NATIONAL CENTER FOR CASE STUDY TEACHING IN SCIENCE

The Dutch Hunger Winter:

Epigenetic Effects on Metabolic and Heart Health

by

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Part I – The Famine

Henkie was hungry. In fact, this 9-year-old had been hungry for several months. He would bring a spoon with him wherever he went just in case there was soup or any food scraps to be found.*

This was not supposed to happen in the Netherlands, one of the most affluent countries in the world, but it did. From December 1944 to May 1945, the country experienced the worst famine in its history under the occupation of Nazi Germany. It was triggered by the Dutch railroad workers' strike called by the exiled Dutch government to help the Allied Forces stop the transportation of Nazi troops.

Unfortunately, the strategy backfired, and Germany retaliated with an embargo that cut off food and fuel delivery to many densely populated regions in the western parts of the Netherlands, including large cities such as Amsterdam, Rotterdam, and The Hague. In this five-month period, people survived on as low as 30% of the normal calories a day at the worst time. This famine, known as the Dutch Hunger Winter, killed an estimated 20,000 people and affected 4 million others. In May 1945, it ended just as abruptly as it began, with the support of air drops by the Allied Forces, including the United Kingdom, Canada, the United States, and Sweden.

This rare but well documented event (including the amount of food ration per person, births, deaths, hospital records, etc.) occurring in a modern and developed country, became a natural case study on how famine influenced human health. Prior to the famine, the nutritional supplies for the entire country, although under German occupation, were considered adequate. The beginning and the ending date of the blockade were clearly recorded. Of those impacted, it was particularly noted that women who were pregnant during the famine gave birth to children who later became more susceptible to diabetes, cardiovascular diseases, and mental health problems.

Throughout human history and across various cultures, the observation has been made that maternal undernutrition may lead to unhealthy children. This observation has been confirmed in lab animal studies, but human studies are difficult to carry out due to ethical considerations. Human studies of this question are also hindered by the presence of other factors mixed in with the nutritional conditions such as education, social economic status, genetics, diseases, and other environmental conditions that cannot be teased apart. The Dutch Hunger Winter was a rare historical event that may be useful for this type of study.

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* Henkie Holvast was just one of many children in Amsterdam who endured the Duth Hunger Winter. Photos of Henkie can be found online by photographer Martinus Meijboom, but cannot be reproduced here due to copyright restrictions.

Questions

1. What makes the Dutch Winter Famine a suitable event for studying the impact of health in children born to mothers who experienced food shortages during pregnancy? (Consider what made this study more feasible to control *confounding variables*, which are variables that are an intrinsic part of the study subjects such as race, sex, social economic class, etc., and are often difficult to control.)

2. What is diabetes? Two common types of diabetes are type I and type II diabetes. What are the major differences between the two?

Years after the famine, many studies have shown that individuals who were conceived during the famine seemed to be more prone to disorders in sugar metabolism and cardiovascular diseases. Table 1 shows the results from one of those studies on individuals who were either exposed or not exposed prenatally to the Dutch famine (Roseboom *et al.*, 2001). In that paper, all individuals who were diagnosed with diabetes were excluded from the study. The categories of exposure during gestation defined by the authors were based on the following criteria:

- Exposed to famine during late gestation: born between January 7 and April 28, 1945.
- Exposed to famine during mid gestation: born between April 29 and August 18, 1945.
- Exposed to famine during early gestation: born between August 19 and December 8, 1945.
- Not exposed (born before famine): born after November 1, 1943 and before the famine.
- Not exposed (conceived after famine): conceived after the famine and born before February 28, 1947.

Table 1. Some characteristics of mothers and their offspring affected by the Dutch Hunger Winter. (Data from Roseboom *et al.*, 2001.)

	Exposure to Famine						
Mother/Infant Characteristics	*Born Before Famine*	*Late Gestation*	*Mid Gestation*	*Early Gestation*	*Conceived After Famine*	*Mean (SD)*	*n =*
Maternal Age	29	30	28	28	28	28 (6.4)	2414
Maternal Third Trimester Weight Gain (kg)	3.2	0.0	4.9	5.7	4.3	3.5 (3.2)	1682
Infant Gestational Age (Days)	285	283	285	287	286	285 (11)	2043
Birth Weight (Grams)	3373	3133	3217	3470	3413	3346 (48)	2414

3. Discuss the characteristics of the mothers and their children among the five categories of exposure to the famine: a) What characteristics in mothers and infants are quite similar across the categories of famine exposure? b) Are there any characteristics quite different among the exposure groups? c) Were the birthweights of any exposure groups considered abnormal?

4. In Table 1, what does "n" stand for? Why is it important to mention it?

In their study, Roseboom and coauthors (2001) traced the individuals who were impacted by the Dutch famine during gestation and those who were born before or conceived after the famine and compared their glucose level, BMI (body mass index), cholesterol level and their self-assessment of health about 50 years after the famine (Table 2).

Table 2. Health characteristics at about 50 years of age in individuals with or without prenatal exposure during the Dutch famine. Types of famine exposure follow the same definition mentioned above. (Data from Roseboom *et al.*, 2001.)

