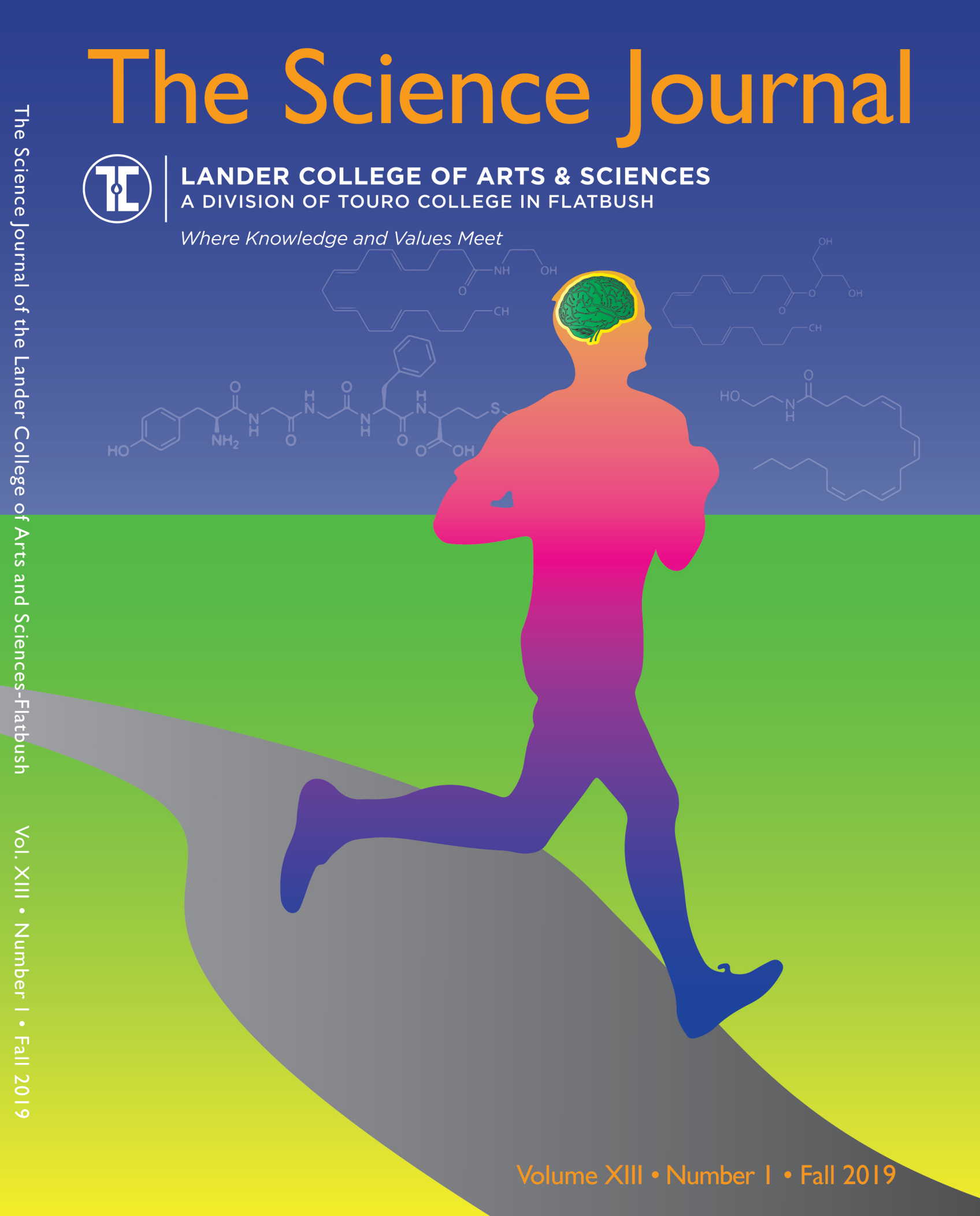
The Science Journal

LANDER COLLEGE OF ARTS & SCIENCES
A DIVISION OF TOURO COLLEGE IN FLATBUSH

Where Knowledge and Values Meet



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LANDER COLLEGE OF ARTS & SCIENCES
A DIVISION OF TOURO COLLEGE IN FLATBUSH
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The cover illustration, created by Professor Antony O'Hara of the Digital Multimedia Design, pertains to the article "Endorphins, Endocannabinoids and Runners' High" by Elisheva Winiarz.

Are Health Problems in Adulthood linked to our Experiences in the Womb? An Epigenetic Approach

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Simone Tendler graduated in January 2020 with a B.S. degree in Biology.

Abstract

The origins of adult disease have been a prime topic for research, as deciphering causes can lead to strategies for preventions and cures. There has been recent intrigue focused on the environment in the womb. Records from England and Wales in 1911 show that those who suffered from cardiovascular disease were geographically correlated with regions high in infant mortality in the past, seventy years before the study. When looking into the cause of the neonatal death rates, low birth weight, poor maternal health, and high maternal death rates during childbirth were clearly associated. Barker inferred that there is much happening in the intrauterine environment that ultimately affects our quality of life. This research helps support the concept that rather than looking at childhood adversity and socioeconomic circumstances to help explain adult disease, we might need to reach further back (Barker, 1990).

Through epigenetic mechanisms, alterations to the physical expression of our DNA can take place without changing the sequences of the actual DNA. Epigenetics is what is responsible during fetal development for the differentiation of stem cells to specific cells by adding or subtracting methyl groups to silence or activate particular genes. When an egg and sperm unite all previous methylation patterns are stripped from the newly formed diploid. However, by the time it becomes a blastocyst, new patterns have already formed. It is during this crucial stage of development that new patterns and changes to our epigenome our founded and passed down (Powledge, 2009).

The purpose of this paper is to determine if chronic health issues in adulthood have their roots in the environment and changes experienced by the fetus in the womb. Environmental exposures like nutrition, stress, and toxicants are evaluated and tested for their potential hand in setting the course for adult disease. Many studies show correlation between malnutrition and the development of metabolic diseases like obesity, type II diabetes, and even hypertension. Likewise, stress during gestation has been linked to anxiety, depression, and posttraumatic stress disorder (PTSD) in offspring. Toxicants like bisphenol-A are the likely culprit for genetically expressed abnormalities that range from cancers of the reproductive system to attention/deficit hyperactive disorder (ADHD) in exposed offspring. The "how" and the "why" are explored in this paper. If we can better understand the origins of adult disease then we are better equipped to defend our future generations against it. Furthermore, the notion that our DNA is not at fault for these outcomes, but rather epigenetic adjustments written on top of our DNA, provides hope that just as easily as it can be added on, we can take it off (Powledge, 2009).

Introduction: What is Epigenetics?

The concept that the in utero environment causes adult diseases later in life was first proposed by DJ Barker in the famous, "Barker Hypothesis," some twenty years ago, but the term, "epigenetics," was originally coined by developmental biologist Conrad Waddington in the early 1940s. Waddington labelled it as, "the interactions of genes with their environment, which brings the phenotype into being." He was referring to the phenomenon that all cells share the same DNA, yet they express complete differences in their phenotype and function. Moreover, skin cells, liver cells, and brain cells are only capable of producing more of their type within their same phenotypic expression, so whatever mechanism is accounting for the differentiation is clearly able to be inherited. These modifications usually take place on certain regions of DNA, cytosine-phosphate-guanine dinucleotides, at the carbon-5' position of cytosine. The most common mechanism seen in the fetal environment includes methylation, the adding or removing of a methyl group to and from the carbon-5' position. The usual methyl donor is S-adenosylmethionine (SAM), which the methyl transferase uses as a source of methyl to the DNA molecule. During methylation, methyl-transferase adds on the methyl group, preventing binding of transcription elements, thereby silencing gene expression (figure 1) (Odom, Taylor, 2010).

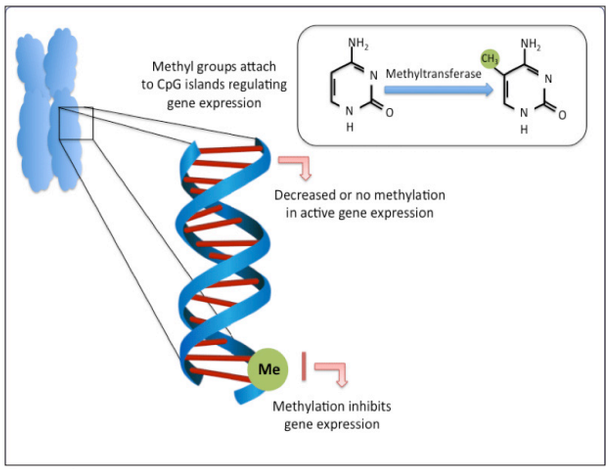

Figure 1

The silencing of genes via methylation occurs by obstruction of the binding of transcriptional machinery that needs contact with the cytosine, in the major groove of the double helix. Methylation can also function by methylating the promoter region of DNA. Methylation can sometimes lead to activation of a gene by obstructing the binding of transcription repressors, ultimately activating certain genes. Another common mechanism involves post translational histone modification, with the

addition or subtraction of different compounds like methyl, acetate, or phosphate groups to histone proteins, causing changes in chromatin packaging. The adding on of these compounds to the four pairs of proteins that make up the "octameric histone core…restricts physical access of nuclear factors to the DNA and alters gene expression" (Odom, Taylor, 2010).

Methylation happens at very critical times in development. During gametogenesis, haploid cells demethylate from the development of primordial germ cells until the period before preimplantation, to return the cell to its pluripotent state. The pluripotency refers to the cells ability to take the shape and responsibility of any cell type in the body. After implantation of the embryo, remethylation begins to differentiate each cell, forming cell specific DNA methylation patterns (Odom, Taylor, 2010).

Methylation and histone modification are seen as adaptive responses. In other words, it is a mechanism to protect the fetus from the volatile environment in which it is developing. For example, in a situation of food shortage, metabolic adaptations are made to increase the energy storage. Problems arise, however, when the adult environment does not match the environment in the womb. When the fetus is subsequently born into a nutrient-rich environment, suddenly its metabolic adaptations can lead it on a path of obesity and all its associated medical complications. (Odom, Taylor, 2010) It is with this idea that we construct a hypothesis that links adult chronic disease to in utero environmental exposures.

Methods

This comprehensive review is based on critical analysis of literature on epigenetics obtained using the Touro College Online library and PubMed publishing, analyzing the information and opinions that were expressed in various experiments. Review articles were useful in providing additional references to source material.

Discussion: Nutrition

Malnutrition

Nutrition is extremely significant when looking at studies to link fetal environment to adult disease. The majority that is known on the epidemiology of epigenetics comes from animal studies, yet the Dutch Famine of 1944-1945 affords a unique opportunity to study the effects of malnutrition on a human population. The period of the famine was clearly defined, the food rations were documented, and registries and health care documentation remained intact. (Heijmans et al., 2008)

Insulin-like growth factor II (IGF-2) is one of the most epigenetically controlled loci. IGF-2 is integral for human growth and development, is known to play a significant role in "metabolic regulation of glucose homeostasis, cardiovascular functions, and lipid metabolism,"(Rijlaarsdam et al., 2016), and is maternally imprinted. This means that while both alleles are inherited, the maternal allele is silenced via methylation. If hypomethylation, the decrease of methylation via subtracting of methyl groups, takes place in these regions, bi-allelic expression of IGF-2 occurs, leading to detrimental outcomes in adult health. (Heijmans et al., 2008)

A previous study involving 372 twins shows that the methylation of IGF-2 largely depends on genetic factors and has methylation marks that are stable at least up until middle age (Heijmans et al., 2008). Therefore, any alterations from the genetic disposition in the methylation patterns done during gestation would be able to be detected many years later. The many different diseases that have been linked to the Dutch Famine are thought to be mediated by abnormal methylation of IGF-2.

A study was done to test methylation patterns of children conceived during The Dutch Famine in comparison to their same sex siblings. The study was careful to segregate those that experienced famine in the periconceptional period and those that experienced famine during late gestation. Blood was drawn from the 122 subjects and their same sex siblings, treated with bisulfite to segregate methylated regions of IGF-2, and then amplified using polymerase chain reaction (PCR). The region contained five CpG islands, areas that are rich in cytosine-phosphate-guanine dinucleotides. In the 60 adults whose mothers experience the famine in early gestation, all but one island showed hypomethylation of between 5-6%. Interestingly, those that were exposed to the famine during late gestation did not have differing methylation patterns than their siblings, suggesting that methylation pattern of the IGF-2 allele are susceptible to environmental influences at a critical window in early development (figure 2). Those exposed during late gestation did exhibit lower birth weight than the other groups, a birth feature that was previously associated with adult disease. This study shows that while low birth weight might be indicative of malnutrition, it is epigenetic modifications that are thought to hold responsibility for deteriorating adult health. (Heijmans et al., 2008)

Coronary artery disease (CAD) is strongly linked to fetuses exposed to the Dutch Famine. Developing organ systems respond negatively to a lack of nutrients available, especially during periods of critical development. Using a registrar of 2,414 infants from the Dutch Famine Birth Cohort born between the dates of November 1, 1943 and February 28,1947, Painter et al. extracted a group of 975 participants that experienced a caloric intake under 1,000 calories for at least 13 weeks of gestation during the famine. Various medical information was collected from participants at age 50 and 58, including blood pressure, glucose tolerance test results, total cholesterol levels, and electrocardiogram information. (Painter et al., 2006)

By the end of this study, 83 candidates developed coronary artery disease, with the highest cumulative incidence of coronary artery disease at 13% seen in those exposed during early gestation. Interestingly, it was those born in mid and late gestation that were born with the lowest birth weights with mothers

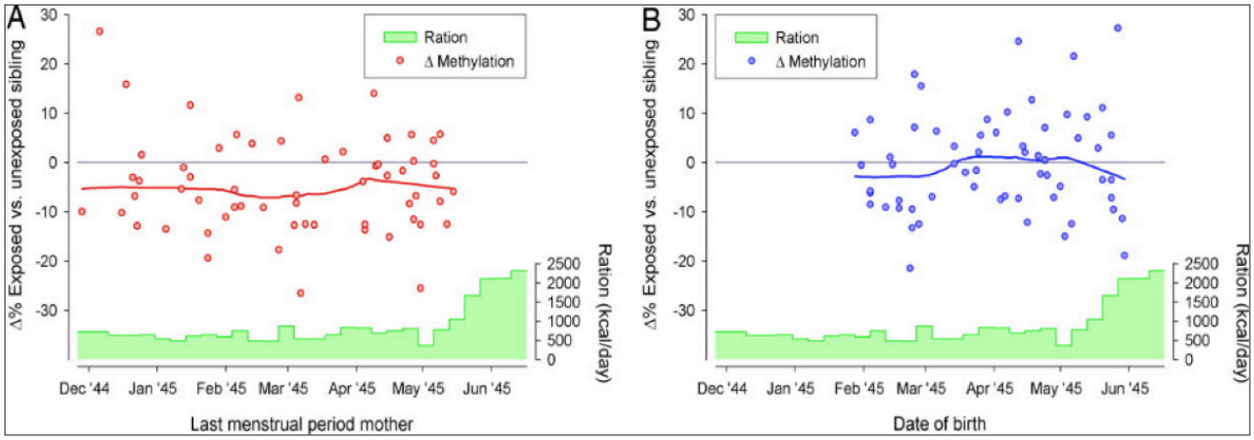

Figure 2, showing different methylation patterns between those exposed periconceptionally and those exposed in late gestation (Heijmans et al. 2007)

that weighed the least. Yet, those that developed coronary artery disease after exposure in mid to late gestation did not show a significant difference in cumulative incidence compared to those that were unexposed. On average, coronary artery disease manifested three years earlier in adults exposed in early gestation than in persons unexposed. (Painter et al., 2006) These results support the previous study that hypomethylation affects fetuses exposed to lack of nutrition in early gestation, thus linking hypomethylation of IGF-2 to adult disease as opposed to small birth weight. (Heijmans et al., 2007) That being said, any exposure to famine correlated with elevated glucose concentrations at 120 minutes and elevated ratios of LDL (low density lipoproteins) to HDL (high density lipoproteins). Most of the subjects were also being treated for type II diabetes and hypocholesterolemia (Painter et al 2006).

Schizophrenia is also linked to this critical window. A cohort of persons born in cities in western Netherlands during 1944-1946 were compared for their risk of schizophrenia. The study included those exposed in early gestation compared with those exposed at other points of pregnancy. A national psychiatric registry of the region was referenced to see the frequency of patients hospitalized at age 24 to 48 years in the two groups of subjects. The results attested to the fact the those conceived at the height of the famine (i.e., that were exposed in early gestation) had a statistically significant twofold increase in the risk for schizophrenia (Susser et al., 1996).

High-Sugar-High Fat Diet

High fat high sugar diets are associated with development of ADHD, conduct problems (CP), and their co-occurrence. IGF-2 may also be linked to ADHD, as it is a major modulator of placental and fetal growth, as well as brain development after birth. Previous animal studies have linked IGF-2 to developmental abnormalities in structure and/or function of the cerebellum and hippocampus, two regions that play significant roles in ADHD. As previously noted, IGF-2 is easily influenced by diet during critical periods of development (Heijmans et al., 2008). Studying a sample of cohorts of mothers and 164 children with early onset conduct problems (EOP) or low conduct problems (CP), researchers considered the link between prenatal diet, IGF-2 methylation status, current conduct problems, and risk symptoms for ADHD (Rijlaarsdam et al., 2016).

DNA methylation information was extracted from cord blood of the children from birth and peripheral blood at age seven. It was then bisulfite converted using the EZ-DNA methylation kit. A large number of probes, 139, were extracted that are mapped to IGF-2 or overlapping regions, with methylation at factor 1 the most relevant to the study. Researchers assessed the mothers' diets during pregnancy via The Food Frequency Questionnaire (FFQ), recording the maternal dietary patterns at 32 weeks of gestation and child's diet at age 3, 4.5, and 7 years of age. The FFQ recorded the frequency of consumption of particular foods with higher frequency indicated by higher scores. High scores in processed foods (chips, fried foods, pasties) and confectionery foods (chocolate bars, cakes, biscuits) indicated a prenatal and postnatal high-fat and sugar diet. At three different occasions (age 7, 10, and 13), ADHD symptoms were assessed with the Development and Well-Being Assessment (DAWBA), a semi-structured interview with open and closed questions directed toward parents about a range of symptoms seen in their children relevant to ADHD, oppositional defiant disorder (ODD), generalized anxiety disorder (GAD), conduct disorder (CD), and major depressive disorder (MDD) (Rijlaarsdam et al., 2016).

Results from this study indicate that youth with early onset persistent conduct problems (n= 83) exhibited higher levels of ADHD symptoms than youth with low conduct problems (n=81). The study also found that early onset persistent conduct problems youth showed high factor 1 methylation at birth of IGF-2 with symptoms of ADHD, but at age 7, methylation levels were negatively correlated with postnatal cumulative risk, with risk domains including life events such as relative's death, contextual risk like financial problems, or direct victimization like

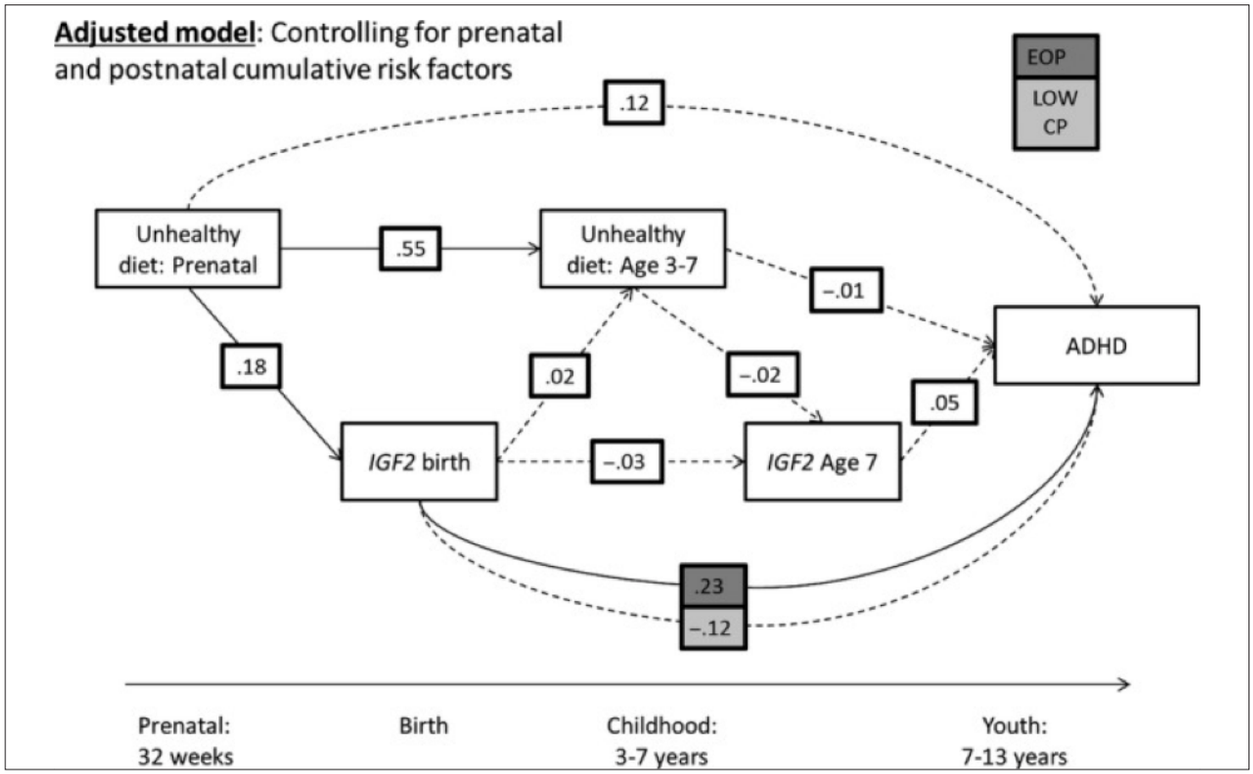

Figure 3, "Prospective interrelationships between unhealthy diet, IGF2 methylation and ADHD for youth with early-onset persistent (n = 83) versus low (n = 81) conduct problems. Multiple group path analysis. Solid arrowed lines indicate standardized path coefficients that survived bootstrap-corrected confidence intervals (i.e. significant paths) for EOP versus low CP youth or averaged across all youth" (Rijlaarsdam et al., 2016).

bullying. This can be seen as further proof that the prenatal environment is the true origin for postnatal disease development, as opposed to what the child experiences after birth. IGF-2 methylation did not correlate with ODD, GAD, or MDD symptoms (Rijlaarsdam et al., 2016).

