing the degree of DNA methylation in the human sperm samples (n=15) in different gene regions, using the merged WGBS data including all three diets: **C)** The region of 1 to 5 kb upstream of the TSS, mean DNA methylation degree of 62.87%; **D)** Promoters, mean DNA methylation degree of 20.69%; **E)** 5'UTRs, mean DNA methylation degree of 13.73%; **F)** Exons, mean DNA methylation degree of 63.40%; **H)** 3'UTRs, mean DNA methylation degree of 82.83%; and **I)** Intergenic regions, mean DNA methylation degree of 77.53%. CpGs (n) is the total number of CpGs analyzed in all samples in each specific region. *WGBS, whole genome bisulfite sequencing; TSS, Transcription Start Site; 5'UTR, 5' Untranslated Region; 3'UTR, 3' Untranslated Region; kb, kilobase pairs; bp, base pairs.*

Ethical Considerations

Ethical considerations in research involve balancing various legitimate interests, such as the pursuit of knowledge, integrity interests, and protection against harm. Although ethical assessment and approval by a body such as the Swedish Ethical Review Authority is mandatory, ethical considerations extend beyond this point. The concept of 'good research practice' also includes reflections on the researcher's own work and professional judgments (97).

The ethical framework for the EU's research funding "The European Code of Conduct for Research Integrity", outlines fundamental principles for 'good research practice'. These principles emphasize reliability in research quality, honesty in conduct and reporting, respect for stakeholders and the environment, and accountability throughout the research process (97).

Study I

Informed consent was obtained from pancreatic donors or their relatives, as approved by the local ethics committee in Lund, Sweden (Dnr 173/2007), which oversees organ donation for medical research.

Study II-III

The TOP-study was approved by the Ethics Committee for the Capital Region of Denmark (January 2009, H-D-2008–119; Hillerød, Denmark) and by the Swedish Ethical Review Authority, Stockholm (Dnr 2022-07072-01) and registered at ClinicalTrials.gov (NCT01345149). Before enrollment, all participants provided written informed consent.

Study IV

The study was approved under the Declaration of Helsinki by the regional ethical board at Linköping University, Sweden (permit number: 2016/183-31, extension: 2019-03080). Written informed consent was obtained before enrollment from all participants.

Discussion

The overall aim of this thesis was to investigate whether lifestyle impacts the epigenome and, thereby, metabolism in humans. The epigenome consists of several epigenetic modifications and mechanisms. Therefore, we limited the content of this thesis to include only DNA methylation, measured in different tissues and in different ways. In Paper I, we analyzed DNA methylation in human pancreatic islets, where we tried to mimic a typical circulation milieu of a T2D patient with elevated levels of glucose and fatty acids—glucolipotoxicity. Here we measured DNA methylation using an array-based method, the Illumina Infinium® HumanMethylation450 BeadChip. In Paper II, we investigated the effect of a Lifestyle intervention during pregnancy in women with obesity and its effect on the offspring. In Paper III, we wanted to explore the effect of weight gain during pregnancy (gestational weight gain, GWG) in women with obesity and its effects on the offspring. In Papers II and III, this was done by measuring DNA methylation in the offspring cord blood using the Illumina Infinium® HumanMethylation450 BeadChip. In Paper IV, we investigated whether diet interventions affected genome-wide DNA methylation in human sperm, by applying Whole-genome bisulfite sequencing.

Interpretation of Main Findings

Study I

Glucolipotoxicity Alters Insulin Secretion via Epigenetic Changes in Human Islets

Elevated circulating levels of glucose and fatty acids are commonly observed in individuals with obesity and seem to contribute to the development and progression of metabolic disorders like T2D (5, 9, 14). In fact, elevated levels of these metabolites are also commonly found in individuals with T2D, accompanied by impaired islet function. In this study, we therefore investigated the impact of high glucose and palmitate levels on islet function, gene expression, and DNA methylation. Our findings reveal that glucolipotoxic treatment leads to decreased insulin secretion and increased apoptosis in human islets. Moreover, we observe extensive changes in gene expression in glucolipotoxic-treated islets, potentially

attributed to DNA methylation changes. Subsequent analysis in clonal beta-cells reveals that these changes influence insulin secretion, suggesting a contributory role of induced epigenomic and transcriptomic alterations in the pathogenesis of T2D.