	Exposure to famine						
	Born before famine	*Late gestation*	*Mid gestation*	*Early gestation*	*Conceived after famine*	*Mean (SD)*	*n =*
Glucose at 120 min. (mmol/L)	5.7	6.3	6.1	6.1	5.9	6.0 (1.4)	702
Insulin at 120 min. (pmol/L)	160	200	190	207	181	181 (2.4)	694
Cholesterol level, total (mmol/L)	6.06	5.83	5.80	6.13	6.00	5.97 (1.06)	704
HDL (mmol/L)	1.35	1.32	1.37	1.26	1.32	1.33 (1.33)	704
LDL (mmol/L)	4.05	3.87	3.81	4.26	4.02	3.99 (1.01)	704
LDL/HDL	2.91	2.82	2.69	3.26	2.94	2.90 (1.53)	704
Percentage with coronary heart disease	3.8	2.5	0.9	8.8	2.6	3.3	736
BMI	26.7	26.7	26.6	28.1	27.2	27.0 (1.2)	
Percentage with poor general health	4.5	6.4	3.7	10.3	5.3	5.5	912

5. If an elevated insulin level suggests a possible metabolic disorder, which group of individuals has such a susceptibility?

6. HDL stands for high-density lipoprotein and is a type of cholesterol commonly known as the "good cholesterol." A high level of HDL reduces the risk of heart disease. On the other hand, a high level of LDL, or low-density lipoprotein, may increase the risk of heart disease. Is there a group that is more susceptible to heart disease using this indicator alone? Is this observation consistent with the percentage with coronary heart disease?

7. A BMI in the range of 26 and 29 is considered overweight (a higher value indicates more overweight). Which group is most overweight?

8. Which group has the highest percentage of self-reported poor health?

Part II – Eukaryote Gene Structure and Control of Gene Expression

Eukaryote Gene Structure

In eukaryotes, DNA is not the only component of chromosomes. Instead, DNA interacts with specific proteins called histones. The complexes formed between DNA and histones are called chromatin. The chromatin may be classified into several categories based on the level of organization (Figure 1). In the most relaxed form, about 200 base pairs of negatively charged DNA wrap around a core of eight positively charged histone proteins, forming a structure called a nucleosome. Single histone molecules, H1, outside the histone octamer, interact with other H1 as well as the histone octamers of other nucleosomes. Between nucleosomes is a stretch of DNA, the linker DNA, which varies in length depending on the species. Nucleosomes along with linker DNA have the appearance of beads on a string.

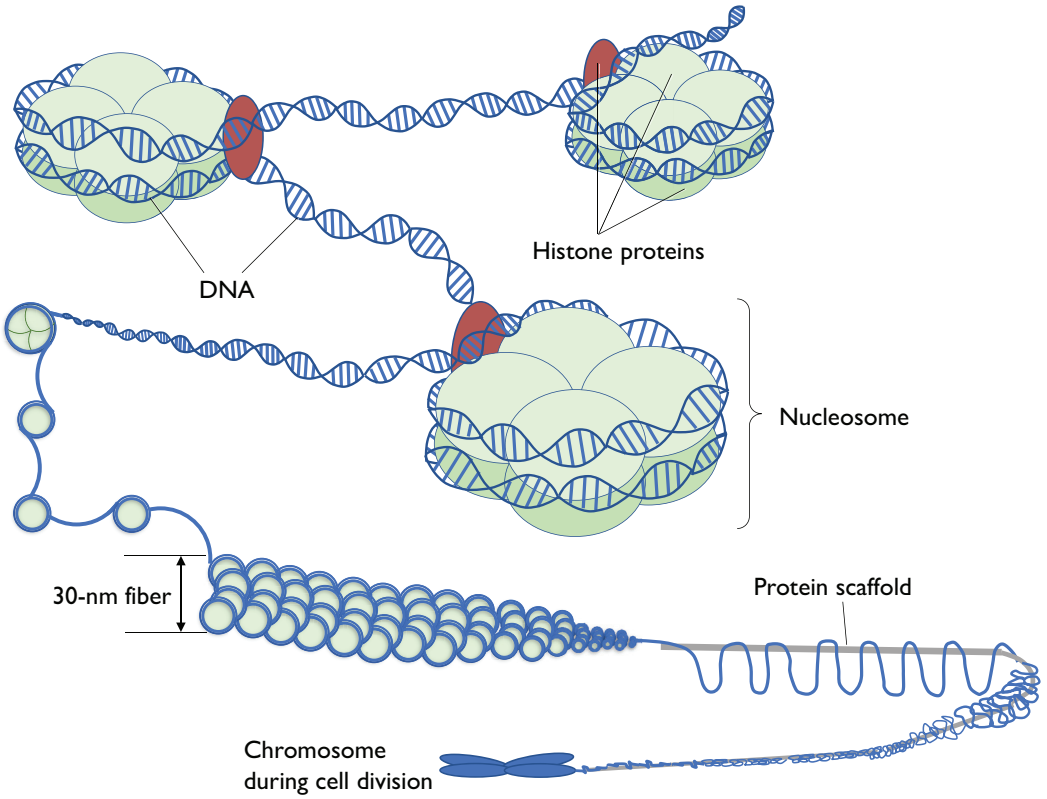

Figure 1. Different levels of DNA-histone organization. H1 histone (shown in red) is required for nucleosomes to form the 30-nm fiber. A stretch of nucleosomes with linker DNA gives the impression of beads on a string. (Image fashioned after Figure 19.2 of *Biological Science* by Freeman *et al.*, 2017).

The 30-nanometer fibers are attached to the scaffold proteins, a type of non-histone protein to hold the entire chromosome in place. Although the structure and function of scaffold proteins are not fully understood, a recent study suggests that specific proteins interact with cations such as Mg^{2+} to form chromosomes, and scaffold proteins are responsible for the chromatin fiber compaction and large-scale chromosome organization (Poonperm *et al.*, 2015). Chromatin's elaborate packaging allows DNA to fit inside the nucleus. The hierarchy of chromatin structure plays a key role in regulating gene expression. To transcribe a gene, chromatin must be decondensed to expose the promoter to allow binding of RNA polymerase.