Cross-lagged associations showed that prenatal diet was associated with IGF-2 methylation at birth for both early onset persistent conduct problems and low conduct problems. However, associations between methylation at birth and ADHD symptoms at ages 7-13 was significantly higher in early onset problem youth than in low conduct problem youth, even though the maternal diet was significant in both domains (figure 3). In early onset persistent conduct problems youth, high methylation did predict ADHD symptoms, linked through the epigenetic modifications that were caused from unhealthy eating habits. It is possible that this vulnerable developmental pathway presents a specific risk factor for EOP children only (Rijlaarsdam, et al., 2016).

Methyl-Supplements

Methylation, the addition of methyl groups to cytosine-phosphate-guanine dinucleotides or to histone proteins, is what causes much of the gene silencing that takes place during fetal programming and early development. For methylation to happen, methyl donors like the B vitamins are vital in our diets. Methylation frequently occurs at transposable elements, DNA sequences that are unique in their ability to "jump" positions on the genome. At first glance, they seem rather parasitic, the source of genetic diseases and mutations by a series of rearrangements and recombination. However, mobile DNA has recently been discovered to have an important role in biodiversity and evolution. A concept known as "molecular domestication" claims that open reading frames, complete RNA, and coding exons are thought to have originated from transposed DNA (Bohne et al., 2008). When methylation patterns differ on transposable elements, they account for epigenetic "mosaicism" and varying phenotypes. They are likely the cause of differential gene expression in identical twins. These regions are also metastable and therefore, very susceptible to nutritional influences (Waterland and Jirtle, 2013).

To analyze the effect methyl donors might have on the developing epigenome, yellow agouti mice were used. The murine agouti gene encodes for a paracrine signaling molecule that instructs follicular melanocytes to switch from black eumelanin production to yellow phaeomelanin, resulting in yellow fur. This is known as the agouti A allele with the pseudoexon 1A pointing away from the agouti allele. The agouti "a" allele is caused from

a loss-of function mutation in A, causing homozygotic "a" allele mice to be black. In contrast, the Avy allele is caused by the insertion of an intracisternal A particle (IAP) retrotransposon to the 5' end of the A allele, within the agouti exon 1A. This ectopic gene is transcribed from a cryptic promoter in the proximal end of the intracisternal A particle (figure 4). The CpG methylation of this region varies largely among different mice, inversely correlated with ectopic agouti expression. This variability is also responsible for a wide variation of hair color, adiposity, glucose tolerance, and tumor susceptibility in Avy/a mice that are otherwise isogenic (Waterland and Jirtle, 2003).

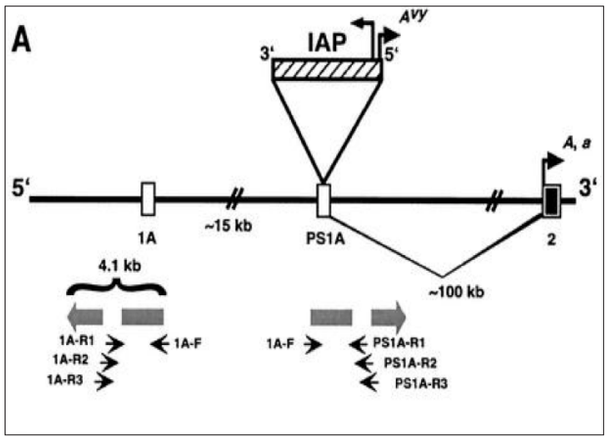

Figure 4, "IAP insertion site in Avy allele. (A) Exon 1A of the murine agouti gene lies within an interrupted 4.1-kb inverted duplication (shaded block arrows). The duplication gave rise to pseudoexon 1A (PS1A)." (Waterland and Jirtle et al., 2003)

If a/a dams are given specific dietary supplementation before mating with Avy mice, their Avy/a offspring exhibit a shift in coat color from yellow to pseudoagouti brown. Because the coat color correlates with Avy methylation status, it is inferred that supplementation influences this phenotypic shift via methylation of the Avy gene. To test this hypothesis, a population of congenic Avy mice that had been sibling mated for forced heterozygosity for 200 generations were prepared. In another group, a/a female mice that were 8 weeks old were randomly assigned to either NIH-31 diets or NIH-31 diets supplemented with methyl donors and cofactors like folic acid, vitamin B12, choline chloride, and anhydrous betaine. The diet was issued for two weeks before mating with the Avy mice, as well as throughout pregnancy and lactation. At age 21 days, the Avy/a offspring were weighed, tail tipped, photographed, and evaluated for coat color of yellow, slightly mottled (a bit of brown mixed in with yellow), mottled (half yellow, half brown), heavily mottled (greater than half is brown), or pseudoagouti. Pseudoagouti refers to the silencing of ectopic agouti expression, causing the brown agouti phenotype of an A/- mouse (Waterland and Jirtle, 2003).

The supplemented dams did in fact express the brown color of pseudoagouti phenotype. Using tail tip DNA, researcher quantified the result from seven different CpG sites of the region, showing methylation at each site, whereas the non-supplemented dams seemed to have methylation distributed bimodally with the epigenetic switch in one of two different states. This observation further proves the notion that methylation of Avy is the mediator of the affects of nutrition on coat color (figure 5). Furthermore, methylation patterns in the tail correlated with methylation patterns in the liver, kidney, and brain, representing the early embryo layers of endoderm, mesoderm, and ectoderm, respectively. This continued to show that such methylation patterns were created at the earliest stages of embryo development and were maintained throughout development (Waterland and Jirtle, 2003).

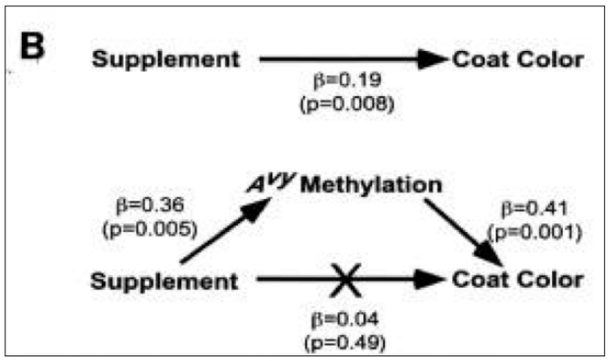

Figure 5 shows that once adding methylation to the model, no correlation was significant between supplement and color alone. Methylation was the clear mediator.

Forty percent of the human genome is made of transposable elements, with copying number exceeding double the magnitude of classical genes. Much variation can be made by simple recombination and transposition of the elements in different areas. Epigenetic modification is likely in these areas (Bohne et al., 2008). Furthermore, many human genes are also transcribed from a cryptic promoter, similar to how the ectopic agouti transcription originates from the Avy intracisternal A particle. This study provides compelling evidence that much of our own phenotypic variability and "epigenetic mosaic" can be influenced by early nutrition (Waterland and Jirtle, et al., 2003).

The restriction of methyl donors has previously been linked to neural tube defects in infants. Pregnant women are careful to take folic acid to avoid this fatal outcome. However, the fetus's epigenome is so sensitive, that even exposure like slightly improper methyl donor levels can bring about its own set of variations. Sinclair et al. experimented by restricting specific B vitamins and methionine within the normal physiological range to explore its effect on adult offspring. He administered a specific diet to adult female sheep from six days before conception until eight weeks after conception. Diets were formed containing all nutrient requirements with decreased vitamin B and sulfur amino acids, yet within the physiological range in both

sheep and human to be considered non-toxic. Because gametes and preimplantation embryos go through extensive methylation reprogramming, this experiment was used to test, "the extent to which periconceptional availability of methyl groups altered DNA methylation of gene-associated CpG islands and affected adult health status" (Sinclair, et al., 2007).

Twenty five ewes that were placed on the 'methyl deficient' (MD) diet showed homocysteine levels in follicular fluid, plasma, and in granulosa cell lysates higher than the 25 ewes that were in the control diet with no methyl deficiency. Most importantly, the incorporation of methionine into S-adenosyl methionine (SAM), as well as S-adenosyl methionine into adenosyl-homocysteine (SAH) were both reduced in ewes on the MD diet. At day 6, the blastocysts were transferred to 203 normal eating ewes. Those that became pregnant gave birth to typical weight babies on both diets, yet the growth weight to weaning for the MD babies were higher, persisting at 22 months of age, resulting in heavier MD offspring. Male offspring of the MD diet experienced an increase immune response to a single intramuscular bolus of ovalbumin both at 1 year of age and in the weeks following the vaccination. The MD males were also proportionately fatter with less muscle mass than control males. MD males also showed the greatest response to an i.v glucose infusion, showing signs of glucose resistance independent of body fat. In terms of cardiovascular function, MD males showed 11 mm Hg higher blood pressure than the controls, as well as a higher systolic, diastolic, and mean arterial pressure response to angiotensin II infusions given in increments.

To test whether these results were linked to epigenetic modification, DNA was processed to look at 1,400 CpG sites using restriction landmark genome scanning (RLGS). Methylation sensitive restriction enzyme, NotI, was used to digest the unmethylated CpG sites, resulting in spots on the autoradiograph. Fifty seven loci were found to be altered in two or more MD animals, which is more than was expected if it were to be distributed randomly between the MD group and control group. Eighty eight percent of these loci were found to be unmethylated or hypomethylated compared to the control group. The other loci appeared to be hypermethylated. Again, a majority of these alterations occurred in males (Sinclair, et al., 2007). These studies continue to support the notion that the etiology of human disease can stem from epigenetic gene regulatory mechanisms that originate early in development from simple aspects of our daily nutrition. This knowledge can be utilized as a cautionary tale for intending mothers to start preventing the conception of unhealthy adults (Waterland and Jirtle, et al., 2003).

Toxicants

Bisphenol A

Bisphenol A (BPA) is an endocrine disruptor used in the manufacturing of polycarbonate plastics found in food and drink containers, baby bottles, and dental composites. Endocrine disruptors are chemicals that mimic endocrine hormones, interfering with the homeostasis of our various organ systems. The endocrine disruptor known as BPA has previously been linked to heavier body weight, increased risk of breast or prostate cancer, and abnormal reproductive function. BPA has been detected in 95% of human urine samples, attesting to the widespread exposure of BPA in the general public (Dolinoy, et al., 2007).

BPA is most known for its effect on genetic expression of homeobox gene Hoxa10, a gene that controls uterine organogenesis. Because BPA is an endocrine disrupting chemical, it has the ability to mimic the actions of these hormones in vitro, disrupting normal development. Hoxa10 gene expression alterations have previously been linked to human cancers, as well as endometriosis in females. BPA seems to work through an epigenetic mechanism to disrupt Hoxa10. In a study by Bromer et al., pregnant mice were treated with an intraperitoneal injection of BPA in sesame oil on days 9-16 of gestation, at a dose of 5mg/kg of maternal body weight. In comparison, mice in a control group were injected with just sesame oil. Offspring were then euthanized at two or six weeks after birth. Genomic DNA was treated with sodium bisulfite with unmethylated regions converted to uracil, while methylated regions remained unchanged. Via quantitative real time PCR, it was concluded the Hoxa10 expression increased by 25% after BPA exposure, as per the mRNA expression seen in comparison to controls. Immunohistochemistry, staining using antibodies specific for a target protein, showed consistent results, as Hoxa10 protein was seen throughout the uterus of female offspring. To determine if these changes were epigenetic in nature, bisulfite conversion, PCR amplicon cloning, and sequencing were done to determine DNA methylation levels. 100% of control mice showed methylation in the promoter region while only 38% of BPA-treated mice showed any sort of methylation. CpG islands in the intronic region showed methylation in 100% of control mice and only 57% of BPA-exposed mice. In summary, demethylation or hypomethylation was observed in all regions in all BPA-treated mice. However, no methylation changes were found in the pregnant mice that were treated with the intraperitoneal dose (Bromer, et al., 2010).

Hoxa10 gene is also known to be a weakly estrogenic compound, with the ability to bind to estrogen receptor α and β. With decreased methylation, Hoxa10 has an increased ability to bind to estrogen receptors (ER) .Therefore, the estrogen receptor element (ERE) of Hoxa10 becomes more occupied by estrogen receptor α and β, causing a heightened reactivity to estrogen levels. The health implications of this were examined by testing estradiol sensitivity in MCF-7 cells. MCF-7 cells are estrogen receptor expressing breast carcinoma cells, with a well characterized estrogen response. In this study, MCF-7 cells were transfected with either methylated or unmethylated ERE

containing Hoxa10-promoter sequence. The cells with unmethylated containing sequences had significantly increased activity with the addition of estradiol, while the methylated cells showed no change in activity (figure 6). These results can explain disease risks in later life due to increased estrogen responsiveness, perhaps explaining some reproduction system abnormalities or cancers of the reproductive system. In summary, these epigenetic changes that are spurred by uterine BPA exposure can be one of the suspects of health problems in adult life (Bromer, et al., 2010).

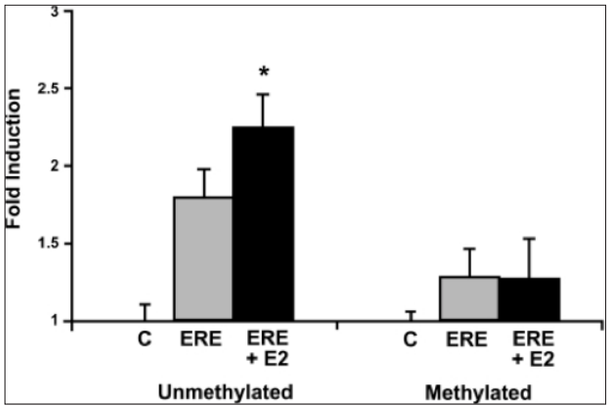

Figure 6 shows the reactivity of unmethylated Hoxa10-promoter to the addition of estradiol, while the methylated Hoxa10-promter shows a loss of response (Broner et al., 2010)

As mentioned previously, although most tissue-specific DNA methylation levels don't vary much across the mammalian genome, methylation is randomly determined at transposable element insertion sites. This can potentially affect the expression of neighboring genes, creating a site of metastable expression. As noted before, this site is particularly susceptible to environmental exposures early in development. The agouti mouse is a frequently studied case because it features the insertion of the intracisternal A particle, causing ectopic Avy transcription via a cryptic promoter at its proximal end, potentially leading to yellow fur, obesity, diabetes, and tumorigenesis (figure 6). Furthermore, methylation of the cytosines near the intracisternal A particle is inversely correlated to ectopic Avy expression and varies tremendously amongst isogenic individuals, causing a wide range of fur colors. BPA was hypothesized to demethylate this area, causing transcription and its phenotypic outcomes (Dolinoy, et al., 2007).

In this study, two groups of female a/a mice were prepared. The control received with one a phytoestrogen-free AIN-93G diet.. The experimental group received the diet with 50 mg of BPA/kg. Both diets were administered two weeks before mating with a Avy/a male until after gestation and lactation. There were 120 resulting offspring in the control group and 124 resulting offspring in the experimental group, with 60 and 73 Avy/a offspring, respectively. Maternal BPA exposure was shown to dramatically shift the coat color of genetically identical Avy/a offspring toward yellow, showing demethylation. 21% of offspring from the group exposed to BPA were labelled as yellow in comparison to 10% of the control group. To assess methylation levels, nine CpG sites in the cryptic promoter regions of the intracisternal A particle were measured using bisulfite treatment and sequencing. The BPA exposed offspring exhibited significantly lower levels in the nine CpG sites, with around 27% methylation compared to around 39% methylation in the offspring control group. After entering BPA dietary exposure, intracisternal A particle methylation, and offspring coat color into a mediational regression analysis model, it was determined that there was little relationship between BPA exposure and coat color directly, but rather methylation was the real mediator of effects. Methylation levels found in the tail corresponded to methylation levels found in the brain, kidney, and liver, revealing that these methylation patterns were established before germ layer differentiation in the embryo. BPA was also seen to affect other metastable epialleles, causing hypomethylation in multiple loci across the genome (Dolinoy, et al., 2007).

In an effort to test BPA levels in human subjects, 244 mothers and their 3-year-old children were recruited for a longitudinal study to measure BPA concentrations in maternal and child urine samples. Maternal urine samples were taken at week 16 and 26 of pregnancy and within 24 hours after delivery. Child urine samples were taken at ages 1, 2, and 3 years old. Behavior and executive functions of the children were also measured using the Behavior Assessment System for Children 2 (BASC-2), a 134-item test of a parent's assessment of a child's behavior in a home and public setting. Parents were also administered the Behavior Rating Inventory of Executive Function-Preschool (BRIEF-P), a 63 question, parental report test that analyzes the child's ability to modulate emotion, control behavioral responses (inhibition), plan for future events, set goals, grasp main ideas, transition from event to event, and hold information to complete a task. For both tests, higher scores indicated more severe impairment. It is intriguing that of the mothers who completed all the assessments, the majority were white, married, between 25 to 34 years old, wealthier, and more educated. Interestingly, these mothers had lower gestational BPA urine concentration than mothers who did not complete the assessment. Children from families of lower maternal education and income also had higher scores on BASC-2 and BRIEF-P.(Braun, et al., 2011)

Gestational urinary BPA concentrations were found to positively correlate with BASC-2 anxiety, hyperactivity, and depression scale scores at three years old, with the magnitude seen greater in females (almost double the association of the whole sample). Lack of emotional control and behavior inhibition suggest that BPA exposure may affect a neurobehavioral domain that is associated with behavioral regulation. Gestational urinary BPA concentrations were also positively correlated with emotional and inhibition scores on the BRIEF-P, with higher scores

attributed to girls again. In general, the effects of gestational BPA concentration were larger among girls than boys and had varying effects (boys had less hyperactivity). Neurobehavior and test scores were not predicted at all by childhood urinary BPA concentration. Only gestational BPA exposure can be implicated in altered neurobehavior, and specifically in females, as well.

The findings of these studies are consistent with previous animal studies that assess the effect of BPA exposure on neurodevelopment. BPA exposure might be responsible for the disruption of sexual differentiation in the brain, altering behavior in a "gender dependent manner," by disrupting endocrine or other neurotransmitter pathways. It is interesting to observe that all of the outcomes of BPA are traced to the exposures inside the womb, with no correlation at all to the postnatal environment (Braun, et al., 2011).

Remarkably, when the formerly mentioned agouti mice of the study done by Dolinoy et al. were supplemented with methyl donors or genistein in the experimental BPA diet, coat color distribution was observed as closer to what was seen in the control group (Dolinoy et al., 2007). Ten to thirteen percent of BPA- exposed -methyl/genistein supplemented offspring exhibited yellow fur color as opposed to the 21% seen in the non-supplemented BPA group, showing that maternal nutrition clearly modified the effects of BPA exposure on methylation patterns. Genistein at high levels had the same effect, even though it is not a methyl donor. Interventions as subtle as maternal nutrition is enough to counteract the effects of an estrogenic endocrine disruptor. This information implies that we are able to reduce disease susceptibility by making small changes in maternal nutrition, giving us the ability to affect generations to come (Dolinoy, et al., 2007).

Nicotine

The in utero effects of maternal smoking are infamous in their detrimental ways. Offspring exposed to smoking after birth do not experience the same adverse affects as those that are exposed as fetuses, hinting to a mechanism that is biologically mediated. It has previously been proven that rodents exposed to nicotine prenatally experienced a direct impact on brain development, reporting "abnormal dendritic morphology, and reduced synapse density in the cerebral cortex and nucleus accumbens" (Chatterton, et al., 2017). Nicotine exposure during gestation is also known to upregulate nicotinic acetylcholine receptors, causing cell death, altered cell size, and increased risk for behavioral impairment. A study done by Chatterton et al. examines the possible epigenetic modifications that are responsible for these developmental differences that have been found to persist even until adolescence. Fetuses aborted during the second trimester were studied and classified based on whether they were exposed to maternal smoking or not. In total, 24 fetuses were tested. The brains of the fetuses were removed to examine the dorsolateral prefrontal cortex, an area known to be involved in decision-making, memory, and neurodevelopment. It is also known to be compromised in function in different psychiatric conditions, including autism spectrum disorder. Methylation microarray analysis was performed to determine differentially methylated regions between the exposed and unexposed groups. Although differences did not remain significant after multiple testing correction (likely due to the small sample size), hypomethylation was found on two genes in the exposed fetuses; SDHAP3 and GNA15. The hypomethylation during second trimester of these two genes is indicative of developmental delays. This resulted in up-regulation of this gene as evident by the increase of mRNA in the samples, an effect mediated by maternal smoking (Chatterton et al., 2017).

Between early and late second trimester, many methylation changes are meant to occur to proceed with development. However, in fetuses exposed to maternal smoking, less change was observed. There was a delay in the upregulation of the SYCE3 gene, a gene that is responsible for synaptic initiation that results in meiotic arrest. As previously stated, SDHAP3 and GNA15 also displayed developmental delays. SDHAP3 is a subunit of the succinate dehydrogenase complex that functions in the electron transport chain. A mutation in this subunit can actually increase levels of oxidative stress. This same differentially methylated region was shown to be hypermethylated in patients with autism spectrum disorder and differentially methylated in the dorsolateral prefrontal cortex of patients with schizophrenia. GNA15 was also found to be differentially methylated in the prefrontal cortex of patients with autism spectrum disorder (Chatterton et al., 2017).

These results show a potential risk that smoking introduces to the fetus in the development of neural abnormalities. All in all, this study supports the concept that smoking exposure during gestation leads to changes in developmental patterns of DNA methylation and gene expression, causing reduced mature neuronal content via nicotine. Unfortunately, the damage can be everlasting (Chatterton et al., 2017).

Smoking during pregnancy has also been linked to obesity because of the shared presence of chemerin, an inflammatory adipokine that is responsible for adipocyte differentiation. It acts as a ligand for chemokine-like receptor 1 (CMLKR1), which is highly expressed in adipocytes. It is found to be elevated in both individuals who smoke and obese individuals.

Obesity has become a national epidemic with 35% of American adults and 20% of children obese, with a total of $200 million is spent each year in obesity-related healthcare costs. Finding the roots to this epidemic can help prevent and treat future cases. The hypothesis that the perinatal environment is responsible for the programming of adult disease is in question as it has been previously shown that fetal exposure to nicotine increases the risk of developing obesity and type II diabetes. The mechanism behind the correlation has yet to be confirmed,

as 15-18% of pregnant mothers continue to smoke throughout pregnancy and lactation (Reynolds et al., 2018).