When exploring the genes uniquely affected by the combined elevated levels of glucose and palmitate, we find several critical processes in insulin-secreting beta-cells, such as pathways crucial for protein metabolism, transport, and secretion. Additionally, a pathway related to the unfolded protein response (UPR), known to impact T2D and beta-cell function (98), was enriched among these significant genes. These findings suggest that alterations induced by glucolipotoxicity may contribute to the deterioration of beta-cell function *in vivo*, a phenomenon not observed with individual nutrient elevations (60, 61). Among the genes exhibiting altered expression in glucolipotox-treated islets, 35 have SNPs associated with T2D risk in previous GWAS (93). Many of these variants or encoded proteins are implicated in islet cell development and/or function (93, 99-101), or influence cell proliferation and/or apoptosis, particularly in beta-cells (93, 102, 103). These findings support the notion that expression changes induced by high levels of palmitate and glucose may heighten T2D risk in obese individuals or further compromise beta-cell function in those with T2D.

Importantly, in our experiments, the exposure to glucolipotoxic conditions was merely 48 hours, while the exposure time in real life may extend over several years or even decades, likely leading to more potent effects. Rescue experiments in the human EndoC-βH1 beta-cell line, for the analysis of whether the epigenetic changes were reversible, showed that the DNA methylation changes largely persisted, even after a 48-hour-long recovery period where the cells were cultured under normal conditions. This implies that restoring circulating metabolite levels may not completely reverse already established epigenetic effects or the recovery time may potentially be longer than the exposure time.

Overall, these findings provide support for the involvement of glucolipotoxicity in the onset and/or deterioration of T2D.

Study II

Lifestyle Intervention in Pregnant Women With Obesity Impacts Cord Blood DNA Methylation, Which Associates With Body Composition in the Offspring

Maternal obesity during pregnancy increases the risk of offspring metabolic disease (104) and is associated with epigenetic modifications in the offspring's cord blood (105). Additionally, distinct DNA methylation profiles have been found in tissues of individuals with obesity compared to lean individuals (106-108). As lifestyle factors have the potential to influence DNA methylation (40, 49), we wanted to

investigate if a Lifestyle intervention during pregnancy in women with obesity influenced the offspring cord blood DNA methylation pattern.

Our findings reveal that a lifestyle intervention comprised of physical activity with and without dietary advice among pregnant women with obesity impacts cord blood DNA methylation in their offspring, with enrichment in genes involved in metabolic processes. Notably, the epigenetic modifications associated with physical activity and a healthy diet in our study appear to be distinct from those previously linked to maternal BMI (105) and GDM (107, 109). Moreover, we find a link between epigenetic markers in cord blood and offspring lean mass and growth. This suggests that adapting to a healthier lifestyle during pregnancy - allowing for offspring epigenetic cord blood modifications - may contribute to improving the offspring's health. This notion is reinforced by studies indicating that newborns born small for gestational age (SGA) exhibit more pronounced differences in lean mass rather than fat mass compared to appropriate-for-gestational-age newborns (110). Being born SGA is associated with an elevated risk of metabolic diseases later in life (111), suggesting that the adverse effects may be due to reduced lean mass. In fact, higher muscle mass and increased metabolic activity may provide beneficial effects on insulin sensitivity and protect against obesity and T2D (112). Additionally, in this study, DNA methylation in cord blood at several sites appears to partially mediate the effect of the maternal lifestyle intervention on offspring lean mass. Notably, the lifestyle intervention group exhibited decreased methylation of *SETD3*, a gene encoding a methyltransferase. Hypomethylation of *SETD3* is associated with increased expression and subsequently higher muscle mass (113), potentially explaining the greater lean mass observed in offspring of the lifestyle intervention group.

Overall, these results emphasize the potential of the intrauterine environment to influence the epigenome, which could subsequently affect metabolism and growth in later life.