Since RNA polymerase cannot access DNA when DNA is in a compact state, in order for genes to express, various processes that change the chromatin structure must take place to unpack DNA at particular genes so that these genes can be transcribed. The process of altering the chromatin structure so that genes can be expressed is collectively called chromatin remodeling.

Many regulatory proteins, collectively called transcription factors, are required to express a eukaryotic gene in addition to RNA polymerase. Specific transcription factors called activators bind to an enhancer, a segment of DNA serving a regulatory role in gene expression, to support the expression and often increase the expression rate of the gene (Figure 2 A and B). Other transcription factors, called repressors, bind to the silencer to inhibit the expression of the gene (Figure 3 A and B).

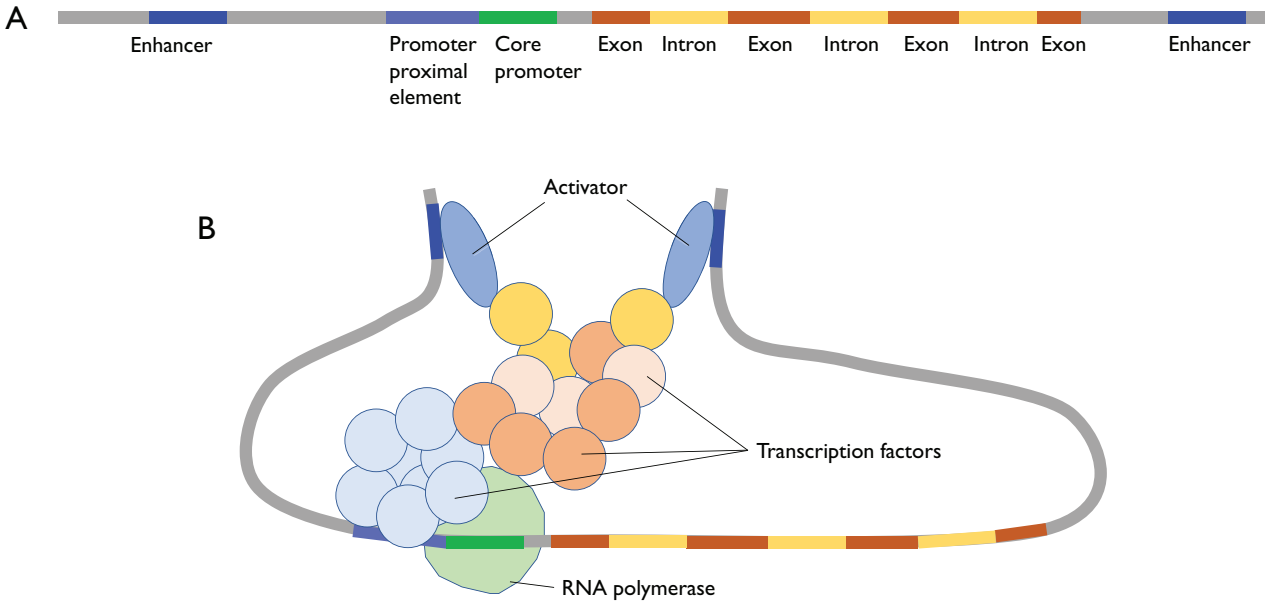

Figure 2. Control of eukaryotic gene expression through regulatory proteins binding to DNA elements around the gene sequence. (A) Schematic of a generalized gene and its regulatory sequences. (B) Binding of activators to enhancers supports efficient interaction of RNA polymerase and transcription factors to promote transcription of the gene. (Note: B not drawn to same scale as A.)

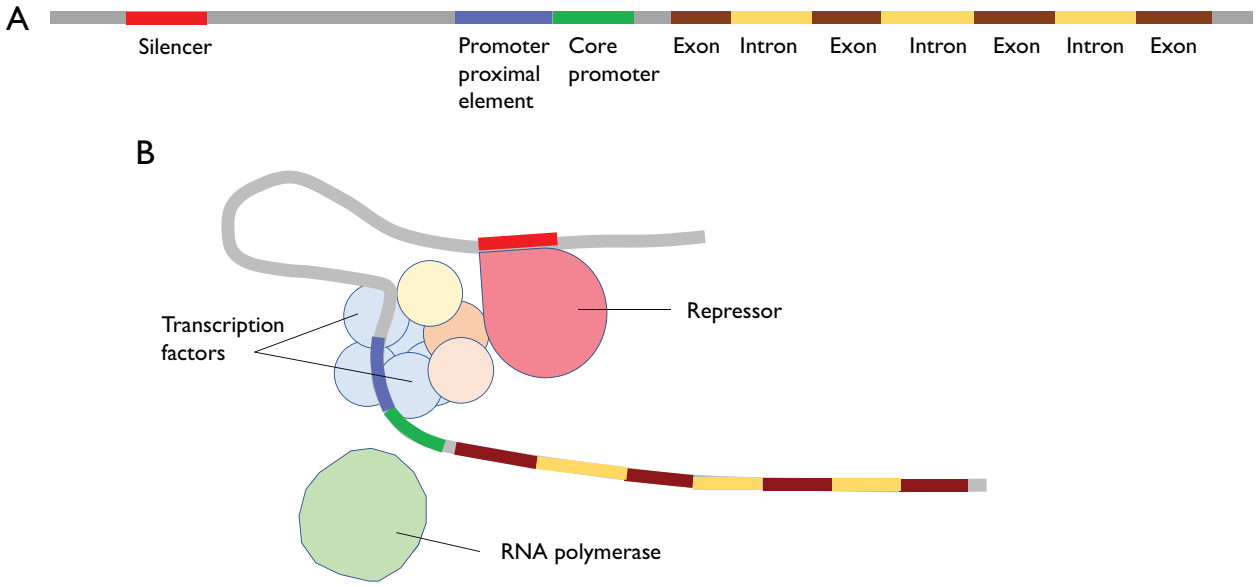

Figure 3. (A) The regulatory element silencer serves as the binding site for the repressor, a type of transcription factor. (B) Binding of the repressor protein on the silencer suppresses transcription by interfering with the assembly of general transcription factors that normally bind to the promoter proximal element and interact with the RNA polymerase. Binding of repressor to the silencer may also interfere with the binding of RNA polymerase to the core promoter. Consequently, the transcription rate of the gene is reduced or shut down.