In this study, two separate cohorts (2012-2013 and 2015-2016) of postpartum women were recruited from the University of Kentucky Chandler Hospital, Labor and Delivery Unit. Subjects were then identified as either smokers or non-smokers. All had full term pregnancies, singletons, and birthed male infants with circumcision performed less than 72 hours after birth. In the first cohort, foreskin tissue was then taken and dissected, snap frozen, and analyzed for chemerin mRNA expression. Of the 46 samples, 31 belonged to offspring of non- smokers with 15 belonging to offspring of smokers. Chemerin mRNA was analyzed in all samples while DNA methylation levels were analyzed in a subset of the samples (12 non-smokers, 7 smokers) (Reynolds et al., 2018).

In the second cohort, foreskin samples were collected from 24 newborns with 13 of them born to smoking mothers. Of these samples, only eight were able to be used due to cell contamination or lack of cell growth. These samples were dissected for the dermal/epidermal layer and incubated. The epidermis was separated from the dermal layer, and the dermal cells were processed and plated. RNA was then collected and isolated from the samples, and chemerin gene expression was analyzed from each sample using qPCR. Maternal and infant characteristics such as weight, height, and body mass index (BMI) were collected for both cohorts (Reynolds et al., 2018).

The results demonstrated that the weight and lengths of infants born to smokers were significantly reduced compared to those whose mothers did not smoke. The foreskin

Figure 7a and 7b compares chemerin mRNA expression and chemerin methylation in non-smoking exposed and smoking exposed infant foreskin samples. (Reynolds et al.,2018)

tissue of babies exposed in utero showed increased chemerin gene expression (figure 7a). Methylation percentages of chemerin CpG sites showed reduced levels of methylation. CpG site 3 of chemerin showed the most significant reduction of methylation with chemerin gene expression inversely correlated with methylation at site 3 (figure 7b).

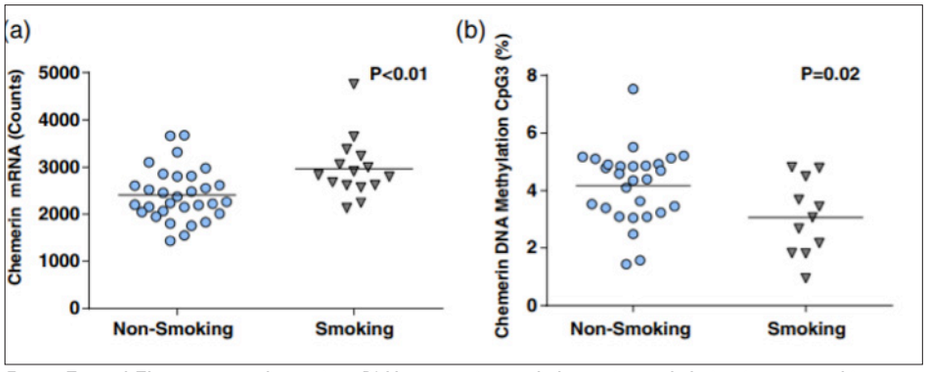

Figure 7a and 7b compares chemerin mRNA expression and chemerin methylation in non-smoking exposed and smoking exposed infant foreskin samples. (Reynolds et al.,2018)

The dermal fibroblasts that grew in cultures for the second cohort were then stimulated with an adipogenic cocktail. Babies of smokers produced cell plates that showed elevated chemerin gene expression, compared to the cells isolated from babies of non-smokers (figure 8). These results correspond with another study that demonstrates that although babies exposed to cigarette smoke in utero tend to be smaller, they have a greater rate of obesity as they get older, suggesting an altered developmental programming. This phenomenon known as, 'catch up growth,' puts babies at risk of developing cardiovascular disease, type II diabetes, or obesity later in life (Power, Jeffries, 2002).

This study involving the two cohorts supports the findings

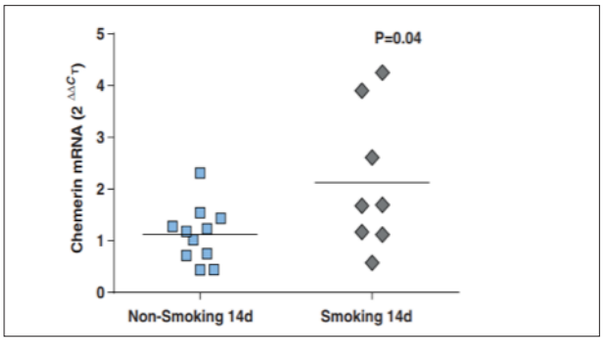

Figure 8, (Reynolds et al. 2017)

of Power and Jeffries et al., further linking cigarette smoking and obesity to methylation and chemerin gene expression. Of course, official causation cannot be completely determined without more research. The study has other limitations, as well: only testing male babies, testing dermal tissue and not adipose tissue, small sample size of the smoking group, and not taking into account second hand smoking are just to name a few. That being said, the overall conclusion is in agreement with other studies on this subject: Smoking alters chemerin gene expression in neonatal tissue of babies exposed to smoking, possibly causing obesity later in life. And perhaps the mediator in this change is epigenetic in nature (Reynolds, et al., 2018).

Trauma

Many people can be exposed to the same trauma, but only some will develop post-traumatic stress disorder (PTSD). So, it begs the question: Is there a biological basis or risk factor for the development of PTSD?

A cohort of 40 Holocaust survivors and their 31 offspring were studied in comparison to individuals of the same age that were not living in Europe in the time of the Holocaust.

Epigenetic changes in the FKBP5 allele, a glucocorticoid binding sequence at intron 7, were measured. Psychiatric analysis was also obtained from the subjects, showing that Holocaust survivors and their offspring suffered more from PTSD and anxiety and/or depression, respectively. After a whole blood examination, bisulfite treatment, and pyrosequencing were performed on the DNA isolates, both Holocaust survivors and their offspring showed differences in methylation on intron 7 of the allele at the bin3/site 6 region, in comparison to the control group. This highlights that Holocaust exposure in the parent was the sole predictor of methylation levels in the offspring. However, survivors showed 10% higher methylation, while their offspring showed significantly lower methylation (Yehuda, et al., 2016).

Offspring methylation levels were inversely related to their wake-up salivary cortisol levels, as well. This might be due to a "intergenerational biological accommodation" (Yehuda et al., 2016). A similar notion is seen with the 11β-HSD-2 allele, another moderator of glucocorticoid action. Increased activity of this allele in offspring was seen as a counteraction to the decreased levels of activity seen in mothers. In mothers, this decrease in activity was an accommodation due to high circulating glucocorticoids. However, in children, upregulation was needed to optimize glucocorticoid levels again. Similarly, because hypermethylation is occurring at the maternal FKBP5 allele to lessen the effects of circulating glucocorticoid, offspring exhibited demethylation to optimize glucocorticoid levels, as well. Although 10% less methylation seems relatively small, differential gene expression has previously been observed with methylation differences between 1%-2%. This lack of glucocorticoid sensitivity can lead to increased risk of psychopathology in the offspring generation, something that the offspring exhibited according to psychiatric evaluation (Yehuda et al., 2016).

A recent study that included Holocaust survivors, as well, discovered that parental PTSD is an important risk factor for the development of PTSD in offspring. Physically, children of survivors had lower 24 hour mean urinary cortisol excretion, an important symptom as lower cortisol levels are also linked to people who have suffered previous traumas. Previous trauma is another risk for the development of PTSD. If cortisol is associated with the risk of developing PTSD, prenatal influences must be investigated as the hypothalamic-pituitary-adrenal axis is programmed during early development. Maternal exposure to glucocorticoids while pregnant can lead to higher levels in the offspring and lower birth weight, a condition linked to various adult diseases including hypertension, insulin resistance, and depression, as well (Yehuda et al., 2005).

To test this correlation, 38 women from a larger cohort of 187 women directly exposed to the World Trade Center collapse on September 11, 2001 while pregnant, agreed to partake in a longitudinal study along with their children. At the ninth month checkup, mothers and infants were asked for their salivary samples at wake up and bedtime to assess cortisol levels. Probable maternal PTSD and severity were assessed with a PTSD checklist, and depression was assessed with the Beck Depression Index. Mothers with PTSD reported more depression than mothers without. Salivary cortisol levels in children of mothers with PTSD were significantly lower than those of mothers without PTSD, a trend that continued throughout the first year of infancy (Yehuda et al., 2005).

It is interesting to note that while the trimester of gestation did not affect maternal PTSD, the effect glucocorticoids had on the baby did differ slightly depending on the trimester of exposure. The babies born to mothers exposed in their third trimester showed even lower cortisol levels. That being said, the severity of PTSD symptoms in the mother correlated with cortisol levels in infants regardless of trimester (figure 9). The results highlight the critical windows of development that are available for reprogramming at each point of gestation, a principle that is important in most epigenetic modifications (Yehuda et al., 2005).

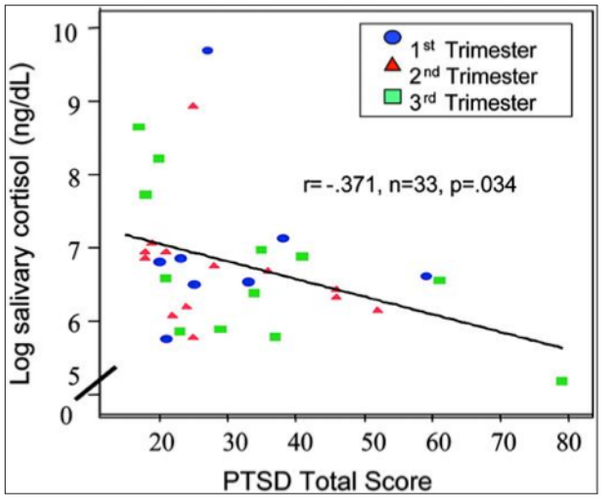

Figure 9, (Yehuda et al., 2005)

Many people are mistaken in assuming that children of Holocaust survivors or of parents with PTSD suffer mentally because of their postnatal upbringing by a traumatized and emotionally incapable parent. However, in Yehuda et al., 2016, the sole predictor for methylation levels in the offspring was parental exposure to trauma. According to the study, emotional abuse and childhood adversity actually did not show any significant correlation to methylation levels and glucocorticoid abnormality. This latter experiment is another testament to the effects the in utero environment can have on the future of the child, as opposed to postnatal "..vicarious traumatization of the offspring by the parents' communication of their trauma to the child or other consequences of parental symptoms (e.g. poor parenting)" (Yehuda, page 4). Babies in Yehuda et al., 2005 were only nine months old at the time of their endocrine testing, so glucocorticoid

programming in utero and/or underlying genetic susceptibility is more relevant then the postnatal environment. Yes, there is always the argument that postnatal care by mothers suffering from PTSD can show inconsistent behavior towards their offspring, affecting their glucocorticoid regulation. However, the stronger correlation to PTSD shown in offspring of the third trimester does implicate prenatal factors (Yehuda et al., 2005).

Stress

The hypothalamic-pituitary-adrenal axis controls growth, reproduction, metabolism, and behavior. It is also extremely active in a human's ability to 'defend' itself during stressful situations via the glucocorticoid receptor (GR). Transgenerational epigenetic modifications can affect the programming of the hypothalamic-pituitary-adrenal axis during early development, affecting glucocorticoid function, as stated previously. In a study that examined intimated partner violence during pregnancy, it was determined that the glucocorticoid promoter gene's methylation status can originate in the womb while a mother's remains unchanged, and sustain itself throughout adolescence. In this study, 25 mother and children pairs were evaluated to determine if gestational maternal adverse experience can cause methylation in offspring that persists for years after pregnancy. Children were between ten and nineteen years old at the time of the study (Radtke et al., 2011).

Mothers were given the Composite Abuse Scale (CAS) test three different times to evaluate intimate partner violence before, during, and after pregnancy (see Table 1). Blood was taken from the mother and child with which she was pregnant with at the time of abuse. The methylation status was evaluated at ten CpG sites found in a transcription factor binding site that are known to have their methylation statuses influenced by early life circumstances. Methylation was detected in 7 of the 10 CpG sites of the glucocorticoid promoter region, with methylation levels ranging from zero to 20%. There was significant correlation of methylation to maternal exposure to intimate partner violence that was experienced during pregnancy only, but no significant correlation was found between offspring methylation and intimate partner violence before or after pregnancy. There was also no correlation between maternal methylation and offspring methylation (figure10). This attests to the prenatal transgenerational influence that stress can have on the hypothalamic-pituitary-adrenal axis. This is consistent with previous studies that have shown that prenatal anxiety has caused sustained elevation of basal hypothalamic-pituitary-adrenal activity, causing behavioral and emotional problems that can continue throughout the lifetime of an individual. Methylation of the glucocorticoid promoter is the potential mechanism in which prenatal stress can influence psychological function. It is interesting that although this is transgenerational in nature, methylation was not inherited along the germ line, as there was no

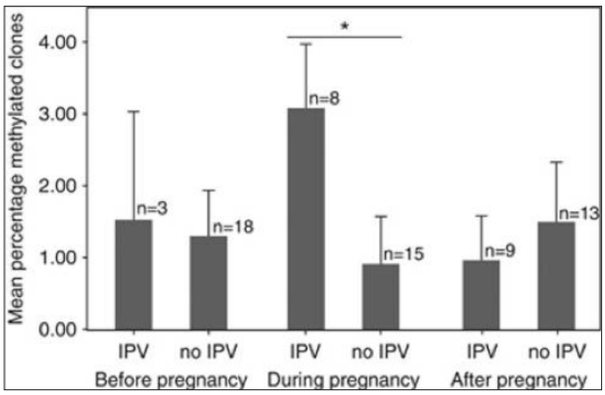

Figure 10

correlation between maternal methylation and offspring methylation (Radtke et al., 2011).

While direct stressors, like the aforementioned intimate partner violence on the mother, have been tested, indirect stress can also prove to be dangerous to a developing fetus. The perinatal environment is extremely impactful on the future of an offspring, with the malleable fetal brain susceptible to even the most subtle of experiences. Mychasiuk et al. studied the phenomenon known as "bystander stress" to demonstrate the effects it might have on the brain, behavior, and development of the epigenome. In this study, eight pregnant dams were separately caged with eight non-pregnant dams. On day 12 and 16 of gestation, the non-pregnant dams were placed on elevated Plexiglass platforms and exposed to bright light for thirty minutes, twice a day. In the control study, the cage mates were moved to another room twice a day for thirty minutes and returned unstressed. To examine data, researchers recorded ultrasonic vocalizations from the rats after the thirty minutes of stress were administered. After the birth of the 120 pups, the pups were also examined in various behavioral procedures to assess their brain development. Afterward their frontal cortex and hippocampus were examined for DNA methylation (Mychasiuk et al., 2011).

At baseline, all ultrasonic vocalizations were the same. However, after the stress procedure, the stressed dams had an increased number of low frequency calls while the bystander dams showed an increased number of high frequency calls. Previous research has demonstrated that low frequency calls are emitted during particular distressing situations, while high frequency calls are emitted in positive situations, such as in times of reward. The researchers suggest that it was the pregnant dam's attempt to soothe the distressed dam by emitting the high frequency call. The stressed dam had a loss of fur on female rats and were more aggressive toward their handlers than the pregnant dams. Once the 120 pups were born to the sixteen mothers, further analysis was done. Brain weight was not affected, but female pups exposed to "bystander stress" showed a decrease in body weight, confirming that the experience proved to be a stressful one (Mychasiuk et al., 2011).

Pups were then tested on their negative geotaxis to assess brain development. Negative geotaxis depends on "vestibular and proprioceptive detection, processing of the vestibular and proprioceptive inputs by the central nervous system, and motor competence to change orientation" (Mychasiuk et al., 2011). This is generally used to detect milestones in the sensorimotor development of rats. After being placed downward at a 40 degree angle, pups were tested on their ability to stay upward on the Plexiglass for the longest amount of time. Pups exposed to "bystander stress" spent less time facing upward on the Plexiglass platform with stressed females specifically receiving the lowest scores in the exercise. The pups were then tested in open field to assess their activity levels. The pups were placed in the center of a transparent Plexiglass box divided into 130 squares. Pups were scored based on how many novel squares they touched with their paw. The "bystander stressed" pups scored less than the control group, with females scores significantly lower than the males. This seems to show the hesitance and unwillingness to explore novel environments exhibited by these young pups. However, as they age, they will lose this inhibition, as seen in previous research (Mychasiuk et al., 2011).

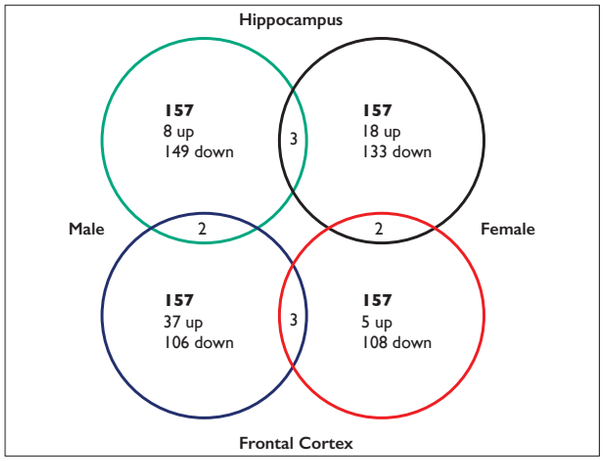

Figure 11 shows the differentially expressed genes found in stressed male and female pups.(Mychasiuk et al., 2011)

In terms of global DNA methylation, all animals in the "bystander stress" cages showed increase in methylation in the frontal cortex and hippocampus with a total of 558 genes differentially expressed between stressed male and female pups combined. There was minimal overlap of differentially expressed genes in brain regions between the sexes (figure 11). This suggests a preexisting difference in brain structure and genetic influence exhibited by males versus females, allowing them to respond differently to "bystander stress." This was clearly seen in their behavioral assessment after birth, as the females did significantly poorer than the males on both negative geotaxis, open field, and lower birth weight. That being said, while the genes did not overlap much, the biological processes that these genes control are very similar between the sexes, with most of genes controlling processes that are important for plasticity of the brain (i.e. cell communication, motion, and transport). Interestingly, a gene that was identical between the sexes in its abnormal expression was the SLC6A1 gene. This gene was downregulated in the frontal cortex. It encodes for a GABA transporter, removing GABA from the synaptic cleft. With its downregulation, GABA would be elevated in the frontal cortex, influencing processes like attention, response inhibition, and working memory. This study is groundbreaking in suggesting that indirect social stressors are just as harmful to the development of offspring as direct stressors, hinting to the intense sensitivity of the fetus. That being said, because blood was not drawn from the mother or "bystander" dam, the mechanism behind this stressed induced methylation cannot be understood completely and is in need of further experimentation (Mychasiuk et al., 2011).

It is vital to realize that while there is much evidence to support the concept that overactivation of the hypothalamic-pituitary-adrenal axis via heightened maternal glucocorticoids puts the infant at a great disadvantage that persists until adolescence, most experiments have been done with animals or small sample sizes. To properly test these results on humans, a cohort of 481 mother-infant pairs were taken from the Barwon Infant Study of Barwon, Australia. Maternal mental well-being was assessed using the Perceived Stress Scale (PSS) questionnaire, a well validated scale that assesses the biological impact of psychological distress. Maternal mental health was also tested using the Edinburgh Postnatal Depression Scale (EPDS) questionnaire at 28 weeks of pregnancy, a 10 item questionnaire used to assess symptoms of depression with a subscale of questions used to assess anxiety. A survey was also distributed to determine information on the physical and mental health of the mother, family medical history, lifestyle, demographic, education, maternal drinking, and other confounding factors. Many exposures were considered to eliminate narrow results that only reflected a specific measure of mental well-being (Table 1). Umbilical cord blood from infants were also collected from participants, as well as blood from the placenta before placental delivery, when possible. DNA was then extracted from the sample of blood and bisulfite converted using MethylEasy DNA conversion kits. Primers were used to amplify a 403 base pair region of the NR3C1 glucocorticoid promoter, spanning 47 CpG sites. After PCR amplification of the region, in vitro transcription and cleavage, DNA methylation was then quantified (Mansell et al., 2016).

The results revealed a strong correlation between the three tests. 65% of depressed mothers were also anxious, while 74% of anxious mothers were also depressed. Methylation levels of NR3C1 in three CpG units were shown to be higher in mothers with increased adverse maternal mental well-being; CpG 1.2, CpG 3.4.5, CpG 47. The aforementioned regions all correlated

to different mental well-being exposures. CpG 1.2 and CpG 3.4.5 were both correlated with perceived maternal distress. Maternal anxiety was correlated with CpG 47. Yet another region, CpG 12.13 was weakly correlated with maternal depression. The covariates like hypertension, preeclampsia, maternal age, antidepressant use, and smoking were correlated with hypo/hypermethylation at different CpG sites, independent of the three that were correlated to mental well-being. That being said, although this was the largest study of its kind performed on the association of methylation and mental-well being, after taking into account the multiple tests and numerous CpG islands affected by the three exposures and the covariates, none of the associations remained significant. This experiment essentially questions all previous experiments that were done with smaller sample sizes and lack of multiple testing. While there is consistency amongst the studies to suggest that it is more than just chance that links maternal depression, anxiety, and adverse well-being to methylation and offspring behavior abnormalities, this study highlights the caution needed in interpreting results and evaluating the studies without bias. The results do support previous findings, like the role of cortisol on fetal "programming," yet it also reveals how the proof is not yet strong enough to withstand a bigger sample size and multiple exposures. In conclusion, more studies, including longitudinal observations, are needed to fully link maternal mental well-being to methylation, as well as to determine the phenotypic implications (Mansell, et al., 2016).

Limitations

Although there has been much speculation and experimentation in the field of epigenetics, no study has yet to link the interplay between genes and epigenetics. When dealing with the cohorts in human studies, genetic dispositions might play a larger role in phenotypic outcome than is given credit. For example, in studies of trauma effect of Holocaust survivors, all subjects were of Eastern European Jewry (Yehuda et al., 2016). It is possible that the effects of methylation on the FKBP5 allele is specific to individuals within the gene pool of Ashkenazic Jewry, while other genetic population would not experience this specific outcome between genes and epigenetics. The fact that there are some Holocaust survivors that have PTSD and not others, also was not explored and hints to a genetic disposition, as well. Furthermore, many human studies relied on questionnaires that were geared toward the mothers or children that were being studied. It is hard to believe that every subject answered with only factual information as opposed to biased and emotional responses. These studies already create a bias from the start, as all subjects consented to the study, thereby aware of what the study was about. Perhaps they agreed because of their feelings of relevance, removing randomness from the population. Another limitation as discussed in Mansell et al. concerns the sample sizes and multiple testing of the experiments. Most of the sample sizes, especially in the human experiments, are not big enough to be relied on completely. Even many of the animal experiments involved less than 100 samples, something that definitely takes away from the credibility of the results. Furthermore, as Mansell et al. clearly stated, multiple testing procedures usually eliminate any significant correlation found. While there is definitely integral information for epidemiology within the field of epigenetics, future experiments and reports should focus on expanding the sample size and designing experiments to survive multiple testing (Mansell et al., 2016).