Study III

Gestational weight gain in pregnant women with obesity is associated with cord blood DNA methylation, which partially mediates offspring anthropometrics

In Paper III, our study reveals associations between GWG in pregnant women with obesity and cord blood DNA methylation patterns linked to BMI, type 2 diabetes, and asthma. Additionally, we demonstrate correlations between GWG and important anthropometric measurements for the future health of offspring, such as lean mass and birthweight. Of note, twenty-five percent of the GWG-associated cord blood methylation sites have also been linked to mQTLs, as well as with traits such as BMI, T2D, asthma, and birthweight, in previous EWAS. Thus, these findings provide evidence that GWG in pregnant women with obesity influences the

DNA methylome in offspring cord blood and impacts anthropometric measurements of likely relevance for offspring health. Furthermore, our research demonstrates that these effects occur within genes related to metabolic disease.

This study is, to our knowledge, the first to identify associations between GWG in pregnant women with obesity and DNA methylation in cord blood using the Infinium® HumanMethylation450 BeadChip. Previous studies have been performed in normal-weight women (114-116). Morales et al. analyzed 1,505 DNA methylation sites in cord blood from 88 offspring, where they found 44 sites to associate with GWG during weeks 0-18 of pregnancy (114). However, these results could not be replicated using a larger sample size and the Infinium® HumanMethylation450 BeadChip (115). Similarly, Sharp et al. did not find any GWG-associated DNA methylation site in cord blood (116). Discrepancies in results between these studies may be due to several factors, including differences in adjusting for cell composition, and GWG measured at various time points during pregnancy (114-116).

High birthweight increases the risk of developing overweight or obesity later in life (117) and being born to a mother with obesity increases the risk of metabolic disease (118). These conditions are likely to be multifactorial, where both the intrauterine environment and genetic factors are important (119). Having a greater proportion of lean mass i.e., more metabolically active tissue, enhances insulin sensitivity and protects against cardiometabolic disease and T2D (120, 121). Children born with higher birthweights are at risk of various long-term health outcomes, including psychiatric disorders, cardiometabolic diseases, and several types of cancer (122). In this study, we show that increased GWG in women with obesity may negatively affect the offspring, leading to less lean mass at birth and a higher birthweight. Methylation sites positively associated with GWG were inversely associated with the offspring's lean mass, while methylation sites negatively associated with GWG tend to be positively associated with the offspring's lean mass. The opposite pattern is observed for birthweight.

Furthermore, several of these methylation sites appear to partially mediate the effect of GWG on offspring's lean mass and birthweight. Six methylation sites are proposed to partially mediate the effect of GWG on both lean mass and birthweight. Among them, we observe a positive association between GWG and methylation of a site residing in the gene body of *KDM1B*, which encodes a histone demethylase involved in regulating histone lysine methylation (123). A negative association was found between GWG and methylation of a site in the gene body of *WNT8B*, encoding a Wnt protein – part of the Wnt signaling pathway and implicated in several developmental processes. Additionally, a negative association is observed between GWG and methylation of a site in the 5'UTR/1st Exon of *CCN4*, encoding a downstream regulator in the Wnt/Frizzled signaling pathway.

Childhood asthma is a global health issue, and preventive strategies are needed to limit its escalating prevalence. Data suggest that childhood asthma is associated with GWG (124), having adequate GWG could, therefore, potentially reduce childhood asthma cases. Interestingly, in our data on GWG-associated cord blood DNA methylation, we found several mQTLs associated with asthma in GWAS and EWAS. Two DNA methylation sites, annotated to *WDR36* and *SIK2* were linked to nine SNPs previously associated with asthma in GWAS. Additionally, six DNA methylation sites annotated to *SLC9A3*, *RNF220*, *TMEM53*, *SLC41A3*, *GRB10*, *TMEM106A,* and *ALG14* were linked to 410 SNPs associated with asthma in EWAS.

These findings underscore the potential benefits of reducing GWG in women with obesity for the future health of their offspring. Furthermore, our results highlight the critical role of the intrauterine environment in programming the methylome, which may have implications for offspring metabolism.