Control of Gene Expression Through Methylation

DNA methyl transferases add methyl groups (–CH_3) to cytosines in DNA. In mammals, these enzymes recognize a C next to a G (noted as *CpG* with *p* indicating a phosphate group between the bases so as not to confuse it with C-G base pairing, Figure 4). The addition of methyl groups reduces transcription of the gene in general.

If a methyl group is added to the promoter region of a gene, it interferes physically with the binding of the RNA polymerase.

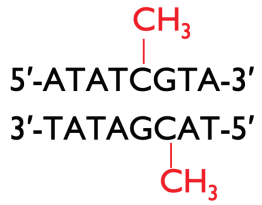

Figure 4. A methyl group is added to the cytosine next to the guanine in mammals.

Control of Gene Expression Through Acetylation

The acetyl group is a chemical group that does not carry any significant charges. However, when it binds to a specific residue on the histone protein, it reduces positive charges in histones and consequently disrupts the interaction between histones and DNA. Therefore, the addition of acetyl groups tends to decondense the chromatin and causes genes to be more actively transcribed. On the contrary, removing the acetyl groups by the action of histone deacetylase triggers the chromatin to be more condensed. This occurs because of tighter interaction between positively charged histones and negatively charged DNA. As a result, the gene is less accessible to RNA polymerase and transcription factors, leading to reduced expression (Figure 5).

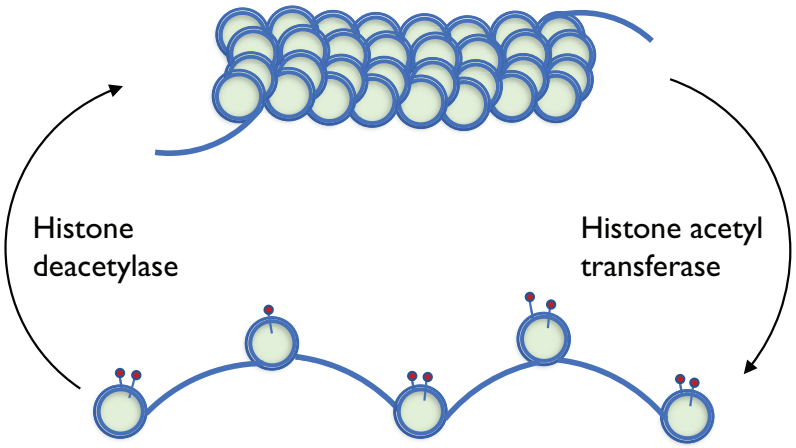

Figure 5. Control of chromatin condensation by the presence and absence of acetyl groups (–$COCH_3$, shown as the red lollipop-shaped symbols on histone molecules in light green). DNA is shown as the blue lines wrapping around the histones shown in light green.

Connection of Methylation and Acetylation on Gene Expression

When methyl groups are added to the regulatory sequences of the gene, they may recruit histone deacetylase to remove the acetyl group on the histones. This triggers the process of condensation of chromatin as a result (Figure 5), which reduces gene expression.

The Epigenetic Control of Gene Expression

The alteration in the expression of a gene using either a methyl group or an acetyl group does not involve a change in the nucleotide sequence of the DNA. Therefore, this type of changes is not considered a mutation, but rather an epigenetic change of the gene. Epigenetics impacts the expression of the existing gene so the quantity of the gene product either increases or decreases.

In mammals, the process of adding a methyl group to regulate the expression of a gene often occurs during the production of gametes, and this process is called *genomic imprinting*. In humans this type of epigenetic change occurs in a small fraction of the genes. Depending on the parental origin, certain genes are imprinted in the egg while other genes are imprinted in the sperm during gametogenesis. The consequence of imprinting is that only the allele from the other parental origin is able to express. After fertilization, the zygote undergoes mitosis, and the pattern of imprinting is maintained in the somatic cells and impacts the expression of that gene.

Part III – Methylation and Its Influence on the Expression of *IGF2*

Sixty years after the Dutch famine, with the advancement of molecular technology, one study (Heijmans *et al.*, 2008) looked at how undernutrition can impact individuals at the level of gene expression. The authors focused on a gene that produces a peptide hormone, the insulin-like growth factor II (IGF2) critical in the growth and development of mammalian fetuses. The gene was the focus of the study because by then, connections between methylation and expression of this gene had been observed in mice (e.g., Sasaki *et al.*, 1992). The *IGF2* gene is maternally imprinted, so often the only copy of the *IGF2* allele that is expressed is from the father. Heijmans and coauthors specifically looked at the methylation in a section of the gene called the *differential methylation region* (*IGF2* DMR). Birth records were searched from three midwife training schools in Amsterdam, Rotterdam, and Leiden; individuals conceived during or outside the famine were identified, interviewed, and their blood samples drawn. The researchers also recruited same-sex siblings of the individuals who were exposed to the famine during their prenatal stages. The term *unexposed* was applied to the control groups that included same-sex siblings who were not exposed to famine *in utero*. The exposed group was defined and divided into groups by the date of the mother's last menstrual period or the birth date of the affected individual. The definition of famine exposure on these groups is detailed below (Lumey *et al.*, 2007; Heijmans *et al.*, 2008):

- Exposed during early gestation (peri-conceptually). The date of mother's last menstrual period was between November 28, 1944 and May 15, 1945.
- Exposed during late gestation, defined by a birth date between January 28 and May 30, 1945. They should have been exposed to famine *in utero* for at least 10 weeks.
- Same sex siblings to the gestationally exposed individuals, not gestationally exposed to famine. They may or may not have been born in the same institution as their affected siblings.
- The official ration during the famine was 900 kcal/person/day.