Conclusion

Epigenetics refers to covalent bond changes that happen above the level of DNA that affects gene expression. Many researchers have speculated about a link between the prenatal environment and adult health. This link is thought to exist because of epigenetic alterations that happen to the fetus because of different environmental exposures. Epigenetic programming takes place during early development in the fetus, thereby presenting a vulnerable and malleable setting for modification.

The purpose of this paper was to investigate the possible link between the in utero environment and adult health, along with the biological mechanism behind it. While there were many limitations to the studies as previously mentioned, there is overwhelming proof that environmental exposures like nutrition, vitamin B, smoking, BPA, stress, and trauma have negative effects on the health and quality of life of offspring. Studies have linked prenatal exposures to obesity, hypertension, coronary artery disease, cancers, and mental illness. According to the aforementioned studies, the events a mother experiences and the decisions that she makes during gestation can seriously deter the path of health for her future adult child. More research and samples are definitely needed to further back this hypothesis, but there is unquestionably enough evidence to support the correlation. It is also important to note that it is possible to avoid some of the detrimental effects of these exposures. As seen in Dolinoy et al., a simple methyl supplement was enough to modify the effects of BPA on an exposed mouse (Dolinoy et al., 2007).

There is no shortage of adult disease victims in America. The Heart Disease and Stroke Statistics of the American Heart Association stated that as of 2019, 46% of Americans suffer from hypertension. The National Institute of Mental Illness stated that 1 in 6 Americans suffer from mental illness. By educating expectant mothers, maybe we can prevent the development of adult disease in at least a percentage of patients. Furthermore, as Powledge mentioned in her article, epigenetics is not permanent like our DNA. More research in this area can help us learn how to rid ourselves of the modifications, or use these modifications to rid ourselves of other undesirable genetic information (Powledge, 2009).

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Impact of Physical Activity on Type 2 Diabetes Mellitus

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Abstract

Type 2 diabetes mellites has become an increasingly prevalent worldwide epidemic. The tremendous economic burden, coupled with the numerous secondary risks associated with the disease, have encouraged researchers to search for causes as well as treatment methods. This research paper focuses on the effect of physical activity as a preventative measure for those at risk for diabetes and as a treatment method for those already diagnosed with the disease. The articles collected included meta-analysis, randomized controlled trials, cohort studies, surveys, etc. The studies that were analyzed have indicated an inverse relationship between physical activity and type 2 diabetes. Since there are multiple variables involved, it is challenging to pinpoint the specific level of effect that physical activity has. However, the majority of the research has shown some level of correlation.

Introduction

The drastic increase in type 2 diabetes mellitus has caused many to research the risk factors associated with this disease. Type 2 diabetes is characterized by the body resisting the effect of insulin or insufficient insulin production. Insulin is a hormone that regulates blood glucose levels. High blood glucose levels over a prolonged period can cause damage to the eyes, heart, nerves, and kidneys. More than 1.6 million new cases of type 2 diabetes arise in the US each year, and millions more are at high risk. (ADA, 2018) It is rated as the seventh deadliest disease in the world by the World Health Organization (WHO). Furthermore, individuals diagnosed with this disease are 2-4 times more likely to develop secondary complications such as stroke, peripheral vascular, and cardiovascular disease.

The various complications caused by type 2 diabetes have created a severe physical and economic burden on our society. Those suffering from this disease have medical costs around 2.3 times higher than those who do not have diabetes. (ADA, 2015). An estimated $21.6 billion is spent in New York each year for the treatment of diabetes and prediabetes. It has thus become a priority to identify those who are at a higher risk and provide preventative measures to help decrease the likelihood of acquiring the disease.

The vast research conducted regarding the numerous risk factors contributing to this disease has concluded that type 2 diabetes results from the interaction between a genetic predisposition and behavioral and environmental risk factors (Tuomilehto et al., 2001). Can lifestyle modification, specifically physical activity, prevent or decrease associated diseases/symptoms of type 2 diabetes mellites.

Methods

The research articles used throughout this paper have been collected through various databases, including Google Scholar as well as the databases available through Touro College Libraries such as PubMed and ProQuest.

Lifestyle Intervention Reducing Incidence of Diabetes

Lifestyle is one of the factors that may impact the presence of type 2 diabetes (Ahmad, Crandall, 2010). Examples of lifestyle characteristics include dietary intake, sleeping patterns, and physical activity. Recent clinical trials, as well as several large cohort studies, have shown that physical activity can reduce the incidence of type 2 diabetes (Sigal et al., 2004).

Exercise and insulin promote the transfer of GLUT4, a glucose carrier protein, which aids in glucose uptake into the muscles. (Colberg et al., 2010). When we exercise, intramuscular glycogenolysis and glucose uptake are increased to meet metabolic demands for energy. There is often an issue with GLUT4 transport for type II diabetic patients. However, exercise allows for an increase in GLUT4 levels and the movement of GLUT4 to the plasma membrane. Therefore, this becomes a non-insulin dependent pathway for glucose to be transported into the skeletal muscle. This glucose uptake has the potential to be equal to normal glucose uptake in non-diabetics.

The Da Qing IGT and Diabetes Study were one of the earliest randomized trials. It analyzed lifestyle interventions which can prevent type 2 diabetes. Individuals with impaired glucose tolerance (IGT) have a high risk of developing NIDDM (non-insulin dependent diabetes mellitus/type 2 diabetes). The study recruited 577 men and women in a Chinese community who had impaired glucose tolerance. They were randomized into four groups, exercise only, diet only, diet plus exercise and control. The 6-year follow up found that the three intervention groups had a risk reduction of 31-46% versus the control group. The long term-follow up had shown that a majority had progressed to the disease, with a lower risk in intervention groups. Although this study may have shown a significant short term (6 years) risk reduction in diabetes, the long-term follow up showed a minor difference between the groups thus indicating that physical activity may not have such a significant long-term effect (Xiao et al., 1997).

The Finnish Diabetes Prevention Study, a more recent study, analyzed the influence of intensive lifestyle changes in the reduction of diabetes). The study randomly assigned 522 overweight (350 women and 172 men), middle-aged (mean age of 55 years individuals who had impaired glucose tolerance to a control or intervention group. The subjects in the intervention group received specific exercise guidelines and dietary recommendations. The 3-year follow-up showed a clinically significant impact of the interventions in the reduction of diabetes. The group had a 58% reduced risk compared to the control group. The study also found

that at least 2.5 hours of moderate to vigorous activity per week caused a 63-69% reduction of diabetes. Furthermore, an extended follow- up revealed that the intervention group maintained a 36% reduction of diabetes incidence. This percentage suggests that benefits can be maintained even after the active intervention was completed. The results of this study clearly show that intensive lifestyle changes (diet and exercise) have the potential to reduce the risk of diabetes in individuals with impaired glucose tolerance (Tuomilehto et al., 2001).

The Diabetes Prevention Program, an additional study of the effect of intensive lifestyle changes, is an even larger randomized controlled trial, recruiting over three thousand overweight, nondiabetic participants with impaired glucose tolerance. The participants were separated into three intervention groups; placebo, metformin (an oral diabetes medicine that helps control blood glucose levels) and intensive lifestyle changes (ILS). The subjects presented with a mean BMI of 34.0 and a mean age of 51 years. Forty-five percent of the subjects were from minority groups, who often display a higher risk for diabetes. The ILS group was given a specific regimen to follow including a low calorie and fat diet and 150 minutes of exercise per week. Furthermore, they joined a 16-week class to learn about diet, exercise, and behavioral modification. Results from a 2.8 year follow up displayed a 58% decrease in diabetes development in the ILS group and 31% reduction in the metformin group compared to the placebo. Results also indicated that weight loss was the main predictor of diabetes reduction (16% reduction per 1 Kg./2.2 lb weight lost). The participants who did not lose the targeted amount of weight but participated in the physical activity still displayed a 44% reduction in diabetes incidence compared to the placebo group. The results support the hypothesis that type 2 diabetes can be prevented or delayed in persons at high risk for the disease. This study shows that specific guidelines, coupled with a wealth of information about health and wellness, can have the potential to impact the incidence of diabetes. The subjects were provided with a lot of knowledge which lasts even after the conclusion of the study. Furthermore, although this thesis does not focus on other forms of diabetes prevention, it is significant to note that physical activity could have a greater effect when combined with additional risk reduction methods such as metformin (Knowler et al., 2002).

A large-scale observational study examined how physical activity impacted the development of type 2 diabetes. The study included 5,990 males from the alumni of the University of Pennsylvania, aged 39–68 years. The subjects were followed for an average of 14 years. A follow-up showed that the disease had developed in 202 of the men. Leisure-time physical activities were measured in kilocalories, and examples included stair climbing, walking, and sports. The study showed that the more the subject participated in leisure-time physical activity, the less likely they were to develop type 2 diabetes. The researchers observed that for each 500 kcal/wk of activity, there was an average of 6% lower risk of type 2 diabetes. It is important to note that since this study is an observational study, it is less conclusive than the previous randomized controlled trials. Furthermore, the study focused on leisure-time physical activities as opposed to more intensive, regimented exercise. This may have been the reason why there was only a 6% risk reduction. On the other hand, it is nice to see that simply engaging in leisure-time physical activity can have a positive impact on one's physical health (Helmrich et al., 1991).

A study was conducted by the use of a questionnaire and a 12-year incidence report to determine if physical activity reduces the incidence of type 2 diabetes in postmenopausal women (age 55-69). During a follow up 1,997 out of the original 350,000 women had reported a new onset of diabetes. There was a negative association with both moderate and vigorous physical activity and diabetes incidence. Furthermore, the most active women had half the risk of diabetes compared to the least frequently active women. The results of this study also showed that reducing adiposity through participating in physical activity can partially prevent diabetes (Folsom et al., 2000).

Lifestyle Intervention Preventing Secondary Risks

Results of these multiple studies have displayed the role of lifestyle modification, specifically physical activity, in the positive reduction in diabetes incidence. The question remains whether physical activity can also benefit those who are already diagnosed with type 2 diabetes in preventing secondary risks.
Recently diagnosed diabetics were grouped to measure the effect of flexibility, aerobic, and resistive exercises on cardiovascular disease risk factors. These risk factors included lipid profile, exercise capacity, flexibility, glycemic control, and body composition. The outcomes displayed that, body fat triglyceride levels and postprandial glucose levels decreased, while aerobic ability and flexibility increased after exercise. These results led to a decrease in the cardiovascular risk profiles in diabetic patients, thus reducing their intrinsic complications. This study showed that even those already diagnosed with the disease can decrease their risk for secondary diseases that are often associated with type 2 diabetes (Acar et al., 2014).

A similar study also focusing on the secondary risk of cardiovascular disease on type 2 diabetic women showed that physical activity allows for a considerable decrease in high sensitivity C reactive protein levels, fasting glucose and total cholesterol (Alizadeh et al., 2012). On the other hand, it causes high-density lipoproteins to increase. Additionally, the study showed that an eight-week exercise schedule could help lower glycemic and lipid profile, thus reduce the risks of cardiovascular disease in type 2 diabetic women.

A thorough 2005 literature review concluded that regular physical activity provides health benefits in individuals who have

already expressed clinical diabetes. Indeed, several prospective observational studies have shown that among individuals with impaired fasting glucose or diabetes, higher levels of activity and fitness are associated with a lower incidence of cardiovascular events and mortality from all causes and cardiovascular disease (LaMonet et al., 2015).

A prospective cohort of 1,263 men, who all had type 2 diabetes, was analyzed. The purpose of the study was to evaluate the relationship of mortality in men with type 2 diabetes, with low fitness and physical inactivity. These men were given a thorough medical examination, which included their fitness level and participation in physical activity. A self-reported baseline was used to document their physical activity while fitness was measured by an exercise test. One hundred and eighty subjects died during a 12-year follow up. Diabetic men in the low-fitness group and physically inactive had a two times greater risk for mortality than fit men and active men (Wei et al., 2000).

Various Forms of Physical Activity

One study meta-analyzed how different modes of exercise training affects glucose control as well as other complications associated with type 2 diabetes. The study included 27 controlled trials involving the estimates for the effect of aerobic training, resistive training, and combined training. There was a total of 1,003 patients with type 2 diabetes that were followed for a 5-104-week period. Results of the meta-analysis indicated that all forms of exercise allow for small-moderate benefits in A1C, the main measure of glucose control. The effects of exercise training seemed to be similar to those of drug, dietary, and insulin treatment. Additional research is imperative to conclude about factors that could affect the outcomes of an exercise program, such as the patient's sex. However, this study indicates that prescribing an exercise program to patients with type 2 diabetes can provide small positive results (Snowling, Hopkins, 2006).

Another study examined the benefits of vigorous activity vs. walking and the incidence of type 2 diabetes in women. The design was a cohort study completed in 1986 (updated in 1988 and 1992). It included surveys of over 70,000 female nurses between the ages 40 to 65 years from the original Nurses' Health Study cohort, which began in 1976. These women did not have a diagnosis of diabetes, cardiovascular disease, or cancer. Although earlier analysis of the original cohort study showed an inverse relationship between vigorous activity and the risk of type 2 diabetes occurrence, there was a lack of evidence regarding the possible role of walking which is a more common form of physical activity in women. In this study, vigorous activity is defined as something which requires 6 METs (metabolic equivalent task-hours) or greater such as jogging, lap swimming, bicycling, etc., as opposed to walking which requires only 2-4.5 METs. The study concluded that both walking and vigorous activity are related to a significant reduction in type 2 diabetes risk in women. These findings support the current guidelines from the National Institute of Health and the Centers for Disease Control and Prevention that recommend a minimum of 30 minutes of moderate intensity physical activity daily (Webster, 2018).

A randomized control trial was conducted comparing the impact of aerobic training (AT) and progressive resistive training (PRT) on the health status of a multi-ethnic Asian population. Progressive resistive training is a program that builds physical strength by lifting progressively heavier weights and is based on the individual's maximum strength. Both programs were completed over 8 weeks with 60 middle-aged adults who had a diagnosis of type 2 diabetes mellites. Health status was measured using the Short-form 36 Questionnaire (SF-36), a self-administered assessment measuring eight aspects of functional health. The subscales are divided into the physical component summary score (PCS) and mental component summary score (MCS). Results of the scoring showed significant improvement in both the PRT and AT groups with both physical and mental health benefits. Furthermore, PRT may have some added benefits since there were significant changes in more domains of the SF-36 than in the AT group. Although the benefits of both forms of physical activity are apparent, it is not necessarily certain that the same positive results would apply to a different population. Each population has many factors that can have an impact on health (Ng et al., 2011).

In an additional study, the same questionnaire, SF-36, was completed by subjects which led sedentary lives and were diagnosed with type 2 diabetes. The purpose of this study was to determine if a 10-week moderate aerobic exercise regimen would affect the quality of life (QOL) as well as the physical health of these subjects. The survey was completed by a group of subjects who participated in the training, three days a week for 20-45 minutes. The survey was also completed by the group of control subjects who did not receive the training. Results of this study showed a decrease in percent body fat and an increase in lactate threshold in the training group. However, there was no significant difference in the mental health component of the quality of life survey (Holton et al., 2003).

A study determined if adults with or at risk for diabetes participate in physical activity. A survey was collected by the Medical Expenditure Panel (a nationally representative survey of the U.S. population) regarding the physical activity of 23,283 adults. Additional information such as health conditions (diabetic vs. nondiabetic) and sociodemographic characteristics where self-reported. Results of the survey showed that 58% of adults without diabetes were physically active versus 39% of adults with diabetes. The number of non-diabetic individuals who responded that they were physically active decreased as the amount of type 2 diabetes risk factors increased. Income level, depression, severe obesity (BMI >40kg), and limitations in physical function were the strongest factors correlating with being physically active or not. The study

concluded that those who have type 2 diabetes or those who are at a higher risk for developing the disease do not participate in physical activity regularly. Thus, it is imperative to create efforts to target this population to increase the levels of their engagement in physical activity by reinforcing its value and ability to help curb rising diabetes and obesity epidemic (Morrato et al., 2003).

As previously noted, most American adults who have type 2 diabetes or those who are at risk for developing the disease do not regularly engage in physical activity. A joint positional statement written by The American College of Sports Medicine and The American Diabetes Association concluded that self-efficacy (associated with the confidence in the ability to exercise) and social support are two of the most reliable predictors of greater levels of engagement in physical activity (Colberg et al., 2010). Family, friends, and health care professionals may be a source of support for these individuals. By taking the necessary precautions, individuals with type 2 diabetes can maintain a safe exercise routine. An exercise program or leisure-time physical activity is critical for the overall health of those suffering from this disease.

Conclusion

Many people assume that high-calorie diets, which are very prevalent in our society (due to fast food, etc.) is the main contributor to obesity and causes a higher risk for diabetes. The studies documented in this research paper highlight another key behavioral factor which contributes to the high rate of this disease. The research has indicated that low physical activity and leading a sedentary life has a great effect on the risk of developing type 2 diabetes. There is also strong evidence that leading an active and fit life has the potential to reduce mortality risk in individuals who already have this diagnosis. Over the past few years, our society has morphed into a technological hub in which the majority of our daily responsibilities can be taken care of with just a few clicks. Thus, it has become common for individuals to be physically inactive and lead sedentary lives. This phenomenon already starts at a young age. For example, our children no longer feel the need to play sports outdoors when they can play the video game version. It is, therefore, imperative to continue researching the effects of physical activity on diabetes risk to bring about global awareness and to halt this fatal epidemic.

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Endorphins, Endocannabinoids and Runners' High

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Abstract

Modern science takes at face value the fact that exercise is beneficial for man's health. In recent years, medical health professionals have begun to harness exercise as a treatment for a broader range of maladies. Since various studies found increased exercise to correlate with higher levels of endorphins, most believed that the endorphins were directly responsible for what the vernacular dubbed "Runner's High." Scientists have sought to either augment or wholly disprove the endorphin hypothesis with further studies. Major Depressive Disorder, or MDD, is an affective condition affecting a significant portion of the general population. Aerobic exercise is increasingly being revealed to be an encouraging treatment for all types of depression and anxiety. The HPA Axis allows one to comfortably exercise without experiencing the extremely stressful symptoms that characterize psychological stress, such as depression, anxiety, fear, etc. Runners' High is the "…happiness, elation…inner harmony, boundless energy, and… reduction in pain sensation" that ensues following prolonged aerobic activity. Endorphins are the body's naturally occurring opiates. The Neurogenesis Hypothesis states that "a decrease in the synthesis of new neurons in the adult hippocampus might be linked to major depressive disorder." Further research implicates BDNF (Brain-Derived Neurotrophin Factor) in exercise-induced neurogenesis. The thought was that beta endorphins are what stimulate increased neurogenesis, in turn causing a decrease in MDD. β-endorphins are large molecules that are too bulky to pass through the BBB and therefore cannot be the cause any changes to occur within the brain. Researchers and scientists have thus turned their attention to endogenous endocannabinoids as the true source of analgesia, sedation, anxiolysis and reduced depression found in endurance exercisers. Chief ligand anandamide (AEA) engenders elevated levels of BDNF (Brain Derived Neurotrophic Factor) during exercise and return-to-baseline levels post-exercise. Endocannabinoids modulate nociception by affecting the Periaqueductal Gray system (PAG). Endogenous cannabinoids and exogenous cannabinoids (marijuana) act in similar fashions, leading to the addictive qualities of exercise. Compelling evidence has ascertained that endogenous endocannabinoids are the underlying cause of the many positive effects of aerobic activity.

Introduction

Modern science takes as face value the fact that exercise is beneficial for man's health. Hippocrates was known to say, "Even when all is known, the care of a man is not yet complete, because eating alone will not keep a man well; he must also take exercise..." While to a contemporary society this concept seems rather primitive and simplistic, it is a truth that forms the basis of modern health sciences. However, it has only been in recent years that medical health professionals have begun to harness exercise as a treatment for a broader range of maladies. Exercise is widely known to help keep one's weight down, tone muscles and enhance physical fitness. Scientists are perpetually expanding their knowledge of exercise and what kind of effects it has on the brain and the body's systems. An understanding of the neuronal activity, neurotransmitter actions and chemical releases that are the responses to varying degrees of exercise can be applied to treat disease and improve lives. One such application is exercise as a treatment for Major Depressive Disorder (MDD). MDD had previously been treated solely with antidepressants, but more recently is being dealt with using a combination of medication and exercise. Various case studies have undertaken to prove the relationship between exercise and positive changes in the MDD brain. Once the neuronal and chemical effects of exercise were established, it naturally led to the conclusion that depressive symptoms lessened due to a proliferation of certain positive hormones that were released into the brain in response to exercise. With further investigations, the popular conclusion was that the feel-good high associated with exercise is caused by endorphins, opioid hormones that appear to increase in response to exercise. Since various studies found increased exercise to correlate with higher levels of endorphins, most believed that the endorphins were directly responsible for what the vernacular dubbed "Runner's High." Since then, scientists have sought to either augment or wholly disprove the endorphin hypothesis with further studies.

Methods

The search engines used include ProQuest, EbscoHost, and GoogleScholar. Tributary information was also obtained using Wikipedia, the New York Times and WebMD.

Discussion

Major Depressive Disorder, or MDD, is an affective condition affecting a significant portion of the general population. In America today, about 5% of all people will eventually be diagnosed with MDD. Quality of life with MDD is radically impaired, and basic life functions such as eating, sleeping and maintaining personal hygiene can be affected. It has been found that roughly 10% of people diagnosed with MDD will probably commit suicide. This is all due to the overwhelming, pervasive feelings of severe depression that characterize Major Depressive Disorder. Deficits in executive function are also consequences of MDD. Patients tested with a Stroop Task, Go/No Go Task, Task-Switching Paradigm and Flanker Tasks all scored low in reaction times, indicating deficits in executive function ability (Ernst, et al. 2006). Previously, MDD had been treated solely with antidepressant medications such as SSRIs (selective serotonin reuptake inhibitors). The anti-depressant medication field is an ever-growing industry, despite limited success and many unwarranted side effects. For examples, anti-depressants stop

working after a while, since the body and brain become accustomed to the drug and gain a tolerance for it. In such a case, the dose would have to continuously be raised in order to have any effect. Furthermore, if one abruptly stops taking the dosage of medication, withdrawal symptoms such as "dizziness, loss of coordination, fatigue, tingling, burning, blurred vision, insomnia, and vivid dreams" may incur. Additionally, the patient may experience "nausea or diarrhea, flu-like symptoms, irritability, anxiety, and crying spells." This tendency is called the "Discontinuation syndrome" and is obviously best to be avoided. Other negative side effects might include insomnia, diarrhea and stomach aches, headaches, joint and muscle pain, reduced blood clotting capacity due to lowered concentration of serotonin in platelets and possible increased tendency towards violence and/or self-destructive behaviors. A Black Box warning was even issued in 2004 by the FDA against SSRIs, warning of the possible risk of "suicidal thoughts, hostility, and agitation in children, teens and young adults" (Harvard, 2019).