Study IV

Impact of Added Sugar on the Whole Genome DNA Methylation Pattern in Human Sperm

Although there are existing studies investigating the effect of physical activity and diet on rodents and human sperm methylome (95, 125-127), to our knowledge, there are no studies investigating the effect of added sugar on the human sperm DNA methylome. Interestingly, Nätt et al. found human sperm to be sensitive to a high-sugar diet (128), with effects on tsRNA levels and sperm motility (128). Using the sperm samples from the diet intervention performed by Nätt et al. (128), we investigated the genome-wide DNA methylome which shows that one week of a sugar-rich diet does not impact the global sperm DNA methylome. The majority of samples had low DNA levels, ranging between 2.2-252.6ng and with a mean of 30.1ng. To make the samples comparable, ≤50ng input was used for the first step of the protocol, the fragmentation, and subsequently, all the material was used for the remaining steps. The mean sequencing depth, for all 42 samples (2 samples had been removed before library preparation due to DNA levels of <2ng, and one sample was removed during QC due to a mean sequencing depth of <1) were 10x, ranging from 1.4 to 20.5x. Due to low coverage, we decided to pull out sites with adequate sequencing depth (≥10x, in all samples) and analyze the effect of the diet on individual CpG-sites in genes linked to male fertility, imprinted genes, the top 1% expressed genes, and CpG-sites with the same genomic coordinates as know tsRNAs. From these analyses we find that the Added Sugar diet leads to changes in DNA methylation levels of sites annotated to genes linked to male fertility, imprinted genes, and genes with the 1% highest expression level in sperm. In fact, 486 individual sites annotated to 151 unique genes show altered DNA methylation

patterns (P<0.05). Although these tests did not withstand correction for multiple testing, the methylation level of several of these sites changed consistently in all individuals, indicating a genuine effect. For example, we found that Added Sugar increased DNA methylation for individual CpG-sites of *ACP1*, *AHRR*, *GNAS*, and *PTPRN2* and decreased methylation of a site in *HDAC4* in all sperm samples analyzed. *ACP1,* encoding a phosphotyrosine protein phosphatase, harbors an SNP representing about 10% of the population linked to lower spermatic concentrations and more atypical spermatozoa (129). *GNAS* is located in a region with intricate imprinted expression pattern. DNA methylation levels in sperm of the *GNAS* gene have been associated with semen abnormalities such as low sperm count and mobility (130) and have been shown to be inversely correlated with sperm concentration and have been found to be linked to follicle-stimulating hormone and luteinizing hormone levels (131). *PTPRN2* encodes a receptor-like protein tyrosine phosphatase that regulates plasma membrane phosphatidylinositol 4,5-bisphosphate levels to facilitate actin remodeling and plays a role in vesicle-mediated secretory processes. Hypermethylation and a negative correlation of expression levels of the *PRPRN2* gene have been found in individuals with low sperm concentrations (132). In this study, we have shown that the Added Sugar diet resulted in a hypomethylated pattern of *HDAC4,* encoding a histone deacetylase. Conversely, our previous research show that regular exercise leads to a hypermethylated pattern and subsequent decreased expression of *HDAC4* in adipose tissue (47). Together, this underscores the significance of lifestyle choices and their impact on the epigenetic control of this gene. These data suggest that Added Sugar may change the methylation levels of sites annotated to genes linked to male fertility, imprinted genes, and genes with the 1% highest expression level in sperm. Still, these data need to be validated in future studies.

Additionally, we provide a comprehensive picture of the DNA methylation pattern in human sperm. We merged the samples from the three diets to obtain adequate sequencing depth, resulting in 15 samples with a mean sequencing depth of 28.7x, ranging from 12.8 to 49.8x. Using these data, we found that the mean methylation level was 77.05%, which is higher than reported by Molaro et al. (~70%) (133) and lower than reported by Chen et al. (~90% in the control group) (134). We found the highest methylation levels, around 82%, in introns and the 3'UTRs and the lowest levels in CpG Islands and 5'UTRs, with approximately 8.5% and 14%, respectively. The discrepancies of results between our study and that of Molaro et al. and Chen et al. may be due to several factors, e.g., methodological in regards to library preparation, sequencing, and bioinformatic analyses, number of individuals included (15, 2, and 8, respectively), and potentially also age and ethnicity (133, 134). Furthermore, we found that exons of non-expressed genes are hypomethylated compared to expressed genes (≥2 mean normalized counts), which is in concordance with what we have found in pancreatic islets (96). Further demonstrating a link

between sperm methylation and RNA levels and the importance of epigenetic regulation of different genomic regions.