Researchers compared the methylation level at *IGF2* DMR region of these famine exposed individuals to their same-sex siblings who were not affected *in utero* by the famine. The authors specifically compared five CpG regions on the *IGF2* DMR between the siblings. Because of close distance between CpG 2 and CpG 3, the authors combined the methylation level into one category. Recall that *IGF2* is maternally imprinted through methylation so only the paternal copy is expressed. Therefore roughly 50% of *IGF2* DMR should be methylated.

Table 3. IGF2 DMR methylation among individuals periconceptually exposed (see definition above) to famine and their unexposed (*in utero*) same-sex siblings.

IGF2 DMR methylation	*Mean methylation fraction (SD)*				*Relative change exposed*	*Difference in SDs*	*p*
	Exposed (n=60)		*Controls (n=60)*				
Average	0.488	(0.047)	0.515	(0.055)	–5.2%	–0.48	5.9×10^{-5}
CpG 1	0.436	(0.037)	0.470	(0.041)	–7.2%	–0.78	1.5×10^{-4}
CpG 2 & 3	0.451	(0.033)	0.473	(0.055)	–4.7%	–0.41	8.1×10^{-3}
CpG 4	0.577	(0.114)	0.591	(0.112)	–2.3%	–0.12	0.41
CpG 5	0.491	(0.061)	0.529	(0.068)	–7.2%	–0.56	1.4×10^{-3}

Questions

Refer to Table 3 to answer the following questions.

1. Were the tested individuals conceived during the famine or were they already a few months into gestation when the famine happened?

2. How do the IGF methylation levels of exposed inviduals compare to those of the unexposed siblings?

In a typical statistical test, scientists set the level of significance at 0.05 (or 5%). This means that the groups of data being compared need to be different enough so that the random chance that caused the experimental group to be different from the control group is equal to or less than 5%. By this convention, if the statistical test results indicate that the *p* values are equal to or less than 5%, the differences between the groups being compared are considered significant.

3. Overall, is there a significant reduction in the *IGF2* methylation level in the individuals with early prenatal exposure to famine? Explain your answer and use the *p* value on the table to support your argument.

4. From Table 3, what is the chance that the difference in CpG 1 methylation level between the exposed individuals and their unexposed siblings is random? Is it a high value or low value? With that, do you think the difference in the methylation level is real or random?

5. Do the data indicate more or less IGF2 protein is produced in the famine exposed individuals?

Heijmans and coauthors (2008) also looked at the *IGF2* gene methylation level in individuals who were exposed to famine during late gestation. Table 4 shows the results of methylation between individuals with late gestation exposure and their same-sex siblings who did not experience prenatal exposure to famine.

Table 4. *IGF2* DMR methylation among individuals exposed to famine late in gestation and their unexposed same-sex siblings.

	Mean methylation fraction (SD)						
IGF2 DMR methylation	*Exposed (n=62)*		*Controls (n=62)*		*Relative change exposed*	*Difference in SDs*	*p*
Average	0.514	(0.045)	0.519	(0.036)	–0.9%	–0.12	0.64
CpG 1	0.460	(0.044)	0.464	(0.048)	–0.9%	–0.09	0.68
CpG 2 & 3	0.462	(0.039)	0.471	(0.039)	–1.7%	–0.21	0.46
CpG 4	0.602	(0.085)	0.612	(0.073)	–1.5%	–0.12	0.30
CpG 5	0.529	(0.060)	0.531	(0.060)	–0.3%	–0.02	0.77

6. Although there seems to be a slight reduction in methylation at all DMR sites in individuals who were exposed to famine during late prenatal stages, were the differences significant? How can you tell?

7. Based on these data what do you expect the expression level of the *IGF2* gene in the participants who were exposed to the famine in late prenatal stages compared to their unexposed siblings?

Heijmans *et al.* (2008) calculated the difference in the methylation level in the periconceptionally exposed individual and his/her same-sex sibling and obtained the value from each of the sibling pairs and plotted them as red dots in Figure 6. For each dot (difference in the methylation level), the mother's last menstrual date was recorded for the famine exposed sibling as well.