Between 30-35% of patients on medication do not even respond to the antidepressant treatment at all. The unpleasant side effects considerably compromise the patients' quality of life in many areas and make the medications undesirable as a primary treatment for depression. Furthermore, there is a high relapse rate found in patients treated with antidepressants, leading to a need for alternative and/or supplementary treatments (Blumenthal, et al. 1999).

Aerobic exercise is increasingly being revealed to be an encouraging treatment for all types of depression and anxiety (Heijnen, et al. 2016). A study was performed testing MDD symptoms in response to exercise. Participants were accepted for the study if they displayed depressive criteria as expressed in the DSM-IV and other symptoms such as "sleep disturbance, weight loss…psychomotor retardation or agitation, feelings of worthlessness or excessive guilt, impaired cognition…and recurrent thoughts of death." They also had to receive a score of at leave 13 on the HAM-D test. The HAM-D is a clinical rating scale used to measure levels of depression. Scores of thirteen to eighteen is mildly depressed, above 18 is severely depressed. Each of the subjects of the study were assigned to one of three groups – exercise treatment, medication treatment, and a combination of exercise and medication treatment. The assignment of groups was a randomized procedure to ensure that equally depressed participants were assigned across the groups. The exercise group met three times a week for 16 weeks, engaging in personalized exercise protocol according to their individual heart rate capability. The classes were overseen by a "trained exercise physiologist." The medication group was administered the SSRI sertraline, specifically used due to its "documented efficacy and favorable side effect profile." Dosages were administered by the staff psychiatrist at intermittent weeks. The combination group concurrently received the same medication and exercise regimens described above. Results were generally positive. Firstly, patients who engaged in exercise showed improvements in aerobic capability, with an 11% increase in the exercise group and a 9% increase in the combination group. Secondly, depressive symptoms improved equally across all three groups. Improvements on the HAM-D scores did not differ across groups, and neither did the scores based on DSM-IV depression criteria. "…The percentage of patients who were no longer classified as clinically depressed at the end of the 4-month treatment period did not differ across treatment groups." The only difference found that they patients benefited more rapidly in the combination group than did the patients in the exercise or medication groups alone. This study demonstrated that exercise treatment as an intervention for depression is equally as effective as medication treatment (Blumenthal, et al. 1999).

Numerous other studies, all of which sought to prove the efficacy of exercise have tested people who suffer from depression across the spectrum. One set of studies has shown that participants, ranging from young to elderly, who engage in daily exercise regimens are far less likely to develop MDD. Subsequent investigation further demonstrated that daily exercise improves depressive symptoms, even in people who have not been diagnosed with MDD. Exercise studies performed with healthy adults with no depressive symptoms also found positive results. These adults reported improved cognitive function, elevated mood and a general sense of well-being. One study tested how well exercise treated "moderate-to-severe" depression, concluding that the depression was highly improved. Another treated MDD patients with a combination of antidepressant medication and exercise regimens. A control group received medication and "health education" lessons. The medication/exercise group responded significantly better than the control group. Yet another tested a group of adults suffering from MDD, exercising with varying intensities of exercise. They found here that the most intense exercise group improved the most. A significant edge that exercise has over medication is that the effects of exercise may extend past the actual regimen, with the positive feelings enduring (in one study) up to almost two years past the experiment (Ernst, et al. 2006). This is in sharp contrast to antidepressant medication, of which the healing abilities curtail immediately with the end of dosage. This is why people on medication require concurrent therapy so that they can manage their life even after their medication dosage is over. On the other hand, exercise is found to be even superior to therapy itself in many instances, with various "young and middle-aged adults" positing that aerobic exercise helped their depressive symptoms more than therapies like psychotherapy, CBT and occupational therapy (Blumenthal, et al. 1999). Aerobic exercise performed by patients with depression and anxiety may improve cognitive function. (Heijnen, et al. 2016) Patients who scored poorly on various task functions, such as Stroop and Flanker Tasks, scored significantly higher following 30 minutes of aerobic exercise (Ernst, et al. 2006). All of these studies have consistently found lower MDD

and anxiety levels in response to exercise training, in addition to higher psychosocial functioning, increased positive affect and elevated mood (Blumenthal, et al. 1999).

The obvious question that troubles scientists and mental health specialists is why exercise has such a potent effect on the human psyche. While the physical action of aerobic exercise is known to cause weight loss and increased fitness due to burning of calories and fat and muscle toning, the reason behind the mental effects are not as simple to extrapolate. Exploring the neuroendocrine response that takes place in response to aerobic exercise may help to ascertain the underlying cause.

Exercise that activates the heart rate from its resting point and raises the maximum capacity of oxygen intake (VO2max) at least 60% can be classified as a physical stressor. In response to such a stressor, the hypothalamus secretes "corticotrophin-releasing hormone, which travels…to the anterior pituitary to induce adrenocorticotropic hormone (ACTH) release" into the general circulation. Arrival of this hormone at the adrenal cortex stimulates cortisol release into the bloodstream. Cortisol acts as an inhibitor and, through the use of medial prefrontal cortex (mPFC) receptors, inhibits "overexcitability of the amygdala" that naturally occurs when a stressor arrives in the bloodstream (Heijnen, et al. 2016). This stress response is known as the Hypothalamic-Pituitary-Adrenal (HPA) Axis, so named for the journey of hormones from the hypothalamus, through the anterior pituitary and into the adrenal cortex, causing relaxation from the body's increased stress levels (Blumenthal, et al. 1999). The elevated levels of cortisol can last up to 2 hours after the actual exercise action, and the amount of cortisol released is proportional to the amount of exercise. Equally important in the stress response is the inactivation of cortisol back into cortisone. This homeostatic cycle ensures that frequent exercisers do not suffer from extreme cortisol levels that can lead to "hypertension, hyperglycemia, major depressive episode and anorexia nervosa" (Heijnen, et al. 2016). Thus, ACTH is a regulatory mechanism that acts as the body's natural stress response. This allows one to comfortably exercise without experiencing the extremely stressful symptoms that characterize psychological stress, such as depression, anxiety, fear, etc. (Angelopoulos, 2001). However, while this neuroendocrine response accounts for the control of increased psychological stressors while exercising, it could hardly account for the flood of "euphoria, anxiolysis (reduction of anxiety), sedation, and analgesia (relief from physical pain)" that characterize what we call Runner's High (Reynolds, 2015).

Runner's high is the reason that America has its plethora of addicted runners clocking miles in their sneakers every day. Every athlete can describe the "…happiness, elation…inner harmony, boundless energy, and…reduction in pain sensation" that ensues following prolonged aerobic activity (Dietrich, 2004). This so-described high is experienced frequently by endurance athletes, but really by anyone who exercises profusely for an extended amount of time. Scientists have struggled for decades with the reason behind runner's high and many hypotheses have been debuted and disproven over time. For years the Endorphin Hypothesis was the most popular by scientists and the overall population, gaining strength through hearsay and seemingly impenetrable evidence.

Endorphins are the body's naturally occurring opiates. When a person exercises, these chemicals are released in the brain, seeming to improve the athlete's mood and promote endurance. While exercise is known to be a healthful practice to engage in for one's physical health, it can cause degrees of discomfort or even pain. Therefore, it would make sense that in response to exercise, endogenous opiates with antinociceptive properties similar to morphine would be released (Reynolds, 2015). Any kind of intense endurance exercise can induce endorphin release, and it seems that the greater the exercise, more mood-altering endorphins are proportionately released (Kolata, 2008). Endorphins are "cleaved from the preprohormone pro-opiomelanocortin (POMC)…a protein found in the pituitary gland and the brain" (Ernst, et al. 2006). The C-terminus of the POMC protein in turn gives rise to hormones such as opiate peptides.

In one experiment, researchers used PET (positron emission tomography) scans to compare endorphin levels in the brains of athletes. Ten athletes did a two-hour run and were tested for opiate levels before and after the run. Additionally, the runners took a psychological test pre and post-run, intended to measure their moods before, and in response to, the flood of endorphins. The researchers did find increased levels of endorphins attaching themselves to the limbic and prefrontal areas of the brain, zones typically associated with emotion. The athletes also reported higher mood and euphoria in a seemingly direct relation to higher endorphin levels in the brain (Kolata, 2008, Heijnen, et al. 2016). Technically, all this study proved was that there is indeed a flood of endorphins in the brain in response to exercise. While there seems to also be a correlation between the elevated opiate levels and mood, the proof was not – yet – impenetrable.

A study performed at the Iranian Academic Center for Education, Culture and Research (ACECER) attempted to ascertain how endorphin levels are affected due to exercise. The researchers included subjects with fibromyalgia (FM) as well as healthy patients. The study was based on the assumption that exercise increases release of endogenous β-endorphins, and that β-endorphins lessen pain perception. Exercise has previously been used as a treatment for diseases such as FM, with a strong role in nociception and elevated pain threshold. Specifically in FM cases, however, the "analgesic effect" or lessening of pain perception seemed significantly dulled. According to the results of the study, this would seem to be due to the fact that FM patients produce less β-endorphins as they exercise and therefore

are less capable of blocking pain. All of the subjects first rested in a supine position in order to attain their basal heart rate, a range of 60 to 100 beats per minute. Blood from the anti-cubital vein was drawn at baseline and at the end of the exercise test in order to compare β-endorphins levels. The exercise test was a treadmill routine that continued as long as the subject was able, until the onset of "fatigue, pain, or physical exhaustion". Every three minutes the incline of the treadmill as well as the speed increased according to specific intervals. As soon as 70% of the patients HRMax (maximum heart rate) was achieved, the subject was told to continue for 15 more minutes only, and their level of exercise intensity was recorded. The post exercise blood was drawn directly afterwards, when the heart rate was still at its maximum. The results were rather interesting. The mean exercise times for the FM and healthy groups revealed that it took a much shorter time for the FM subjects to achieve HRMax than the healthy group. The majority of subjects who were able to reach their HRMax at later stages of exercise were from the healthy group. Ninety three percent of the healthy group, as opposed to the mere 30% of FM subjects, reached HRMax during stage four of exercise. This indicated that FM patients were unable to exercise to the same extent that the healthy participants were. While the blood samples indicated that both groups had amounts of β-endorphins, the FM subjects has significantly lower levels in both their baseline blood and in the post-exercise blood. This means that while both groups experienced a rise in β-endorphins due to the exercise, the FM patients were unable to produce the same influx of β-endorphins as the healthy group. These blunted levels of β-endorphins may explain why FM patients are more immune to the analgesic effects of exercise that usually take over post-exercise. Usually, aerobic exercise stimulates elevated β-endorphins levels which in turn seem to stimulate decreased nociception, elevated mood and psychological stability. However, due to "failure of normal elevation of [endogenous] β-endorphins," FM patients seem less able to produce the same analgesic effects. Therefore, they might be more prone to develop chronic allodynia (pain from a non-painful stimulation of the skin) and heightened nociception with the absence of normal β-endorphins levels (Bidari, et al. 2016).

Scientists have explored how endorphin levels rise in response to exercise. A study sought to verify the elevated levels of endorphins, with and without naloxone (opiate antagonist). It has already been proven than exercise with an intensity of 75% of VO2max and 80% of HR (heart rate) causes an elevated release of beta endorphins into circulation. However, this study examined whether longer lengths of activity cause even further increased endorphin levels, and how long one must exercise to stimulate endorphin release. The study included "nine healthy, fit males." In this double-blind experiment, some subjects were injected with naloxone, and some with a mere placebo. They all took a graded exercise test (GXT) to assess their physical capabilities and then performed exercise on treadmills, running until they were too exhausted to go on. Phlebotomy techniques were used to draw blood and measure beta endorphin levels. Levels of VO2max, HR (heart rate) and hematocrit (ratio of RBC volume in blood) were measured during 10 min, 20 min and 30 min intervals for both trials. No changes in Hct levels were found between trials.

β-endorphin concentration across the trials were recorded and trials showed significant differences. Before exercise, β-endorphins levels were at roughly 40 ml; across 10, 20 and 30-minute intervals, influxes of 80 to 90 ml of β-endorphins were recorded. Of note is that after the initial rise in β-endorphins, the volumes leveled off and stopped rising across time during exercise - it stayed constant. β-endorphin levels were significantly higher under naloxone administration. To summarize the results of the study, a significantly large increase in β-endorphin were found during and throughout exercise as compared to resting levels. They also found that the men injected with Naloxone experienced even higher levels of β-endorphins. Additionally, tests with low to moderate levels of exercise showed no changes at all in β-endorphin levels. Therefore, this suggests that it is only intense aerobic exercise that induces significant increases in β-endorphins. This elevation occurs rapidly from the onset of exercise and remains level through the duration. This test found much higher β-endorphin levels in the naloxone-injected participants as compared to the placebo participants. However, other experiments did not find much of a difference between naloxone test and placebo test. This is an obvious disparity that may be due to "differences in the exercise protocol, method of administration of the antagonist, and in the stereo-specificity of the antagonist used in the respective experimental protocols" (Angelopoulos, 2001).

Now the question stands, what is it that causes this drastic β-endorphins rise during exercise? The researchers suggest that Naloxone attaches to the opioid receptor, blocking any other opiates and signaling the pituitary gland to release more β-endorphins. These β-endorphins do glucoregulation during exercise, "augmenting glucagon levels and attenuating insulin release." Glucagon keeps blood glucose at high enough levels to allow the body to function normally (Glucagon, Diabetes. Co). This is a positive feedback loop – more β-endorphin is released, it does more glucoregulation, and so more β-endorphin is released. This may be why endorphin levels were higher with Naloxone injections.

ACTH also increases under stressful condition, just like β-endorphins. ACTH is also secreted by the pituitary and regulates cortisol in the bloodstream. Just like the endorphins in this study, ACTH levels rise with an injection of Naloxone. Thus, it seems that both β-endorphins and ACTH are regulatory mechanisms, acting to ensure the body's homeostasis despite outside

stressors. However, ACTH regulates the psychological stress response and endorphins normalize the physical response. This study demonstrated how and why β-endorphins levels were significantly elevated over the course of aerobic activity (Angelopoulos, 2001).

It is clear that aerobic exercise indeed causes an influx of β-endorphins to be released into blood circulation. The next step would be to secure as fact that it is indeed the flood of endorphin opiates that lowers depressive symptoms and induces a euphoric affect. The following study attempted to ascertain the possible link between aerobic exercise and the subjects' emotional state. The study involved middle aged subjects engaging in physical activity. First off, the subjects' emotional states were evaluated using FaceReader. FaceReader is a facial analysis software that assesses emotional state based on 6 primary emotions - happy, sad, angry, disgusted, scared, and surprised. The subjects were also interviewed personally to assess how they were feeling just then. After the facial analysis the researchers drew blood from the median cubital vein of each subject to have a baseline sample of blood plasma levels. The physical activity was measured using a veloergometer load test. A veloergometer is similar to an elliptical exercise bike, however it is equipped with specialized hardware capable of measuring physical prowess. This exercise regimen is called a load test, and it commenced for a half hour, with increasing load every three minutes. After the load test, the pre-test procedures – FaceReader, interview and phlebotomy – were repeated and analyzed. The pre and post blood measures were tested for plasma beta endorphin levels to see if there was an increase in endorphins in response to the exercise. If an increase was accompanied by a similar increase in mood, this would provide evidence that it is endorphins that cause the elevated mood associated with physical activity. The results showed an endorphin increase in only half of the subjects. However, in regard to positive affect, the data found a general increase across the subjects. A 20% increase in happiness was found and all except one showed a decrease in negative emotions. (One subject showed an increase in disgust due to unpleasant lab conditions) (Kundzina, et al. 2014). These findings definitively supported the hypothesis that extended aerobic exercise of at least 60% VO2 intensity is directly linked to increased positive affect and decreased depressive symptoms. It is of significance to note, however, that only half of the subjects showed a marked increase in circulating beta endorphin levels. Here we introduce a hypothesis that closely allies with the subject at hand. This is the Neurogenesis Hypothesis, which states that "a decrease in the synthesis of new neurons in the adult hippocampus might be linked to major depressive disorder (MDD)." Anti-depressant medications such as SSRIs have been used for years as treatment for MDD. Anti-depressants act to stimulate synthesis of new neurons in the adult brain. Eventually, a link was drawn between the anti-depressants and neurogenesis, since the time it takes for medication to have an effect is the same span of time it takes for the newly synthesized neurons to gain functionality. Returning to our exercise hypothesis, an experiment with lab rats found a 2 to 3-fold increase in neurogenesis in rats who had "regular access to a running wheel when they are compared with control animals." This discovery naturally led to the awareness that exercise assuages depressive symptoms by inducing increased neurogenesis, the "growth of new neurons in the adult [mammalian] brain." While it was once thought that neurogenesis is limited to the developmental stages of growth, it is now known that certain areas of the adult mammalian brain retain active progenitor cells that are constantly synthesizing new neurons and glial cells. There are two major sites of adult neurogenesis, the dentate gyrus of the hippocampus and the subventricular zones adjacent to the lateral ventricles. Based on experiments performed with lab mice and rats, roughly 9,000 new neurons are synthesized daily in the dentate gyrus alone. Research indicates that these numbers are reflective of human neurogenesis as well (Ernst, et al. 2006). One site of adult neurogenesis is the subventricular zone (SVZ) that is adjacent to the lateral ventricles of the brain. "Numerous proliferative precursor cells" line the walls of the ventricles that are composed of ependymal cells. The precursor cells are contained in this zone and eventually mature into new neurons and glial cells. The second site is the dentate gyrus (DG), located on the hippocampus in the brain. The hippocampus, a "bilateral limbic structure that plays a role in…learning and memory...." has two distinct areas – the Cornu Ammonis (CA) and the dentate gyrus. The CA has three subfields filled with pyramidal cells, multi polar neurons that act as primary excitation neurons in the brain. The dentate gyrus is connected to the CA and is "composed of small, round granule cells" that form a C shaped area. It is underneath these granule cells that active progenitor cells are housed and give rise to new neurons and glia, giving the area its name – the subgranular zone (SGZ) of the dentate gyrus. The two zones are closely adjacent to each other but develop differently. Active progenitor cells in the dentate gyrus give rise to daughter cells that migrate into the granule layer above. There, they develop and send out axons into the Cornu Ammonis region 3 (CA3). Over the course of four to five weeks, these daughter cells mature and act like fully grown granule cells. The daughter cells in the subventricular zone give rise to daughter cells that migrate into the olfactory bulbs to act as local interneurons. Thus, there is adult neurogenesis occurring daily at a rapid rate in the healthy mammalian brain. The neurogenesis theory states that if this complex process should somehow be impaired and fewer neurons would be created, the result would be the expression of depressive symptoms. The introduction of anti-depressant medications would promote neurogenesis and get the brain back on track. The plot thickens with the discovery that exercise too, promotes neurogenesis in the hippocampus of the brain. This strongly links exercise as a reliable therapeutic intervention for treatment of MDD (Ernst, et al. 2006).

Several research experiments have sought to augment the proof with concrete data. Firstly, MRI (magnetic resonance imaging) data have shown that hippocampal volume is severely compromised in the brains of depressed patients. This would give strength to the idea that it is reduced neurogenesis in the dentate gyrus that gives rise to depressive symptoms. Other studies showed that the corticosteroids released from the adrenal gland as part of the natural stress response (HPA Axis) negatively impact neurogenesis in the hippocampus, thus causing the "decreased hippocampal volume associated with MDD." The stress response is thus amplified, sustaining a vicious cycle of continuous depression and stress response. It's important to note that the decreased size of the hippocampus is not due to the degeneration of pre-existing neurons. Therefore, it would follow that fewer neurons are being created in the first place. Comparatively, researchers found increased hippocampal volume in direct relation to aerobic exercise. Lab rats that engaged in aerobic exercise consistently displayed large increases in hippocampal volume due to subsequent supplementary neurogenesis. As compared to control mice and rats with no access to a running wheel, active mice displayed double the number of new neurons in the subgranular zone of the dentate gyrus of the hippocampus. Furthermore, it has been ascertained that treatments for MDD, such as SSRIs (selective serotonin reuptake inhibitors) or electroconvulsive shock therapy, also cause an increase in neurogenesis. This expresses a direct relationship between neurogenesis and lessening depressive symptoms. A third proof involves the timing. Newly generated neurons typically require four to five weeks to become fully functioning neurons. This is a "latency similar to the onset of therapeutic benefit for most…antidepressant medication[s]." This information also supports the idea that proper neurogenesis is integral to reducing depressive symptoms. Research has even shown that in certain cases, aerobic exercise can foster the same therapeutic effects as cognitive therapy (Ernst, et al. 2006).

Further research implicates BDNF (Brain-Derived Neurotrophin Factor) in exercise-induced neurogenesis. BDNF promotes "neuronal survival and regeneration" in the adult brain. Aerobic exercise causes elevated levels of BDNF to be produced specifically in the CNS. This specificity indicated that BDNF plays a particularly central role, namely in the brain. One study found that exercise in mice led to increases in BDNF. They also noted that in a case with depressed lab animals, exercise led to an increase in both BDNF levels and hippocampal neurogenesis. On the other hand, it is known that it is neurogenesis that causes the anti-depressant effects of exercise; therefore, it was concluded that elevated levels of BDNF contribute to increased neurogenesis, thus playing an indirect role in lowering the depressive symptoms. BDNF stimulates cell proliferation specifically in the subgranular zone of the dentate gyrus, and also supports long-term survival of these new neurons. A positive cycle can thus be recognized: exercise increases BDNF, BDNF enhances neurogenesis and neuronal survival, causing increased positive affect, probably leading to further exercise to prolong the effect (Ernst, et al. 2006).

The obvious question is: why does exercise stimulate neurogenesis; what endogenous substance causes the brain to up the ante on neurogenesis when the body is engaged in aerobic activity? This returns us to the endorphin hypothesis. The thought was that endorphins are what stimulate increased neurogenesis, in turn causing a decrease in MDD. Studies have found that not only do infusions of opiate peptides seem to cause an increase in neurogenesis in the dentate gyrus, but also that "opiate receptor antagonists" seem to cause a decrease in adult neurogenesis. Therefore, endorphins may have a role in the survival and/or formation of new neurons and glia (Ernst, et al. 2006).