Methodological Considerations

The relationship between epigenetic modifications and observed phenotypes is complex and intertwined, with both factors often influencing each other in a bidirectional manner. Epigenetic modifications may influence the observed phenotype through their effect on gene expression and cell functions, but environmental factors may also modify epigenetic modifications through phenotypic traits. This may present a challenge to establishing causality in epigenetic studies. This can be addressed by the study design and/or by the use of specific statistical methods. All four papers try to address this challenge through the study design; in Papers I and IV, we use within-subject design, meaning participants serve as their own control; in Papers II and III, which is based on a randomized control trial, and considered the golden standard for establishing causality, we also employ causal mediation analyses in an attempt to establish causality partly due to the fact that we have meddled in the randomization.

In this thesis, we have used two different techniques for studying DNA methylation patterns: the Illumina Infinium® HumanMethylation450 BeadChip Array and WGBS, each with its advantages and limitations. Array-based methods are a cost-effective approach for assessing predetermined CpG-sites, i.e., they are limited to the CpG-sites covered by the array. WGBS allows for profiling of DNA methylation across the entire genome at single-base resolution, and identification of DMRs. WGBS is quite costly and computationally intensive compared to array-based methods and requires higher sequencing depth to get adequate coverage, particularly so for samples with low DNA input.

Control of confounding variables is a critical step for finding true associations and establishing causal relationships. In Studies II and III, we therefore considered all maternal and offspring variables that might influence the relationship between the independent and dependent variables. However, as sample size often presents challenges in EWAS, and this holds true for our studies as well, as well as reducing the risk of overfitting the model, we decided to only include variables with a $P<0.25$ in univariate analyses in the final regression models, based on the work on a purposeful selection of covariates by Bursac et al. (135).

In epigenetic studies, it is also important to consider the cell-type composition of the tissue under study. Tissues are made up of various cell types, all with their own distinct epigenetic profile. Controlling for the cell-type composition ensures that observed differences are not only due to differences in cell proportions. In most cases, the correct cell-type composition cannot be determined, but it can be

estimated using two main approaches: reference-based and reference-free methods. Reference-based methods rely on pre-existing profiles of cell types and their epigenetic signatures. These references are often based on a small sample set and, thus do not have the ability to account for the variance that may exist in a larger population. Reference-free methods infer cell-type composition by the application of advanced computational and mathematical methods, such as unsupervised learning algorithms that cluster or decompose the data.

Replication and validation steps in science are essential processes to ensure the credibility of the research results. Replicating findings, preferably in independent cohorts, will showcase the robustness of the results, help reduce the likelihood of Type I errors, and assess their generalizability. Validating epigenetic marks using alternative methods or functional assays enhances the reliability and biological relevance of the study results. In Study I, we validated our results by functional follow-up experiments in cell lines. In Study II and III, we evaluated the robustness of analyses by sensitivity analyses, and in Study III we additionally split the cohort into a discovery and validation cohort. Study IV can be considered a pilot study and as such, the results need to be validated in further studies.

EndoC-βH1 is a human pancreatic beta cell line of fetal origin with different DNA methylation patterns compared to mature beta cells (136). As such, treatments modifying DNA methylation may exhibit distinct effects in EndoC-βH1 beta cells compared to mature beta cells. However, despite their differences, the EndoC-βH1 beta cell line remains the most reliable *in vitro* model available for studying human beta cells.