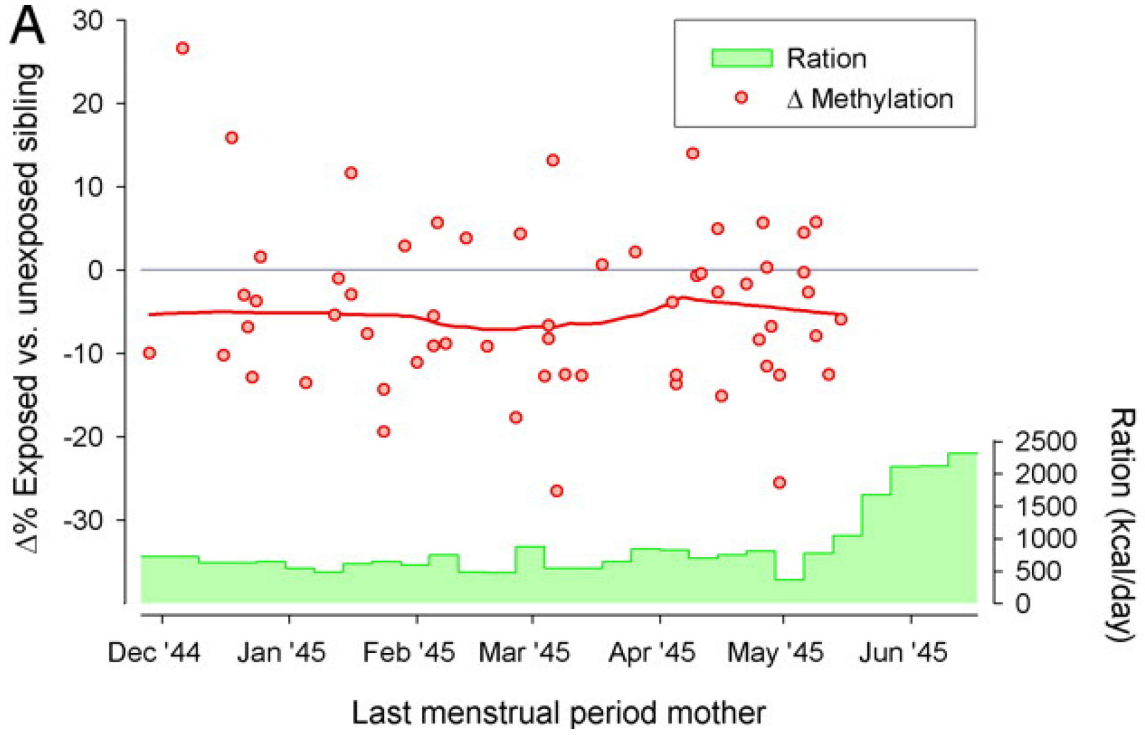

Figure 6. Periconception exposure: difference in methylation on *IGF2* DMR in famine-exposed children according to mother's last menstrual period. Unexposed siblings are shown in straight grey line. Each red dot represents percentage difference (Δ) in the methylation level in the exposed individual to his/her same-sex, unexposed sibling with the scale on the left (n = 60). Red drawn line is locally weighted scatter plot to help visualize the difference in the methylation level. The green area describes the official food ration in kcal per person per day averaged per week with the scale on the right. (Figure from Heijmans *et al.*, 2008, © National Academy of Sciences.)

Questions

Refer to Figure 6 to answer the following questions.

8. What is the purpose of using the last menstrual period of the mother indicated in Figure 6?

9. If the normal daily caloric value is about 2000 kcal for women and 2500 kcal for men, was the entire span of time (from December 1944 to the middle of May 1945) a famine? (*Hint:* take a look at the rations provided to people.)

10. Is there a difference in *IGF* methylation levels between exposed and unexposed siblings?

11. Are the red dots processed data or raw data?

12. Compare Figure 6 to Table 3. What are the advantages and disadvantages of describing data using this graph compared to a table format?

Similarly, Heijmans *et al.* (2008) calculated the difference in the methylation level in the late-gestationally exposed individual and his/her same-sex sibling and obtained values in relation to the exposed sibling's date of birth. The results are plotted in Figure 7.

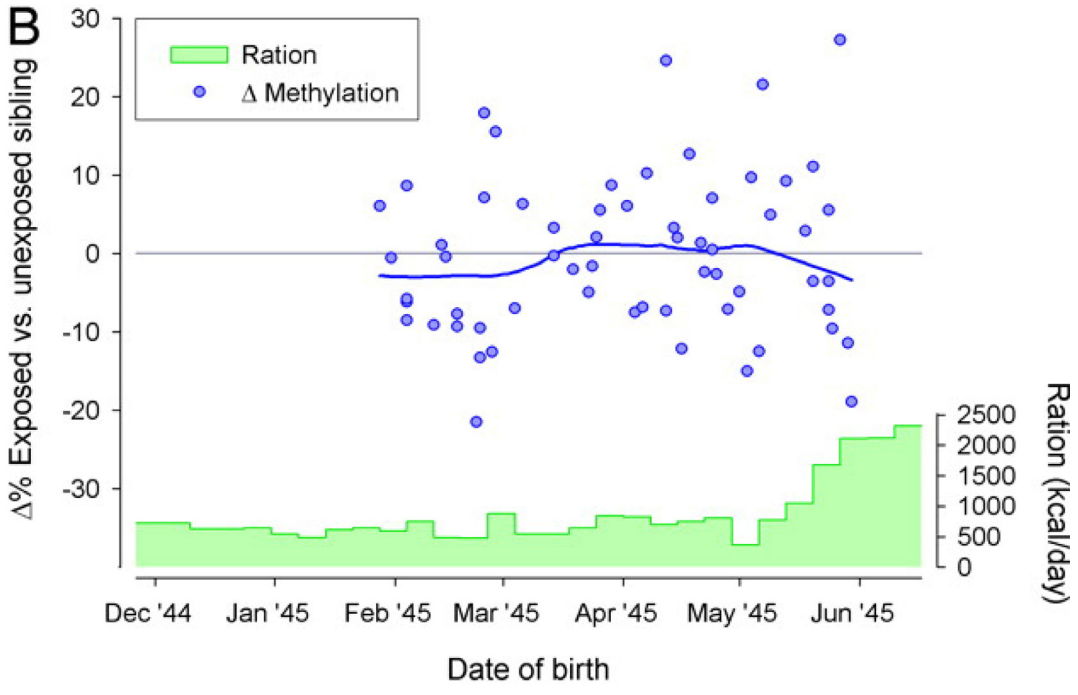

Figure 7. Exposure in late gestation: difference in methylation according to the date of birth of the famine-exposed individuals to their same-sex siblings who were not exposed during gestation. The blue drawn line describes the difference in methylation level (n=62) between the experimental group and the control group that is shown in gray. (Figure from Heijmans *et al.*, 2008, © National Academy of Sciences.)