The Endorphin Hypothesis seemed sound, and was sustained for decades in the medical arena and among lay athletes at large. However, significant holes in the hypothesis were sprung that threatened the authenticity of its claims. Among other problems, the most substantial issue was that of the Blood Brain Barrier (BBB). Blood Brain Barrier describes the precise "microvasculature of the central nervous system." It is a semipermeable border separating the blood circulation from the brain and ECF (extracellular fluid of the CNS). This system of tightly packed endothelial cells (in the capillary wall), astrocytes (covering the capillaries) and pericytes (embedded in the basement membrane of the capillaries) is able to control the movement of substances to and from the brain and blood circulation. Substances such as "molecules, ions, and cells" are screened heavily by the BBB to ensure that no harmful microbes or other destructive substances reach the brain. This system ensures proper neuronal function and protection of the brain and spinal cord against "toxins, pathogens, inflammation, injury, and disease" (Daneman, R, et al. 2015). Since the cells of the BBB are so tightly packed, certain molecules that are too large are unable to pass through to gain access to the Central Nervous System. An example of such large molecules are beta endorphins. They are too bulky to pass through the BBB and therefore cannot be the cause any changes to occur within the brain. So, while endorphins are indeed released during exercise and may staunch peripheral muscle pain that results from exercise, they cannot be the source of any Runners' High or diminished depressive affect (Reynolds, 2015, Fuss, et al. 2015, Dietrich, 2004). Other "methodological confounds" inherent in the endorphin hypothesis are as follows:

1. Firstly, the possibility of cross reactivity is very strong. Cross reactivity occurs when two antigens are very similar in their amino acid makeup and therefore an antibody raised against one antigen might also bear an affinity for the similar antigen. The antibody is "programmed" to find the first antigen but it also recognizes the second. Beta endorphins

bear almost identical amino acid sequences with adrenocorticotropic hormone (ACTH) and other pro-opiomelanocortins. Therefore, any detecting antibody would possibly cross-react, making it difficult to detect which is which. (Proteintech, 2018) This is a serious confound for scientific experiments involving the authentication of the beta endorphin hypothesis and possibly invalidates much of the proof presented from these experiments. The fact that endorphins have similar structure specifically to ACTH is a further confound, since ACTH is also known to increase with endurance exercise.

2. Furthermore, when beta endorphins bind to a μ opioid receptor, it activates the "endogenous opioid system" that further activates the "analgesic and euphoric properties" inherent in the opiate system. It is also known, however, that symptoms such as "severe respiratory depression, pinpoint pupils, and inhibition of gastrointestinal motility" are all characteristic of endogenous opioid activation (Dietrich, 2004). If it were beta endorphins that were activating the opioid system, these symptoms would also be present in runners and endurance athletes. Since they are not, we are forced to reevaluate what it is that truly causes Runners' High.

Endocannabinoids

Researchers and scientists have now turned their attention to endogenous endocannabinoids as the true source of analgesia, sedation, anxiolysis and reduced depression found in endurance exercisers (Dietrich, 2004, Heijnen, et al. 2016). More recent studies are focusing on endocannabinoids and what is it about them that makes exercise "mildly intoxicating" (Reynolds, 2015). Endogenous endocannabinoids are the body's natural chemicals that improve mood and reduce nociception. They are endogenous lipids that "engage cannabinoid receptors, producing analgesic and euphoric effects just like those of exogenous cannabinoids, namely marijuana (or cannabis - Δ-9-THC ((–)-trans-Δ9-tetrahydrocannabinol;THC) (Lu, et al. 2016, Reynolds, 2015). While we now know that opiate endorphins mediate analgesia on a peripheral level, we have seen that there are still analgesic effects that occur irrespective of a working opioid system. This supports the evidence that cannabinoid induced analgesia also takes place at a central level (Dietrich, 2004). Endocannabinoids are what tell the hypothalamus to induce the release of endorphins. Therefore, any analgesic effects that endorphins do have are actually mediated by endocannabinoids (Heijnen, et al. 2016). Unlike endorphins, endocannabinoids are small enough to cross the Blood Brain Barrier and bind to brain receptors, thus enabling them to induce peripheral as well as central effects (Fuss, et al. 2015). An interesting feature of cannabinoid function is that way they are produced. Classic neurotransmitters are created ahead of time and stored in their respective synaptic vesicles for whenever they might be needed. Cannabinoids, on the other hand, are created and released almost immediately on demand. When called upon, for example, by G protein coupled receptors or during depolarization, endocannabinoids will be "liberated in one or two rapid enzymatic steps and released into the extracellular space" to produce a rapid effect on the system (Lu, et al. 2016). There are two types of identified endocannabinoids, CB1 and CB2. CB1 can be found in the CNS, "densely concentrated" in the cerebral cortex, hippocampus, basal ganglia; amygdala, hypothalamus and cerebellum (Dietrich, 2004). Most CB1 receptors are found on axon terminals and pre-terminal axon segments, not on the active zones (Lu, et al. 2016). This further elevated endocannabinoids over endorphins, for while endorphins cannot even cross over in to the brain, endocannabinoids are located in the places one would assume pain relievers and analgesics would be found. The hippocampus is a site for memory, as well as neurogenesis which reduces depression; the amygdala regulates the fear response; the hypothalamus is the "master gland" of the brain and orchestrates all incoming and outgoing activity. The cerebral cortex is a massive conglomeration with numerous functions that would be affected by endocannabinoids. The frontal lobe of the cortex cares for "higher executive functions" such as "emotional regulation, planning [and] reasoning…" The parietal lobe is responsible for sensory integration such as touch, temperature and pain perception. Thus, is makes sense that these areas would abound with CB1 receptors. CB2 receptors are mainly located in the PNS, expressing themselves on immune cells, microglia and vascular elements to protect the CNS. (Lu, et al. 2016) There are also cb1 receptors on peripheral nerve terminals. For example, "pain sensing [small diameter] C fibers, large diameter Aß and Aδ fibers… [and] dorsal root ganglia" (Dietrich, 2004). Therefore, we see that ECBs are synthesized both centrally and peripherally, unlike endorphins which hare only synthesized in the Peripheral Nervous System. The possibility of a third cB receptor is currently being investigated and may turn out to account for some of the Runners' High effects.

Chief ligands of CB1 and CB2 receptors are anandamide (arachidonoyl ethanolamine) and 2-arachidonoyl glycerol (2-AG) (Lu, et al. 2016). They are derived from fatty acid derivatives. Anandamide is known to bind with cb1 receptors over cb2 receptors, indicating higher central activity over peripheral activity (Dietrich, 2004). Anandamide (AEA) is a "highly lipophilic" fatty chemical and therefore can easily cross in to the brain proper (Heijnen, et al. 2016). This would implicate anandamide in activating analgesic effects in the brain (Dietrich, 2004). AEA increased gradually when cortisol is released during the HPA Axis, demonstrating a role in regulation of stress and amygdalar over excitability (Heijnen, et al. 2016). Rats showed an increase in hippocampal anandamide after running on a wheel for an extended period of time, indicating that anandamide plays a role in exercise

and homeostatic regulation (Fuss, et al. 2015). Additionally, anandamide acts as a vasodilator and produces hypotension, facilitating blood flow during exercise (Dietrich, 2004).

Anandamide (AEA) also engenders elevated levels of BDNF (Brain Derived Neurotrophic Factor) during exercise and return to baseline levels post-exercise. As mentioned above, BDNF is part of the neurotrophin family and plays a strong role in exercise-induced neurogenesis. In addition to promoting positive affect due to neurogenesis, BDNF also "exerts beneficial effects on cognition through its ability to enhance neurogenesis, synaptic plasticity and long-term potentiation, the basis of learning." Therefore, enhanced release of AEA due to exercise stimulates BDNF to stimulate heightened cognition and learning. Indeed, psychological stress and mood disorders have been found to reduce BDNF levels in the brains of rats and mice. Physical stress, or aerobic exercise, does just the opposite. A study among an elderly population showed that "moderate intensity walking" over a year served to intensify BDNF levels, further causing task switching functioning to remain constant. The control group of sedentary elderly showed a diametric decline in BDNF and in task switching as well (Heijnen, et al. 2016). Other studies have shown that aerobic activity "ranging from 20 to 90 min of 40–75% of maximal power output or 40–60% of VO2max or 75% of maximal heart rate" causes noticeable increases in BDNF levels (Heijnen, et al. 2016). Thus endocannabinoids play a role in cognition and mood as well.

Scientists noted that there were elevated levels of both endorphins and endocannabinoids in the plasma of mice, post run (Reynolds, 2015). In one experiment, the cB receptors in mice were blocked, noticeably inhibiting the Runners' High that is usually induced post run. The mice were anxious and sensitive to pain perception. Blocking the endorphin receptors had no effect and the mice experienced the calming and antinociceptive effects of runners' high as usual (Fuss et al., 2015, Reynolds, 2015). A similar study also involved lab mice running in a wheel. First off, increased running directly increased levels of endocannabinoids, effectively inducing a reduction in anxiety and pain. Furthermore, ablation of the mice's cb1 receptors completely inhibited the runners' high effect, eliminating anxiolysis. When the mice were injected with blockers of their cB receptors, the analgesic effects were further inhibited (Fuss, et al. 2015). Yet another study with mice divided the mice into running and non-running groups. The running mice were at ease to spend more time in bright light areas, something anxious mice don't do. This indicated that the running induced sedation. The mice were also tested for pain perception with a hot plate test. The runners did not jump in pain or lick their paws as much as normal, indicating that running induced antinociception, endowing the mice with heightened "thermal pain sensitivity." All of these studies found that reductions in anxiety and elevated sedation in mice are due to increased CB1 receptors in forebrain GABAergic neurons, while antinociception was caused by cb1 and cb2 receptors in the PNS (Fuss, et al. 2015). This antinociceptive effect can even rival that of morphine (Dietrich, 2004).

Another study engaged healthy male athletes running at "varying degrees of intensity" over a period of four days. Each running session induced a 70-80% raise in basal heart rate, and was performed at 50-90% of maximal intensity capacity. A sharp increase in cB1 receptors was found in the "frontal cortex, amygdala, hippocampus, and…hypothalamus" all known to be essential areas that regulate affective homeostasis (Heijnen, et al. 2016). A similar study involved male college athletes exercising on treadmills or stationary bikes at 70-80% maximal heart rate. After about an hour, drastically raised levels of anandamide were measured in their blood circulation (Dietrich, 2004).

Another way that endocannabinoids modulate nociception is by affecting the Periaqueductal Gray system (PAG). The PAG serves an important role in modulating ascending pain transmission, fielding "afferents from nociceptive neurons in the spinal cord [and sending] projections to thalamic nuclei that process nociception." In regard to descending pain inhibition, the PAG inhibits neurons in the dorsal horn of the spinal cord. The major functions of the midbrain periaqueductal gray (PAG) include suppressing pain, fear and anxiety, and promoting analgesia and cardiovascular control" (Bebehani, 1995). "Electrical stimulation of [both] the dorsal and lateral periaqueductal gray system" causes not only bindings to cb1 receptors but a subsequent release of anandamide into circulation. Additionally, an experiment revealed that "subcutaneous injection of the chemical irritant formalin" caused a direct infusion of the ligand anandamide into the periaqueductal gray. This implicates anandamide as a nociceptive agent for central chemogenic pain (Dietrich, 2004).

Upon observation, it can be noted that runners' high is only found in response to endurance exercise, as opposed to exercise in spurts. Sports such as "sprinting…weightlifting…track, soccer, football, tennis [and] basketball" doubtfully engage the endocannabinoid system since they do not involve long term exertion (Dietrich, 2004). Running, dancing or biking are examples of activities that would engage the ECB system and induce a runners' high. As mentioned earlier, endorphins play a role in moderating only peripheral chemogenic pain in the muscles. In addition to the central effects of anandamide, we find that cannabinoids also act similarly to inhibit edema and muscle inflammation that might occur during intense exercise. This moderation is important for endurance athletes because muscle pain is a cause and effect of lactic acidosis, which in turn introduces symptoms like muscle ache, nausea and stomach pain (Dietrich, 2004).

Endogenous cannabinoids and exogenous cannabinoids (marijuana) act in similar fashions. "The psychoactive constituent of marijuana, Δ-(9)-tetrahydrocannabinol (THC), exhibits high affinity for the CB1 receptor, which is densely expressed in brain

regions implicated in the control of emotion and cognition." Therefore, just like marijuana, ECBs can induce sedation, analgesia, reduced anxiety, euphoria and even impaired spatial learning (Dietrich, 2004). This essentially means that prolonged exercise produces the same effects as marijuana.

This leads into the addictive quality of endurance exercise. The endocannabinoid system is closely linked with the dopaminergic reward system of the brain. Dopamine is the brain's pleasure hormone, and dopamine receptors D1 and D2 are modulated by endocannabinoids. The two main ECB ligands anandamide and 2-arachidonoylglycerol (2-AG) proliferate in dopaminergic pathways as a "retrograde feedback system" for GABA nerve terminals. There, they modulate dopamine transmission (Bloomfield, et al. 2016). Additionally, cBs affect dopamine activity by increasing firing rates in the medial forebrain bundle, ventral tegmentum and substantia nigra. In the same vein, withdrawing the cB presence would induce withdrawal symptoms similar to that characteristic of marijuana withdrawal. This is due to reduced firing of the pleasure hormone dopamine. Therefore, endogenous as well as exogenous cannabinoids (CB1) are what cause the influx of dopamine release into circulation, thus causing a feel-good high or a "Runners' High." Just like exogenous ECB cause drug addiction, so too endogenous ECBs engender an addiction to that which brings on the dopaminergic feelings, namely aerobic exercise. This explains exercise addiction; exercise causes elevated levels of ECBs, ECBs in turn cause increased firing of dopamine, inducing an addictive "high." Indeed, it has been found that even just blocking dopamine receptors inhibits Runners' High. Athletes who are forced to refrain from their routine endurance exercise (for example, in the case of an injury) report withdrawal symptoms not unlike those of drug withdrawal (Dietrich, 2004).

Conclusion

The analgesic, euphoric and anti-depressant effects that take hold in response to aerobic exercise have been a major source of contention among the medical, scientific and lay community for decades. The endorphin hypothesis certainly took center stage for quite a time as the source behind what the vernacular aptly termed "Runners' High." However, compelling evidence, experimental proofs and biological veracities have enabled scientists to ascertain that endogenous endocannabinoids are the underlying cause of the many positive effects of aerobic activity. Included in these positive effects are: elevated ACTH to regulate the stress response, heightened BDNF production to promote neurogenesis and neural survival, analgesia, anxiolysis, decreased anxiety and depression, euphoria, enhanced cognition, and numerous others. Endorphins do not cause Runners' High; however, they do engender other positive effects during exercise. Exercise has been seen to induce Runners' High in all ages and stages, be it depressed or emotionally healthy, young or elderly, and male or female. With this knowledge, the proof holds true that enhanced release of endogenous endocannabinoids due to aerobic activity reliably and consistently induces a Runners' High, and thus decreases the depressive symptoms inherent in Major Depressive Disorder.

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The Relationship Between PCOS and Obesity: Which Comes First?

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Abstract

Polycystic ovary syndrome (PCOS) is recognized as the most common reproductive disorder in women. Obesity is believed to play a central role in the development of PCOS, as many women with this condition are reported to be overweight or obese. A strong correlational relationship exists between PCOS and obesity. This paper examines the relationship between PCOS and obesity in order to determine whether PCOS causes obesity as opposed to obesity causes metabolic changes that lead to PCOS. Analysis was conducted by reviewing and comparing many studies related to the topic. Factors such as insulin resistance, hyperandrogenemia and body fat distribution were examined in obese and non-obese PCOS subjects. Most studies included in this review could not conclusively determine whether PCOS contributed to obesity or vice versa. The important points raised in the literature showed that obesity could be an important factor to predict PCOS. In women who are predisposed to PCOS, the metabolic and hormonal issues that are present such as insulin resistance and hyperandrogenism, can lead to weight gain and eventually obesity. Obesity in turn can exacerbate the symptoms of PCOS such as further metabolic issues and reproductive abnormalities.

Introduction

Polycystic ovary syndrome (PCOS) is a common metabolic and endocrine disorder effecting 15- 20% of women of reproductive age. This disorder, originally known as Stein-Leventhal syndrome, was discovered in 1935. Its clinical features include obesity, hirsutism, acne, infertility, and oligomenorrhea. PCOS is also attributed to several hormonal and metabolic disturbances, including increased androgen production and disordered gonadotropin secretion leading to menstrual irregularity, hirsutism, and infertility. Aside from interfering with reproductive function, PCOS also disrupts the metabolism of women, affecting insulin action and β-cell function, increasing the risk for glucose intolerance and type 2 diabetes (Liu et al. 2017; Raisbeck 2009).

The origin of PCOS remains unclear, but research has shown that one of the characteristics of this disorder is the excess production of androgens in the ovaries (Alanbay et al. 2012). Androgen secretion is the result of abnormal response of the ovary to gonadotropins, insulin and insulin-like hormones such as insulin-like growth factor-1 (IGF-1), which enhances LH-stimulated androgen secretion by theca cells. Although the condition is not life threatening, the lack of treatment could lead to more serious health issues in the future, such as increased risk of infertility, dysfunctional bleeding, endometrial carcinoma, obesity, type 2 diabetes mellitus, dyslipidemia, hypertension, and increased risk for cardiovascular diseases (Raisbeck 2009).

Among the risk factors associated with PCOS, overweight (body mass index (BMI) 25–29.9 kg/m2) and obesity (BMI ≥30 kg/m2) have been considered as major contributing factor to overall health concerns among women worldwide. Obesity has also been a determined as a contributing factor to reproductive health problems such as anovulation. As body weight increases, incidence of anovulation also increases significantly. Another contributor to reproductive dysfunction is the accumulation of abdominal fat, indicating a higher risk associated with insulin resistance (IR). IR in obese women has been associated with anovulation and increased androgen secretion (Kuchenbecker et al. 2011).

Several studies relate obesity as a risk factor of PCOS (Reinehr et al. 2005; Soydinc et al. 2013). Some studies report that overweight and obesity incidence in females with PCOS is as high as 80%. The mechanisms by which obesity influences PCOS ' pathophysiology and clinical expression are not fully understood, but obesity is independently associated with IR (Rojas et al. 2014) and sex steroid imbalances that may lead to an increased risk of menstrual irregularities and hyperandrogenemia, similar to PCOS symptoms (Pasquali and Gambineri 2006). On the other hand, others proposed that regardless of physical condition PCOS could occur. PCOS may develop in women with a BMI in any range including both underweight and overweight women. (McEwen and Hartmann 2018). The consistent association between PCOS and obesity suggests a biological basis for this observation. Obesity exacerbates many of the reproductive and metabolic abnormalities associated with PCOS. Considering the close association between PCOS and obesity, the question remains whether PCOS causes obesity or does obesity cause metabolic changes that lead to PCOS?

Method

A critical appraisal of the literature on the topic was conducted. A search for related studies was conducted using the access of Touro's library in various databases, such as EBSCO, ProQuest, PubMed, MEDLINE, CINAHL, Cochrane Reviews, and Sage online publications. Other information was sourced from Google Scholar. Keywords used to obtain the relevant documents included PCOS, obesity, insulin resistance, reproductive health abnormalities and PCOS, metabolic anomalies and PCOS. In addition, a library search was also conducted for references in text books, relevant articles and other research findings using the same keywords.

Discussion

Metabolic Factors

One of the main contributors to PCOS is insulin resistance (IR). Insulin is an important hormone produced by beta cells in the pancreas. It is responsible for the metabolism of glucose reduction of blood glucose levels by stimulating the glucose intake in insulin-sensitive tissue such as those found in the skeletal muscles.

IR is common and an early predictor of metabolic diseases. IR is the "inability of insulin to optimally stimulate the transport of glucose into the body's cell" (McEwen and Hartmann 2018).

Several studies investigated IR among women diagnosed with PCOS regardless of BMI. Fasting insulin levels in both lean and obese women diagnosed with PCOS were determined and compared with control subjects. A total of 64 women with PCOS and 20 healthy subjects were evaluated using anthropometry, oral glucose tolerance tests, and insulin tolerance tests. Glucose levels were then measured using glucoseoxidase, whereas serum C-peptide and insulin levels were obtained using immunoradiometric assays. Insulin sensitivity and β-cell function were derived from fasting values of insulin and glucose or from oral glucose tolerance tests or insulin tolerance tests measurements. Results revealed that β-cell function is elevated in both lean and obese women with PCOS. In obese women suffering from PCOS, reduced values in fasting state-derived disposition index were found, whereas lean women suffering from PCOS were found to have increases in these variables when oral glucose tolerance tests were determined. The authors suggested that insulin hypersecretion may be an important mechanism in the pathogenesis of PCOS. Further, prevention of serious metabolic complications could be achieved with the early screening of impaired insulin action and secretion in women with PCOS (Vribikova et al. 2002).

Under normal conditions, the relationship between insulin secretion and sensitivity is constant to maintain normal glucose tolerance. If the sensitivity of insulin varies, the secretion of insulin also changes. Obese and lean females with PCOS have lower production of insulin sensitivity compared to the amount of insulin secreted in response to blood glucose levels than weight-matched healthy women (Dunaif 1999).

Another study investigated the association of IR to the increased risk for cardiovascular disease among women with PCOS. They examined the effects of IR on myocardial microcirculation and peripheral artery function in patients with PCOS. A total of 55 women (28 with PCOS without IR, 18 with PCOS and IR, and 11 normal controls) participated in the study. All subjects were examined using high-resolution vascular ultrasound and real time myocardial contrast echocardiography (RTMCE). Results indicated that women with PCOS and IR had depressed replenishment velocity and myocardial blood flow reserve, but no changes in endothelial dysfunction or intima–media thickness. By contrast, women with PCOS but without IR were found to have isolated depression in replenishment velocity, suggesting that this condition could be an early indicator of myocardial flow abnormality (Aldrighi et al. 2015).

IR and hyperinsulinemia are exacerbated in obese individuals. The accumulation of excess intra-abdominal fat increases IR because of its sensitivity to lipolysis and releases more free fatty acids in the circulation and produces several cytokines (i.e. tumor necrosis factor-α [TNF-α], IL-6, leptin, resistin) that occur in IR (Carpentier 2008). Circulating free fatty acids can accumulate in non-adipose tissues, causing lipotoxicity and insulin resistance. In obesity, IR is also related to TNF-α that enhanced serine phosphorylation of IRS-1 and inhibits insulin receptor signaling (Hotamisligil et al. 1996). Furthermore, IR associated to obesity induces leptin resistance and reduced adiponectin levels, which are two factors that may reduce fatty acid oxidation and promote lipotoxicity (Carpentier 2008).