Clinical Implications

Through Studies I-IV, we hope to highlight the importance of lifestyle choices in human health. How we as individuals and as a society can affect human health by making and facilitating healthy lifestyle choices. Moreover, we want to emphasize that epigenetics may serve as an important mechanism linking lifestyle choices and human health. Studies II and II, and potentially also Study IV, illustrate how lifestyle choices not only impact our own health but also seem to exert a direct effect on the health of the offspring. The findings from these studies demonstrate the individuals' ability to influence their and their offspring’s health outcomes through lifestyle choices. However, it's of the utmost importance to recognize that the responsibility for maintaining a healthy lifestyle should not solely rest on the individual. Instead, society must provide support and assistance and facilitate healthy choices. These are essential components in creating a culture of well-being and enabling individuals to follow a healthier lifestyle.

The results from Studies II and III, indicate that there are ways to reduce the negative effects of obesity during pregnancy. Increased physical activity in the form of increased step counts per day, with and without dietary advice on a low-fat, low-calorie Mediterranean diet, as well as lowering the GWG, in fact, show positive effects on the offspring. Based on these findings, it would be desirable for midwives or similar healthcare professionals to encourage pregnant women with obesity to do more physical activity and be provided with support from a dietitian to enable them to maintain a healthy diet.

Study IV indicates that paternal lifestyle choices are also important. However, in this study, we are unable to make true connections to fertility or offspring health outcomes and can merely speculate. However, in the same subjects, Nätt et al. have seen effects of the dietary intervention on sperm parameters important for fertility (128), indicating that diet influences the likelihood of conception. During development, the DNA methylation pattern in mammalian cells goes through two waves of reprogramming. The first wave occurs in the germline and the second after fertilization. Sperm promoters are bound by transcription factors (TFs) but are transcriptionally inactive (137). TF-bound sites might hence escape the second wave of DNA methylation reprogramming. The TF distribution may be affected by environmental factors, potentially serving as a mechanism for the transmission of epigenetic patterns across generations (137). In fact, DNA methylation patterns have been found to be heritable (138). It is, therefore, possible that diet-induced DNA methylation patterns are transmittable to the offspring. This could potentially encourage men to maintain a healthy lifestyle.

Strengths and Limitations

All four studies included in this thesis have strengths and limitations. Since we have discussed these quite well and in-depth in the individual articles, I will only address some general strengths and weaknesses and some personal reflections.

The methylation changes identified in these studies may be considered subtle, however, we argue that the cumulative impact of several minor changes, each contributing in a small part, could potentially have a large effect on the overall outcome.

Study I is the only *in vitro* study included in this thesis. *In vitro* studies have several inherent strengths and limitations. They are great as they can help circumvent many ethical concerns and allow for precise control over experimental conditions. However, *in vitro* models are unable to reflect the complex system of a living organism and, as such, may induce artifacts or lead to biased results.

Some of the great strengths of intervention studies in human subjects (Studies II-IV) are their direct clinical relevance and ability to find causality. Compliance and adherence, on the other hand, are major limitations of human studies. Researchers are reliant on the participants' cooperation, and we must assume that the participants comply with the study protocols. However, it's important to acknowledge that this may not always be the case. This uncertainty naturally affects the study's outcome and is something we are currently unable to fully account for.

Summary and Conclusions

Study I

We identified glucolipotoxicity-induced methylation and expression changes in human pancreatic islets. These changes seem to contribute to impaired insulin secretion and increased apoptosis. Collectively, our results support the involvement of glucolipotoxicity in the onset and progression of type 2 diabetes, highlighting its potential impact on disease development and exacerbation.

Study II

In this study, we show that a lifestyle intervention among pregnant women with obesity has an impact on the DNA methylome of offspring cord blood. Additionally, we are able to link cord blood epigenetic markers with offspring lean mass and growth. These findings underscore the importance of the intrauterine environment in programming the epigenome, potentially influencing metabolism and growth trajectories later in life.

Study III

GWG in pregnant women with obesity was found to be associated with cord blood DNA methylation of 441 sites, several of which have previously been linked to BMI, T2D, and asthma. Furthermore, GWG is associated with offspring lean mass and birthweight - anthropometric measures important for offspring health. These results show the importance of managing GWG in pregnant women with obesity to facilitate favorable health outcomes in their offspring. Moreover, our study highlights the influence of the intrauterine environment on the methylome, potentially affecting metabolic profiles in the offspring.