Questions

Refer to Figure 7 to answer the following questions.

13. Assuming full term pregnancy is 9.5 months, about when were the study participants born in February 1945 conceived? How many months into gestation were these participants when the famine started? Similarly, when were those born in June 1945 conceived and how many months into gestation were these participants when the famine started?

14. Is there a difference in the *IGF2* methylation levels between those who were exposed to famine several months into gestation and their siblings who were not exposed to famine *in utero?*

15. In comparing Figure 7 to Figure 6, what conclusion can you draw with regard to the reduction of methylation at *IGF2* DMR sites?

16. Do the observations above indicate that a higher level of IGF2 hormone was a direct cause of poor health in individuals who were exposed to famine during early prenatal stages or is it more like a correlation (i.e., a connection between poor health and higher IGF hormone)? What evidence do you need or what experiment can you design to find support for a direct causal relationship between high IGF2 and poor health?

Part IV – Impact of Excess IGF2 Hormone to Lipid Production in Mice

Because of the complexity and the ethical concerns of conducting human trials to determine causes that may lead to a disease, not many studies on human health can point to a direct and clear causality. These are some of the reasons why model organisms are often used to study biological science and human health. Kessler and coauthors (2016) wanted to see if a higher level of IGF2 hormone can induce the growth of certain tissues or molecules that have health implications. They introduced extra copies of the *IGF2* gene to the liver cells of mice. Note that cells already have their original copies of *IGF2;* the introduction of extra copies of the gene could potentially increase the quantity of the gene product, the IGF2 hormone. They first measured the *IGF2* mRNA level to see if the native *IGF2* gene copies were able to express themselves (Figure 8A). They also wanted to make sure that the introduced *IGF2* gene copies were able to express (Figure 8B). They then finally observed the difference in the quantity of fat production in the liver cells and compared the results to mice liver cells that did not receive extra copies of the gene (Figure 9).

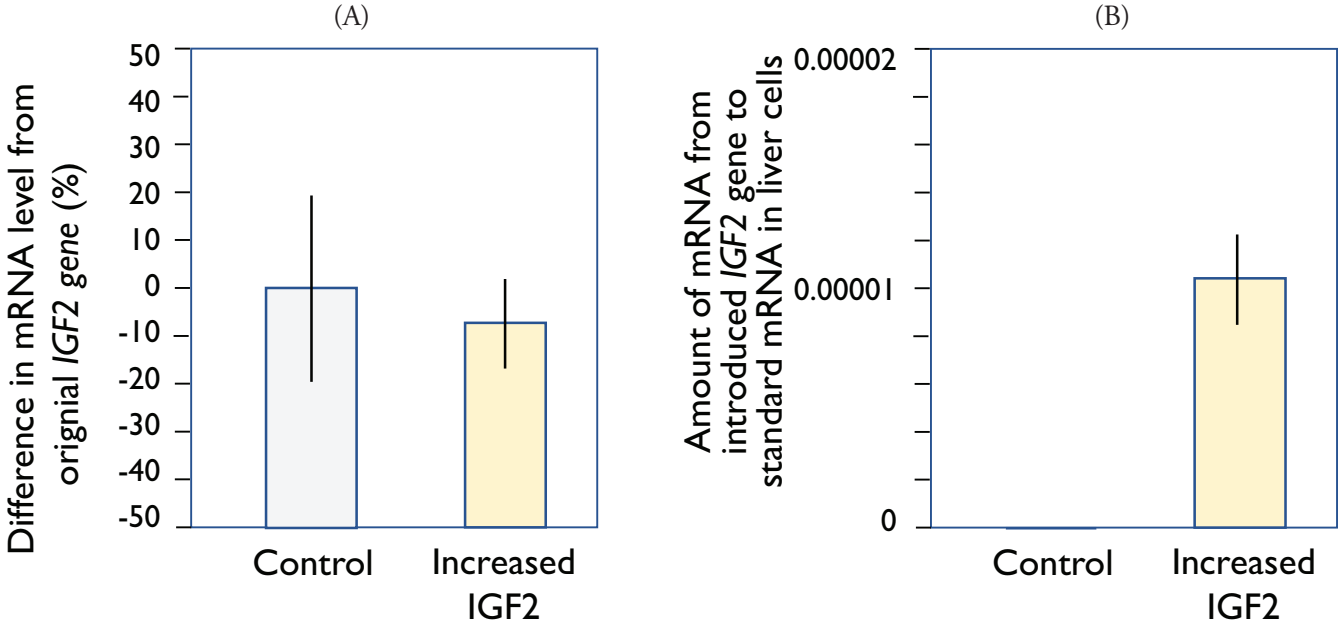

Figure 8. (A) Level of the original *IGF2* gene expression in the (control) group of mice that did not receive additional *IGF2* gene and the (increased *IGF2*) group that received additional *IGF2* gene ($p = 0.76$). (B) Amount of mRNA expressed from the additional *IGF2* gene between the control group (no additional *IGF2* gene copies) and the group that received additional *IGF2* gene ($p = 0.006$). (Figures adapted and information from Kessler *et al.*, 2016.)