The mechanisms causing insulin resistance in PCOS have many similarities with those seen in relation to visceral adiposity (Kabir et al. 2005). Excess free fatty acids derived from lipolysis/hydrolysis of acylglycerol in adipocytes accumulate in the hepatic portal veins, and this induces hepatic dysfunction. This condition contributes to elevated glucose secretion, stimulates pancreatic insulin secretion and glucose uptake in adipose tissue (Bergman et al. 2000). This specific insulin resistance, also known as hepatic insulin resistance, is only present in obese women with PCOS and not in healthy women of comparable body weight (Dunaif 1999). The results of these studies demonstrate only a correlational relationship rather than a causal one between obesity and PCOS on glucose production, which may highlight an important factor of the pathogenesis of glucose intolerance.

Another approach was taken to determine the effects of insulin resistance on PCOS. Researchers investigated the relationship between neuropeptide Y (NPY) and insulin resistance. NPY enhances appetite and is structurally and immunologically similar to the pancreatic polypeptide, which has 36 amino acids. NPY is found in the central and peripheral nervous system. NPY controls nutrient intake and body weight. NPY also has an inhibitor effect on the hypothalamohypophyseal-ovarian axis. A total of 45 patients with PCOS and 44 healthy reproductive age individuals participated in the study. At early follicular phase in patients with PCOS, insulin, fasting blood sugar, follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, testosterone, dehydroepiandrosterone sulfate (DHEA-S), thyroid stimulating hormone (TSH), cortisol, estradiol, and NPY levels were determined. Meanwhile, insulin, fasting blood sugar, prolactin, DHEA-S, TSH, cortisol, and 17-OH progesterone levels were obtained from the control group. Fasting insulin levels and homeostatic model assessment for IR (HOMA-IR) were more elevated in obese patients with PCOS than in patients with normal weight and healthy controls. NPY levels were also higher in obese-overweight women with PCOS compared with the control and normal weight patients, but they were not statistically significant. NPY levels did not differ in patients with and without IR (Koseci et al. 2019).

A study confirms the strong association between metabolic aberrations such as IR and PCOS. IR is clearly manifested among women with weight gain and who are genetically predisposed to develop PCOS. Conversely, weight reduction of women with PCOS reduces the negative impact of PCOS. Excluding obesity and fat mass, PCOS is related to metabolic aberrations

such as IR, dyslipidemia, and non-alcoholic fatty liver disease. Mechanisms attributed to the development of IR in PCOS have been established in previous studies. For example, the relationship between visceral adipose tissue and IR has been proven in other studies. Further investigation suggests that abdominal mass in women is proportional to the total fat mass regardless of PCOS status. This condition alone cannot completely explain the occurrence of IR in PCOS, however, fat distribution in women suggests an explanation as to why IR worsens as women with PCOS gain weight (Barber et al. 2016).

Biochemical Indicators

Many studies have used biochemical indicators as an approach to better understanding PCOS. One examined the impact of hyperandrogenemia on IR. Research has shown that impaired metabolism of glucose and IR is partially explained by high androgen concentrations. Women with PCOS have both hyperandrogenemia and IR. By activating androgen receptors, testosterone caused IR in subcutaneous adipocytes. This impairs glucose metabolism due to defects in downstream protein phosphorylation, kinase-C protein, which usually mediates insulin's impact on glucose transport (Corbould 2007).

In another study, the rationale for IR in PCOS patients was investigated. Adipocytes of women with PCOS had enhanced glycogen synthase kinase-3 (GSK3) action. Consequently, insulin-stimulated glucose transport was impaired, and IR developed. Furthermore, overexpression of GSK3 promoted androgen biosynthesis through direct stimulation of P450c17 enzyme activity (Chang et al. 2008). Results from these two studies verify that continuous exposure to androgens impairs insulin action and contributes to the development of IR.

Androgens have an important role in body composition because they determine the location of fat stored in the body. Androgens influence adipocyte function and distribution by the inhibition of adipocyte differentiation, which modulates lipolysis and lipogenesis (Dicker et al. 2004). In general, males have greater distribution of body fat in the upper portion of the body, whereas females accumulate fat in the posterior region of the body. Among women, distribution of adipose tissue differs between women with and without PCOS. Adipose tissues of women with PCOS have been found to be dispersed viscerally similar to that of males. For a given waist circumference, abdominal subcutaneous fat in women has been found to be higher than that of men regardless of age (Kuk et al. 2005).

To determine the possible developmental origins of PCOS, a model was constructed to trace the pathophysiology of PCOS involving excess androgens (including the signs and symptoms) at every stage of the disease. Androgen excess occurs in a vicious perpetual cycle. The gene expression of excess androgens in utero results in high LH and insulin concentrations, enhancing the enzyme activities in theca cells and encouraging the progression of primordial to preantral and small antral follicles. These interactions elevate androgen levels. The process is completed and revived when a female with excess androgen procreates and bears a female fetus, suggesting the genetic path of the syndrome (Homburg 2010).

Body fat distribution was researched among females with and without PCOS. The subjects were matched for age and BMI. They also looked at the connection between concentrations of androgen, IR, and fat distribution. A total of 31 women with PCOS and 29 healthy subjects as controls participated in the study. Women with PCOS demonstrated higher fat mass value in the trunk and arms. Fat accumulation occurs during puberty in the hips, thighs, and buttocks and is preserved throughout women's fertile stage. However, there is more upper body fat distribution for females who have been diagnosed with metabolic illnesses and PCOS. Excess androgens were ascribed to this disease. As a consequence of obesity, the impact of high androgens is increased due to lower concentrations of sex hormone-binding globulin (SHBG) and greater concentrations of freely circulating bioactive androgens. Insulin increases testosterone's impacts by suppressing SHBG. It functions as a co-gonadotropin and inhibits SHBG's hepatic synthesis excluding sex steroid impacts that contribute to hyperandrogenism. Insulin stimulates the synthesis of ovarian androgen and decreases the concentrations of SHBG circulation due to the high concentrations of complete serum and free testosterone. Consequently, there was more upper body fat distribution among females with PCOS. This research suggests that lowering the distribution and structure of fat may be useful in decreasing PCOS-related metabolic aberrations (Cosar 2008).

The relationship between abdominal and upper-body fat, glucose and lipid metabolism markers, and serum androgens in women with PCOS were investigated. The study included 40 women aged 19-49 years with BMI 18.7 – 53.8 kg/m2. The participants had at least two of the following features of PCOS, namely, oligomenorrhoea or amenorrhea, clinical and/or biochemical evidence of hyperandrogenemia, and polycystic ovaries in ultrasound imaging. All obese subjects had increased abdominal fat. A significant correlation was found between obesity and serum fasting glucose and insulin levels. The same result was found in homeostatic model assessment index of IR. A positive correlation was observed between estimates of obesity and serum triglycerides and between obesity and blood pressure. Subsequently, the researchers confirmed direct positive correlations between free androgen index, body weight and BMI (Kozakowski and Zgliczynski 2013).

Others also recorded the elevated incidence of upper-body obesity as evidenced by enhanced waist circumference and waist-hip ratio compared with BMI-matched control females, confirming the same results. Studies using dual-energy x-ray absorptiometry have disclosed enhanced accumulation of core fat

in females with PCOS in accordance with these results (Douchi et al. 1995). Chronic exposure in females with PCOS to greater concentrations of testosterone may alter the distribution of body fat in these females. Studies of androgen administration in non-obese females to male transsexuals leading to increased visceral fat and adverse impact on insulin sensitivity provide support for this hypothesis (Elbers et al. 1997). In both obese and normal-weight females, exposure to androgens increases visceral fat (Rosenfield 1999). In rats, administering a single elevated dose of testosterone early in life contributes to the growth of insulin resistance and centralization as an adult of adipose tissue mass (Nilsson et al. 1998). Early androgen exposure may adversely affect future distribution of body fat with higher core fat accumulation. Studies of isolated abdominal fat cells from females with PCOS have shown that there is a preferential abdominal accumulation of adipose tissue in both obese and non-obese females with PCOS compared to control females (Dunaif et al. 1992). In obese females with PCOS, femoral adipocytes are fewer than reproductively ordinary females, consistent with a change to the distribution of upper-body fat in females with PCOS. Observation of the increased distribution of visceral adipose tissue in PCOS can be ascribed to the impact of androgens, their metabolism, and tissue-specific steroid receptor expression (Blouin 2009). Since this visceral fat is biologically active (Kuchenbecker et al. 2011), it most probably contributes to further metabolic and endocrine disorders in PCOS.

Women with upper-body obesity have PCOS symptoms such as reduced sensitivity to insulin and are at greater danger of cardiac illness and diabetes (Vague 1956). It was observed that in females with upper-body obesity, the incidence of diabetes, hypertension, and atherosclerosis was greater than in lower-body obesity. Furthermore, it has been noted that the incidence of upper body obesity rises in females after menopause and females with upper body obesity tend to have hyperandrogenic characteristics such as hirsutism.

PCOS is defined by anomalies in the hormone releasing gonadotropin (GnRH), a pulse generator that leads to a rise in the release of LH over the follicle stimulating hormone (FSH). These abnormalities are obesity-independent. Healthy obese females have no defects in levels of 24-hour LH and FSH. Excess insulin stimulates hypothalamic GnRH secretion up to a certain point, thus inducing gonadotropin secretion (especially LH) from pituitary cells, which in turn stimulates the development of androgen in the ovaries. When hyperinsulinemia is found, the pituitary is led to secrete big quantities of LH, which tends to boost the LH / FSH ratio. LH stimulates androgens synthesis in the ovaries, and an absence of FSH impairs the aromatization of androgens in granulosa cells to estrogens. These modifications cause the tiny ovarian follicles to grow and hinder the maturation needed for the development of the dominant follicle that then appears as polycystic ovaries (Dunaif 2003).

Obesity and PCOS

Although it is true that obesity is a risk factor, the disease has also been diagnosed in lean females, although reproductive issues are generally discovered more frequently in obese females, regardless of PCOS. Obese females were more probable to have irregular menstrual cycles and anovulatory infertility compared to ordinary weight females. The risk of anovulatory infertility rises by 24 kg / m2 at BMI and continues to increase as BMI rises. But even a slight proportion of decrease in body fat can restore these women's menstrual cycles.

To understand which comes first, obesity or PCOS, studies have investigated this relationship in girls and adolescents. It was noted that girls with a high BMI in childhood had an increased risk of oligomenorrhea and a diagnosis of PCOS in young adulthood (age 24), yet the possibility that features of PCOS were already present in these girls cannot be excluded. The researchers investigated if PCOS (or its features) in adolescents is predictive of later class III obesity. Despite not using pelvic ultrasonography, PCOS was diagnosed using the Rotterdam criteria in 12 (40%) of 30 oligomenorrheic girls at age 14 years. Of these girls, 33% displayed class III obesity by 24 years of age versus 8.4% of girls without PCOS. Other predictors of class III obesity included low sex hormone binding globulin (SHBG), oligomenorrhea, high childhood insulin levels, increased cFT and MetS, all of which are recognized as PCOS phenotypes (Glueck et al. 2011). Meanwhile, others conducted a prospective study on 244 randomly selected postmenarchal girls from a large population-based birth cohort to investigate the influence of obesity on the development of abnormal ovarian morphology. They found PCOS in 61.1% of the obese girls, but only in 32.1% of the normal- weight girls, suggesting that obesity is a contributing factor (Hickey et al. 2011). These studies illustrate that obesity and PCOS are correlative in their pathogenesis.

Additional trials explored the differences between obese and nonobese Croatian females with PCOS in clinical, hormonal and metabolic characteristics. The research included a total of 74 obese and 208 nonobese females with PCOS. Obese females with PCOS were discovered to be at greater danger of developing oligomenorrhea, but at a lower risk of developing hirsutism and acne. Furthermore, obese subjects were more likely to develop hyperandrogenemia, insulin resistance, hypercholesterolemia, hypertriglyceridemia, and high serum CRP concentrations than nonobese females. Among obese females with PCOS, metabolic problems were more probable to happen than their healthy counterparts (Baldani et al. 2013).

Especially in females with upper-body obesity, the discovery of enhanced androgen production in obese females was revealed. Androgen clearance rates have also risen, however, the circulation of bioavailable androgens remains within the normal range. Similarly, due to hyperinsulinemia, SHBG concentrations are decreased in this state. SHBG concentrations in females

with or without PCOS are negatively associated with the circulating insulin concentrations or with the degree of insulin resistance. Reducing insulin concentrations with diazoxide in obese PCOS females, a drug that only reduces insulin secretion without altering insulin sensitivity, has been discovered to increase SHBG concentrations. This finding indicates that insulin may directly suppress the secretion of liver SHBG and compensatory hyperinsulinemia as opposed to IR, explaining low concentrations of SHBG in obese females with PCOS. Due to reduced SHBG secretion induced by insulin, hyperandrogenemia is further exacerbated. Consequently, obesity-related hyperinsulinemia is a significant contributor to ovarian production of androgens in PCOS. By directly stimulating steroidogenesis in ovarian theca and granulosa cells, hyperinsulinemia may lead to hyperandrogenemia. Similar studies have been done on mice and have shown that liver and muscle cells display insulin resistance during constant hyperinsulinemia, while insulin receptors remain sensitive in pituitary and ovarian cells, an adaptation that improves the secretion of pituitary hormones and ovarian androgen production (Nestler, et al 1991). This observation was used as a model to describe the insulinemic contribution to the advanced androgen production to PCOS in obese women.

A systematic and meta-analytical review on existing literature was conducted to determine the prevalence of obesity among women diagnosed with PCOS. Moreover, the researchers intended to determine whether ethnicity, geographic regions, and the diagnostic criteria of PCOS had confounding effects on this relationship. A total of 106 studies conducted before 2010 were included in the review. Among them, only 35 studies were included in the meta-analysis because the rest did not include a control group. They found that compared to non-obese women, obese women with PCOS were more likely to have poor clinical reproductive presentation. Evidence also suggested that PCOS contributed to obesity. Increased androgen levels in women regardless of PCOS status were found to affect the appetite for high-fat and carbohydrate rich foods. However, other metabolic factors such as hyperinsulinemia, reduced postprandial thermogenesis, and basal metabolic rate and alterations contributed to weight gain in women with PCOS. However, the question remains whether PCOS contributed to obesity or vice versa. Some studies showed that women with PCOS had a greater tendency to accumulate fat in the upper body. This effect had also been found even in normal weight women. Overall, it was concluded that women with PCOS were more likely to be overweight or obese compared to healthy counterparts. Caucasian with PCOS had greater risk for obesity than Asian women with PCOS (Lim et al. 2012).

Conclusion

In most health issues around the world, obesity is a significant characteristic. Such observation does not exclude PCOS. However, most of the research in this review has not been able to determine conclusively whether PCOS contributed to obesity or vice versa. However, the significant points raised in the literature showed that PCOS could be predicted by obesity as a significant factor. Overweight or obese females were at a higher risk of PCOS than ordinary weight females, but the weight problem does not exclude ordinary weight females from getting PCOS and its problems.

Researchers found that an elevated BMI among girls during childhood increases the risk of oligomenorrhea and a diagnosis of PCOS in young adulthood (age 24). They also investigated whether PCOS or its features were present in these adolescents, which could predict later class III obesity during adulthood. Among 12 of 30 oligomenorrheic girls at age 14, 33% of the girls were predicted to have class III obesity in a decade compared to 8.4% without PCOS (Glueck et al. 2011). Another study found PCOS in 61.1% of the obese girls, but only in 32.1% of the normal- weight girls, suggesting that obesity is a contributing factor (Hickey et al. 2011). These studies illustrated that obesity and PCOS are correlative in their pathogenesis.

By contrast, it was found that both lean and obese women with PCOS had elevated βF regardless of BMI. Lean PCOS subjects had elevated DI values when OGTTs were determined. Aside from body weight, insulin hypersecretion could be an important mechanism in the pathogenesis of PCOS. Early screening of insulin action in women with PCOS regardless of BMI could prevent serious metabolic complications (Vribikova et al. 2002). Meanwhile, others found that both obese and lean women with PCOS have lower product of insulin sensitivity compared to the amount of insulin secreted in response to blood glucose levels than weight-matched healthy women (Dunaif 1999).

Nevertheless, being overweight or obese is a significant contributory factor that aggravates the conditions of women with PCOS. IR among women with PCOS had been found to increase the risk of cardiovascular disease. Women with PCOS and IR demonstrated depressed replenishment velocity and MBFR, but no changes in endothelial dysfunction or IMT. However, women with PCOS but without IR were found to have isolated depression in replenishment velocity, suggesting that this condition could be an early indicator of myocardial flow abnormality (Aldrighi et al. 2015).

Other studies indicated that only a correlational relationship (rather than a causal one) exists between obesity and PCOS on glucose production. It was noted that IR and hyperinsulinemia are intensified in obese individuals. Accumulation of excess intra-abdominal fat increases IR because of its sensitivity to lipolysis and releases more FFAs in circulation and produces several cytokines (i.e. tumor necrosis factor-α [TNF-α], IL-6, leptin, resistin) that occur in IR (Carpentier 2008). The mechanisms causing IR in PCOS have many similarities with those seen in relation to visceral adiposity (Kabir et al. 2005). A specific IR

also known as hepatic IR is only present in obese women with PCOS (Dunaif 1999).

Researchers concluded that evidence in the literature suggested that PCOS contributed to obesity. Increased androgen levels in women regardless of PCOS status were found to affect the appetite for high-fat and carbohydrate rich foods. However, other metabolic factors such as hyperinsulinemia, reduced postprandial thermogenesis, and basal metabolic rate and alterations contributed to weight gain in women with PCOS. They also noted that women with PCOS were more likely to be overweight or obese compared to healthy counterparts. Caucasian with PCOS had greater risk for obesity than Asian women with PCOS (Lim et al. 2012).

The literature reviewed yielded significant information on PCOS. However, no conclusive evidence pointed to obesity as the main cause of PCOS or PCOS could cause weight gain. Biochemical and metabolic features that were impaired because of PCOS were suggested in these studies. Metabolic rate anomalies found in women with PCOS were more likely to cause their weight gain. Aside from weight gain, other PCOS features such as insulin hypersecretion, overproduction of androgens, reduced SHBG, relationship between IR and myocardial flow abnormality have been mentioned as contributing factors to more serious health issues among women with PCOS.

PCOS is not an exclusive condition for females who are overweight or obese. Normal weight females also exhibited PCOS, especially females deemed to be PCOS candidates due to biochemical and metabolic dysfunctions. Due to the confounding factors connected with body fat and PCOS, weight gain is feasible for these females. Nevertheless, it was discovered that more than half of the females with PCOS studied in this research were overweight or obese. To determine the connection between body mass and PCOS, a more comprehensive research should be performed.

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The Biophysical Study of the Efficacy of Various Phospholipid Membranes on Daptomycin

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Abstract

Daptomycin is an important lipopeptide antibiotic used in the treatment of systematic and life-threatening infections of the skin and underlying tissue caused by Gram-positive bacteria. Calcium and phosphatidylglycerol (PG) must be present on the target cell membrane for daptomycin's mechanism of action to proceed. Calcium and PG also promote oligomerization, a formation that has been assumed to aid in the bactericidal process. The purpose of the experiments conducted was to understand the basic biophysical properties of membrane phospholipids as they exist in their pure and mixed monolayer forms. Furthermore, the experiments conducted attempted to discern how daptomycin penetrates the different lipids that were used. Data collected would be useful for future experiments that aim to understand the naturally occurring bacterial membranes and how daptomycin interacts with them. Using precise biophysical approaches, specifically monolayer studies involving a KSV NIMA-Langmuir Trough and Kibron Langmuir Trough, our lab conducted basic research which could prove to be useful in revealing daptomycin's ambiguity. Preliminary results revealed differences in isotherms between phospholipids with anionic and zwitterionic head-groups. Further data collected revealed daptomycin's degree of insertion in phospholipids with and without the presentation of calcium. Given the limitations of our retrospective studies, additional studies are needed to make definitive evaluations with these results. Because resistance to daptomycin is rising, it is particularly imperative to conduct further research to understand its unsolved mechanism of action.

Introduction

Daptomycin is a lipopeptide antibiotic used clinically against various Gram-positive bacterial infections. It is a naturally occurring compound found in the soil saprotroph streptomyces roseosporus. Daptomycin was approved for clinical use in the United States in 2003 to treat complicated skin infections such as methicillin-resistant staphylococcus aureus, commonly known as MRSA. The molecule is larger and structurally more complex than most other antibiotics, with its mechanism of permeabilization still not completely understood (Taylor et al. 2016).

At present, it is believed that daptomycin works by disrupting Gram-positive cytoplasmic membrane function by creating oligomers in the outer membrane of the bacterial cell and then transferring them to the inner layer of the membrane. This causes leakage of ions, such as potassium, which ultimately leads to loss of membrane potential that is fatal to the cell (Pogliano et al. 2012).

The most current research suggests that the antibiotic binds easily to cluster lipids (lipids with short, branched, and/or unsaturated fatty acyl chains). During the binding, the cell membrane structure alters significantly causing many peripheral membrane proteins, especially phospholipid synthase PlsX and lipid II synthase MurG, to lose their ability to bind effectively (Muller et al. 2016). This provokes rapid cell membrane depolarization and a potassium ion efflux, which effectively causes DNA, RNA, and protein synthesis cessation (Steenburgen et al. 2005).

Although the mechanism of action of daptomycin has yet to be elucidated, it was discovered that daptomycin is calcium dependent (Zhang 2015, Ho et al. 2008). Additionally, binding and oligomerization are mediated by phosphatidylglycerol, or PG (Taylor et al. 2017). A lack of PG can result in bacterial resistance. Phosphatidylglycerol (PG) is anionic at a neutral pH. Because daptomycin is negatively charged, its insertion into an anionic material would be inhibited. However, it has been shown that calcium will facilitate the interaction of daptomycin within anionic membranes by acting as a middleman, or electrochemical bridge, to assist binding of oligomers of daptomycin to the membrane (Zhang 2015, Ho et al. 2008, Taylor et al. 2017).

Cardiolipin (CL) is a membrane lipid associated with bacterial resistance to the antibiotic. The reason for this is that CL confines daptomycin to the outer membrane leaflet, thus preventing the drug from penetrating the inner membrane leaflet and hindering pore formation. It was also discovered in previous studies that CL reduces the number of oligomer subunits by approximately half. Subsequently, bacteria may become more resistant to daptomycin by increasing the content of CL in their cell membranes. However, inhibition of pore formation may not suffice to prevent bactericidal action. This is because daptomycin may damage bacteria by more than one mechanism, which has yet to be proven (Zhang 2014).