Study IV

While our findings indicate that a one-week sugar-rich diet does not significantly alter the overall sperm methylome, there is nominal evidence suggesting potential changes in the methylation levels of specific CpG sites associated with male fertility and imprinted genes after consuming a diet rich in added sugar. This study also offers a thorough exploration of the global DNA methylome in human sperm, highlighting their correlation with gene expression and the significance of epigenetic control across various genomic regions.

In conclusion, these data support the notion that DNA methylation is modifiable by our lifestyle choices, potentially affecting the metabolism and the risk of developing metabolic disease.

Future Perspectives

We are moving towards an era of personalized treatments, where healthcare is more individualized with diagnosis and treatments being more effective. This is possible in part due to biomarker discovery, where, e.g., an individual's genetic makeup and protein levels are considered when selecting treatments. This will allow for the best possible drug effect and minimize the adverse effects of the treatment. Epigenetics also poses as a potential biomarker for treatment response and tolerance, as has been shown by García-Calzón et al. regarding metformin - the first-line drug therapy in type 2 diabetes patients (139). Additionally, epigenetic editing is a promising approach in developing novel treatments for many diseases with aberrant gene expression. However, optimizing epigenetic editing technologies' efficiency, specificity, and safety is needed before implementation in patient care is possible.

Using digital health solutions makes health care more accessible, both in terms of geographical location and the effort it requires. It may enable more frequent follow-up care due to its convenience. Today, there are multiple remote monitoring devices and even more mobile health apps, making it possible for individuals to track their health metrics in real-time. This could allow healthcare providers to step in in a timely manner and provide advice on necessary or enhancing treatment adjustments, potentially improving adherence to treatment regimens.

Adherence to lifestyle interventions can be measured to some extent by biomarker and biochemical analyses. For example, it is possible to measure lipid profiles and glucose levels in blood, and in urine or hair, it is possible to attain information about nutrient intake which may provide the researcher with information regarding compliance with dietary recommendations. Here, epigenetics may serve as biomarkers one day, as we have seen effects in several different tissues based on lifestyle factors such as diet and exercise (38, 40, 41, 47-49, 69, 91). Although these methods are not widely used for these purposes today, they may become more common as costs decrease and the reliability of the analysis increases. This would allow for more reliable information regarding adherence and, thus also, causality within lifestyle interventions.

As mentioned in the discussion of Study IV, the diet intervention analyzed in the human sperm was short, only two weeks, of which only one week was with excess sugar. The process of human spermatogenesis is, however, estimated to be much longer, in fact, up to 2.5 months (140). Due to the importance of proper DNA

methylation in sperm, it is possible that the DNA methylation pattern already formed in sperm will not be affected by a change in diet. Therefore, it would be of interest to perform a diet intervention extending over the lifespan of the sperm. This would ensure all sperms have formed during the intervention and would potentially allow for an effect on the overall DNA methylome. However, this would probably be difficult to carry out in humans from an ethical perspective. A 50% excess of sugar for such a long period would lead to quite a substantial weight gain in the participants increasing their risk of, e.g., T2D and CVD.

Furthermore, it would be of high interest to perform lifestyle intervention studies with multi-omic levels, i.e., combining several omics layers such as epigenomics, transcriptomics, and proteomics, together with deep phenotype data. Preferably in several different tissues to be able to see the effect of the lifestyle in different parts of the body but also to analyze what tissues mirror or are in discordance with each other. This could allow for unraveling complex biological networks and examining the intricate interplay between these layers. Additionally, it would generate and deepen our knowledge about how our lifestyle choices affect our health and the risk of developing metabolic diseases, such as obesity and T2D. Enhanced understanding of how our lifestyle impacts health can potentially help motivate individuals at high risk of disease to adopt healthier habits. Furthermore, it may allow for identifying molecular and cellular changes that can be targeted for precision medicine for the development of more effective prevention strategies and treatments for metabolic diseases.

In addition, I hope that in the future, we, as a research community, will be better at including the sex chromosomes in DNA methylation analyses. Evidence shows marked differences in the DNA methylation pattern in different tissues between men and women and studies in animal models suggest that these differences are due to the sex chromosomes and not the hormonal differences between males and females (141).

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