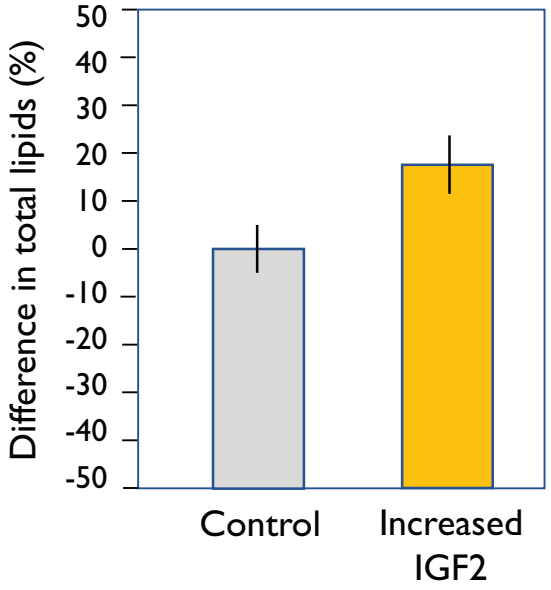

Figure 9. Amount of total lipids between mice without receiving extra copies of *IGF2* gene and the mice that received the gene in their liver cells ($p = 0.015$). The increased total lipids included cholesterol. (Figure and information adapted from Kessler *et al.*, 2016.)

Questions

Refer to Figure 8 to answer the following questions.

1. Why do you think it was necessary to measure the mRNA level of *IGF2* gene of the control group and the experimental group (results shown in Figure 8A and B)? What do the mRNA levels represent?

2. What does Figure 8A indicate? Are the results from the two groups of mice significantly different?

3. What does Figure 8B indicate? Was the difference in the expression level of the additional *IGF2* gene significant?

Questions

Refer to Figure 9 to answer the following questions.

4. Combining information from Figure 8B, what does the information in Figure 9 indicate?

5. Do the results about the lipid production and the quantity of IGF2 hormone suggest a direct causality or correlation?

Part V – Connection Between IGF2 and BMI

There is a well-established connection between obesity and type 2 diabetes, especially for individuals whose BMIs are greater than 30. It is however not very clear if IGF2 levels are connected with the BMIs. Frystyk and coauthors (1999) investigated the difference of IGF2 circulating in the blood after overnight fasting in people of different (optimal or obese) BMIs with or without type 2 diabetes. The diabetic individuals were instructed to suspend their medication overnight before the blood draw next morning.

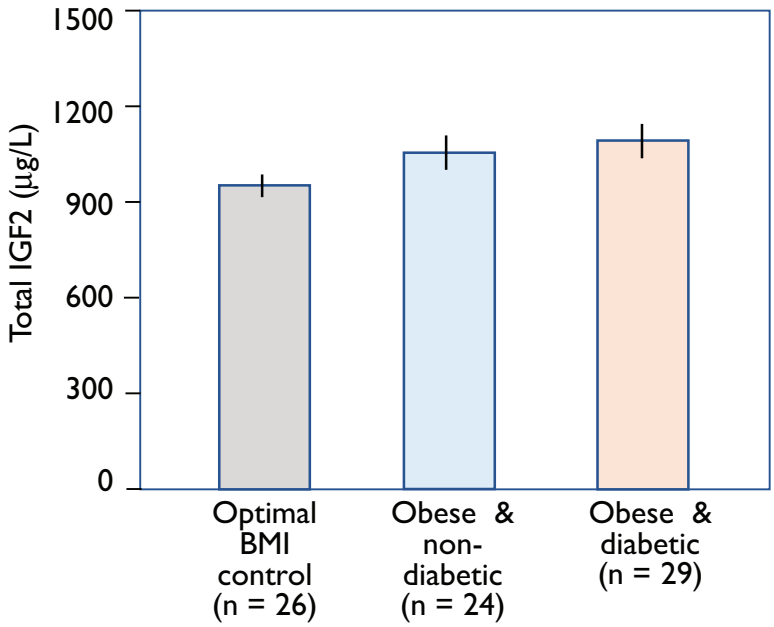

Figure 10. Fasting serum IGF2 level in three groups of participants: first group of participants with optimal BMI and healthy; participants in the second group were obese but were otherwise healthy; and the third group of participants were obese and diabetic. There is a significant difference in the IGF2 level between participants with optimal BMI and the obese groups either with ($p < 0.002$) or without diabetes ($p < 0.006$). Between the obese groups with or without diabetes there is no significant difference in their fasting IGF2 level in the serum.

Questions

1. What does Figure 10 indicate about the IGF2 hormone level in the blood among participants of different BMI and diabetic conditions?

2. Do the results in Figure 10 suggest a causality or a correlation? Explain.

Part VI – Putting It All Together

Use the information provided in previous sections to answer the following questions.

1. In general, what does methylation at specific sites of the regulatory sequences of a gene do to the expression of that gene?

2. In the *IGF2* gene, what does undernutrition do to the expression of this gene that is important to growth and metabolism? What is the overall impact to the individuals from the change of gene expressions?

3. From the case story, maternal nutrition to the metabolic health of offspring is best described as genetic, epigenetic, or phenotypic? Explain.

4. Use the following concept terms and action/linking terms to produce a concept map connecting the findings of the studies together.

   Concept terms: Early gestational exposure to undernutrition, *IGF2* gene, IGF2 protein, fat production, coronary heart diseases, metabolic health (e.g., type 2 diabetes)

   Possible action/linking terms: increase, decrease, reduce, methylation, expression of, correlates with, causes, leads to

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