Focus in the laboratory enhanced our understanding of daptomycin and how it interacts with various lipids. A phospholipid layer arranges itself so that its polar, hydrophilic head group cooperates with the water. The fatty acid hydrocarbon tails act in a hydrophobic manner by resisting the water and directing themselves toward the nonpolar air. Daptomycin is amphipathic, which means it contains both polar and nonpolar parts. When injected, it can either interact with the water or the membrane. This certainly aids the antibiotic in integrating effectively and quickly with a bacterium because it does not require a carrier to enter the cell. Studies show that Daptomycin is effective in as little as one hour and this can be attributed to its quick insertion into the cell (Steenburgen et al. 2005).

It would be necessary to research how many molecules of daptomycin interact with the water, and how many molecules interact with the membrane. If the antibiotic does, in fact, insert into the membrane, an increase in surface pressure would be expected due to a more compact and crowded surface. Based on pressure, it can be determined how effectively daptomycin inserted.

The lipids used in our laboratory were sought to mimic the

bacterial cell membrane. The primary subphases used in our studies and in other studies as well were Ultrapure water and buffer. The buffer was used as a comparative subphase commonly exposed to bacteria and blood in the human body. pH, in both subphases, are similar enough to suggest that no inconsistency will result, even with the slight differentiation between a pH of 7 for the water and 7.4 for the buffer. Langmuir monolayer studies provided us with the information necessary to determine the nature of the lipids and how daptomycin interacts with the various lipids.

Langmuir monolayer studies provide useful information other than just surface pressure. Despite the 2-D nature of the monolayer, the molecules in monolayers exist in states that are analogous to that of the 3-D nature of liquids, solids, or gases. Just as changing the pressure of a gas can change its phase, changing the surface pressure of monolayer can do so as well. Surface pressure is a measure of the various forces between the molecules of a monolayer. As pressure is often plotted against volume at a constant temperature, so too surface pressure is plotted against the mean molecular area. The mean molecular area is the average free space each molecule in a monolayer must occupy. It can be viewed as the analog of volume- just as surface pressure is the analog of pressure. As the molecules get closer, increased interactions change the surface pressure. A surface pressure-area isotherm gives information about a monolayer such as its phase and degree of interaction between the molecules. When the molecules in a Langmuir monolayer are spread very far apart, they exert little force on one another. This, conceptually, is similar to that of a gaseous state. In the gas phase, surface pressure is almost undetectable. The behavior of the gaseous phase is treated as a parallel to the ideal gas law, where PV=nRT. As the monolayer is condensed, the molecules can enter a transition into the liquid state. There are two stages in the liquid phase- the liquid-expanded phase (LE phase) and the liquid-condensed phase (LC phase). In the LE phase, the π-A isotherm displays some curvature and liftoff from π=0. As compression progresses, the molecules enter a coexistent LE-LC phase, where the LE and LC phases are at equilibrium with each other, displayed by a somewhat horizontal line. The LC phase is typically marked by a steep slope, when the molecules are compressed even further. Increased interaction between the tails occur, thereby causing a more "solid-like" phase to transpire. Further compression of the monolayer can result in an actual solid-like phase. However, further compression is extremely difficult and the molecules strongly interact with each other until they reach the final phase where they collapse. The place of collapse informs us of the extent of pressure the molecule can withstand (Zhang 2014).

Materials and Methods

All experiments took place on a Langmuir Trough, which consisted of movable barriers. The Langmuir trough is filled with a liquid called the subphase, typically water or buffer. The lipids were deposited on the surface dropwise. This dropwise technique is called "spreading" and involves a certain degree of manual skill (Zhang 2014). Movable barriers compress and expand over the spread monolayer, altering the density of the molecules on the surface. This change in density resulted in changes in the surface pressure, which was detected and evaluated.

To ensure that the machine would consistently hold pressure, the barriers were tested and then assigned to lie in a specific orientation during every experiment. The softwares used along with our MicroTrough were the complementary KSV NIMA and Kibron softwares (Figures 1A and 1B). Ultrapure, distilled, and R1 water were used for both cleaning (the machines and the probe) and for the subphase. Ultrapure water and R1 were obtained through the Millipore Direct-Q 3 with UV Ultrapure. The KSV NIMA Langmuir trough was disassembled and thoroughly cleaned with R1 water, ethanol, and rinsed off with R1 once more before and after every run. The probe was rinsed with R1, ethanol, and R1, as well. Once water or buffer was added to

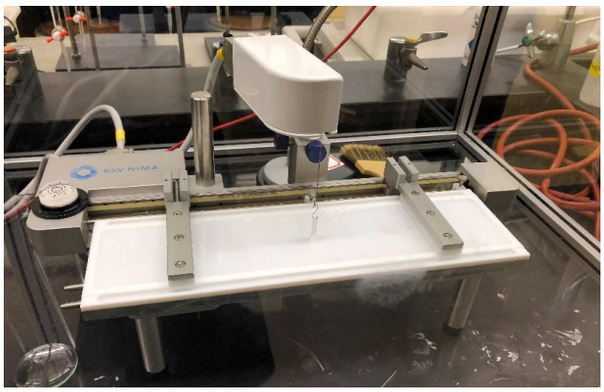

Figure 1A. KSV NIMA Langmuir Trough used in our laboratory for the experiments conducted.

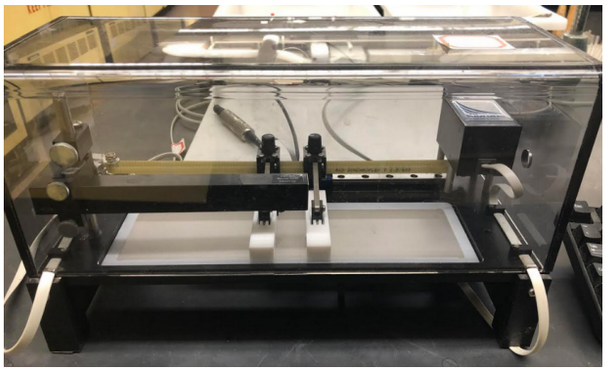

Figure 1B. Kibron Langmuir Trough used in our laboratory for the experiments conducted.

the trough, the machine was calibrated by zeroing the balance. The probe was raised out of the subphase to verify accurate reading, after which it was inserted back into the subphase. The surface was aspirated to remove any foreign substances. The Kibron Langmuir trough followed a similar protocol, however,

the probe was sterilized via a bunsen burner.

In addition to the standard trough used to carry out the procedures in the laboratory, a modified trough was used, as well. This trough contained a reduced and depressed area upon which the subphase could reside. It included a small cavity that was useful for insertion of daptomycin into the subphase. The cleaning measures did not differ from the standard procedures. Buffer or water served as the subphase and was applied to the trough. A mini stirrer was positioned within the indented area in order to stir the subphase. Lipid was meticulously applied to the surface until a surface pressure of 5 mN/m or 10 mN/m was achieved. Following that, 16 µl of 10 mM daptomycin was injected into the subphase, using a Stuart Pipette. Once stabilization of surface pressure was attained, 5 µl of 1.6 M calcium buffer solution was injected into the subphase using a Stuart Pipette with a changed tip.

Daptomycin was acquired from Teva Pharmaceutical. The buffer that was used was a concentration of 100 mM NaCl and 10 mM Hepes, reaching a pH of roughly 7.4. To accomplish this, 2.383 grams of Hepes and 5.844 grams of NaCl were measured out. They were then combined to dissolve until the neck of a 1L volumetric flask filled with Ultrapure water. The solution was then placed in a 1L beaker and evaluated with an electronic pH meter. Via a Pasteur pipette, the pH was adjusted by adding NaOH and HCl, respectively.

All lipids used were obtained in pure form from Avanti Polar Lipids Inc. The lipids were prepared by dissolving 10 mg of lipid in a 10 mL flask containing 3:1 chloroform methanol, resulting in 1:1 mg/mL of lipid. All pipettes and other volume measuring materials were tested for precision and accuracy.

Hamilton syringes were used to apply the lipid solution. Chloroform was used to clean the syringes which were cleaned consecutively approximately 5-10 times. For the KSV NIMA, lipid solution was distributed by using a 50 µL syringe which was filled with 15 µL of lipid. To distribute solution to the Kibron, we used a smaller syringe filled to 8 µL. While administering the lipid solution, the individual applying it would be cautious to do so quickly and with minimal contamination (i.e. breathing, sneezing, and coughing). This is crucial because the machine cannot distinguish between molecules of the lipid and molecules of the contaminant. Start was delayed by 10 minutes to allow for the lipid to spread.

The experiments focused primarily on lipids such as cholesterol, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2- dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3- phospho-(1'-rac-glycerol) (DPPG), 1,2- dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DMPG).

Our preliminary experiments sought to calculate the surface pressure (π) over $Å^2$/molecule for pure cholesterol, pure POPC, pure DPPC, pure DMPC, pure DPPG, 50% cholesterol/50% DPPC, 50% cholesterol/50% DMPC, and 50% cholesterol/50% DPPG. Our research then shifted its focus toward DMPG and its interaction with the daptomycin.

Results/Discussion:

Cholesterol Monolayer

Figure 4 shows the surface pressure-area (π-A) isotherm obtained for a pure cholesterol monolayer. The curvature of

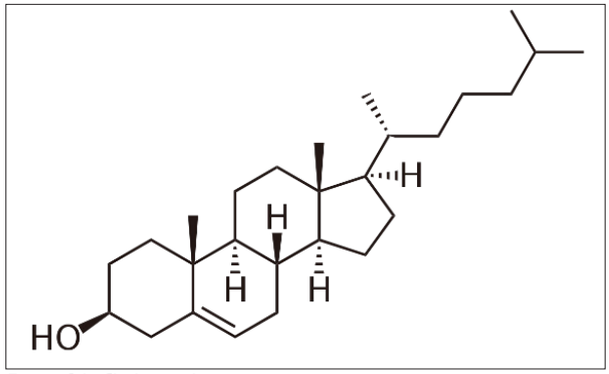

Figure 3A. Cholesterol.

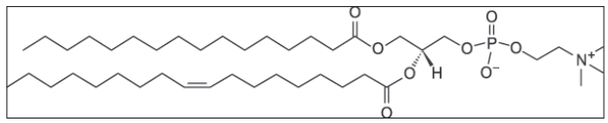

Figure 3B. POPC.

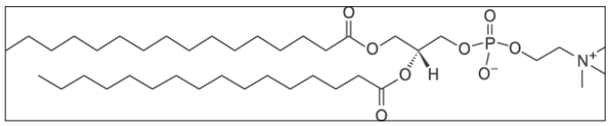

Figure 3C. DPPC.

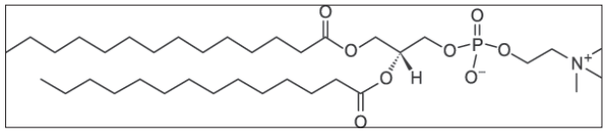

Figure 3D. DMPC.

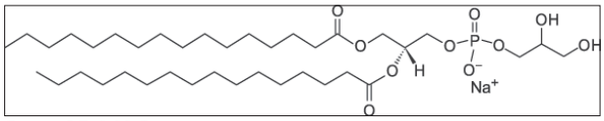

Figure 3E. DPPG.

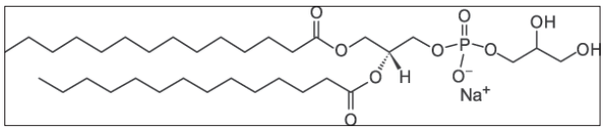

Figure 3F. DMPG.

the isotherm indicates that cholesterol molecules remained in a gaseous state upon compression until they sharply enter a more condensed phase as the compression proceeded.

Following evaluation of our results, it was noticed that a somewhat steep rise in surface pressure occurred at 45.5 Å2/molecule. Collapse occurred at 44 mN/m. The relative domain of cholesterol is quite minimal, attributing to the smaller values of Å2/molecule.

POPC Monolayer

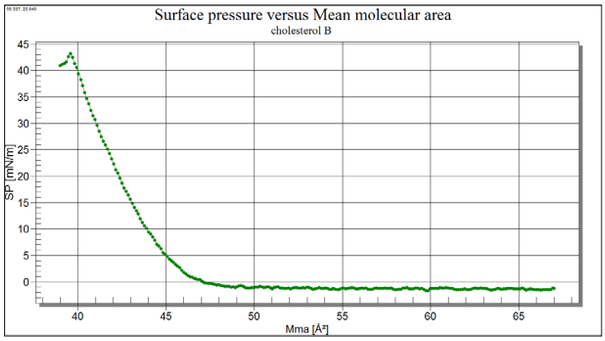

Figure 4. Surface pressure (π, mN/m) vs. area per molecule (MMA) isotherm of cholesterol monolayer on water obtained in our lab.

Figure 5 shows the surface pressure-area (π-A) isotherm obtained for a pure POPC monolayer. Under experimental conditions, POPC exists in a liquid-expanded (LE) phase during compression. Further compression would cause the POPC to exist in a liquid-condensed (LC) phase (Faye et al. 2013). The isotherm revealed a rise in surface pressure at around 107.5 Å2/molecule, with a steady and smooth curve until a collapse at around 44 mN/m.

Figure 5 exhibits the curve of a typical unsaturated molecule. POPC contains a double bond (Figure 3B), rendering it unsaturated. Since unsaturation hinders effective packing of molecules, the isotherm displays a smooth and continuous curve, lacking

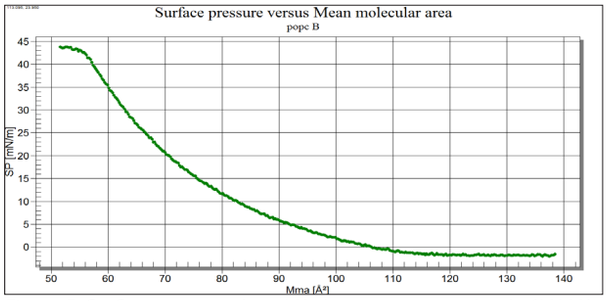

Figure 5. Surface pressure (π, mN/m) vs. area per molecule (MMA) isotherm of POPC monolayer on water obtained in our lab.

phase changes.

DPPC Monolayer

Figures 6 illustrates the surface pressure-area (π-A) isotherm obtained for a pure DPPC monolayer. Five distinct regions, corresponding to different phases, were seen in the isotherm in a varying study (Takeshita et al. 2017). These phases have been assigned as gaseous, LE, the coexistence of LE and LC phases, and the collapsed phases. The isotherm can be interpreted as follows: in the gaseous phase, the molecules are random and disordered, thereby giving rise to insignificant surface pressure. Once in the LE phase, the molecules are more condensed than they are in the gaseous phase, yet they are still considered disordered. In the LC phase, the molecules are more ordered and densely aligned than they are in the LE phase, causing them

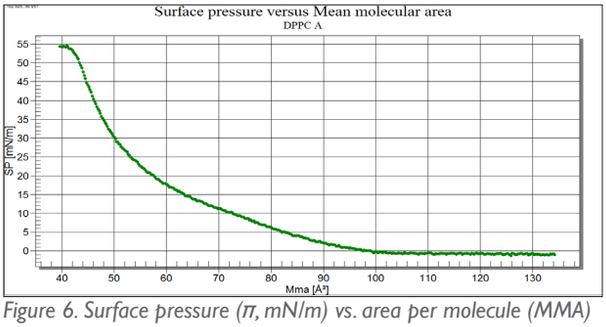

Figure 6. Surface pressure (π, mN/m) vs. area per molecule (MMA) isotherm of DPPC monolayer on water obtained in our lab.

to adopt a somewhat crystalline configuration (Takeshita et al. 2017). Finally, a kink in the isotherm suggests that the monolayer collapsed.

Due to the saturated nature of DPPC (Figure 3C), the tails can pack efficiently against one another, causing a phase change to take place. The isotherm begins with minimal to no surface pressure, which is indicative of the fact that the molecules are in the gaseous phase. The DPPC then entered the LE phase at around 96 $Å^2$/molecule, where the molecules rearranged themselves in a more disordered fashion. Moving onward, it is difficult to determine where exactly the molecules entered the LE-LC phase, although perhaps it occurred from 56-72 $Å^2$/molecule. Nonetheless, that area of the curve is not distinct enough to be certain that the coexisting phase happened then. It seems that the LC phase, i.e. the area where the slope steepened, occurred at 70 $Å^2$/molecule. Finally, the monolayer collapsed at around 55 mN/m.

DPPG Monolayer

Figures 7 displays the surface pressure-area (π-A) isotherm obtained for a pure DPPG monolayer. According to other studies, a "knee" is present at around 75 Å2, until a plateau forms. Above this plateau, a steep rise in surface pressure occurs upon decreasing area. There is an apparent coexistence of the LE phase and LC phase. These observations agree with the typical anionic nature of DPPG (Figure 3E). Although it should be noted that the exact position of these transition phases can vary based on compression rates, subphase conditions, or temperature, as with every experiment (Kim et al. 2012).

Figure 7 displays an increase in surface pressure at 75 Å2/

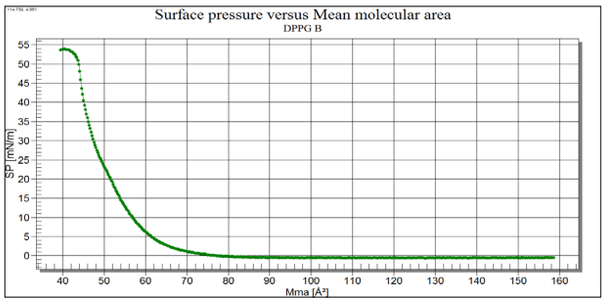

Figure 7. Surface pressure (π, mN/m) vs. area per molecule (MMA) isotherm of DPPG monolayer on water obtained in our lab.

molecule. It is difficult to tell where the coexisting LE-LC phase resides. Still, the slight elevation from 48-56 Å2/molecule may be attributed to that. The area past 48 Å2/molecule represents the LC phase.

In another study conducted in our laboratory, 1 M daptomycin solution was tested without lipid. No reaction occurred. Then, an additional solution of daptomycin with 4 µL DPPG and 2 µL of calcium was prepared. In this experiment, the (π-A) isotherm increased immediately and dramatically, illustrating that daptomycin requires PG and calcium ions to work effectively.

DMPC Monolayer

Figure 8 establishes the surface pressure-area (π-A) isotherm obtained for a pure DMPC monolayer. As illustrated by the isotherm, the DMPC monolayer exhibits a smooth and continuous curve due to its shorter acyl chain (Figure 3D), characterized by a steady and constant liquid phase for the lipid, lacking phase changes. The results obtained in our lab follow this pattern of DMPC's molecular nature of interaction, with an even curve beginning to rise at 105 Å2/molecule and a collapse at around 47 mN/m.

Cholesterol-DPPC (1:1) Monolayer

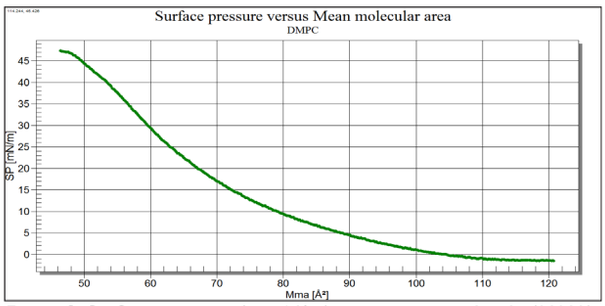

Figure 8. Surface pressure (π, mN/m) vs. area per molecule (MMA) isotherm of DMPC monolayer on water obtained in our lab.

Figure 9 showcases the surface pressure-area (π-A) isotherm obtained for a DPPC:Cholesterol (1:1) monolayer (red curve), relative to pure cholesterol (green curve) and pure DPPC (blue curve) monolayer isotherms. Due to the cohesive forces between cholesterol (Figure 3A) and saturated DPPC (Figure 3C) (Ohe et al. 2007), the isotherm values are lower than those expected by the rule of additivity (Cruz Gomes da Silva et al. 2017), however the values are only insignificantly lower. The rule of additivity informs us of the area that would be occupied if the lipids were to be mixed ideally. The interaction between the smooth hydrophobic part of cholesterol and DPPC is due

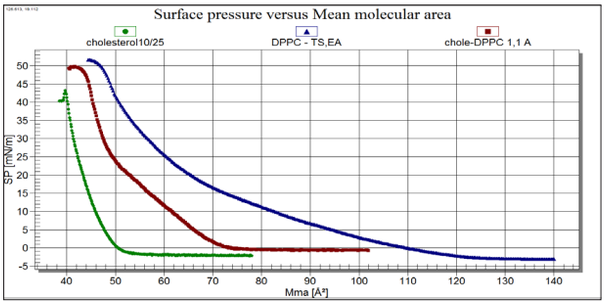

Figure 9. Surface pressure (π, mN/m) vs. area per molecule (MMA) isotherm of DPPC:Cholesterol (1:1) monolayer on water obtained in our lab.

to van der Waals forces (Sabatini et al. 2008). Nonetheless, due to DPPC's saturated and rigid acyl chain, it is difficult for bulky cholesterol to penetrate, thus interfering with the molecular packing and causing DPPC:Cholesterol to occupy less area per molecule at constant pressure, but not significantly.

Additionally, it can be perceived from the results obtained that the collapse pressure for pure cholesterol lies at around 40 mN/m, while pure DPPC lies at 52 mN/m, and DPPC:Cholesterol lies at 50 mN/m. Pure DPPC creates the most stable monolayer since it is able to withstand the highest surface pressure, as indicated by the higher collapse pressure value. DPPC:Cholesterol withstood pressure at a value that lies slightly lower than pure DPPC, due to destabilization of the film via cholesterol.

Lastly, the phase change observed in the DPPC:Cholesterol monolayer at 48-72 Å2/molecule, has a broader range than the phase change observed in the pure DPPC monolayer, occurring at 56-72 Å2/molecule. This is because cholesterol serves as a contaminant or impurity, which broadens the phase change upon compression.

Cholesterol-DMPC (1:1) Monolayer

Figure 10 displays the surface pressure-area (π-A) isotherm obtained for a DMPC:Cholesterol (1:1) monolayer (green curve), relative to pure cholesterol (red curve) and pure DMPC (blue curve) monolayer isotherms. There must be cohesive van der Waals forces present since the isotherm for DMPC:Cholesterol illustrates that the DMPC:Cholesterol occupies less area per molecule at constant pressure, thereby exhibiting lower surface area values than those expected by the rule of additivity. Despite the fact that bulky cholesterol interferes with the saturated

DMPC acyl chain (Figure 3D), the chain is nevertheless shorter

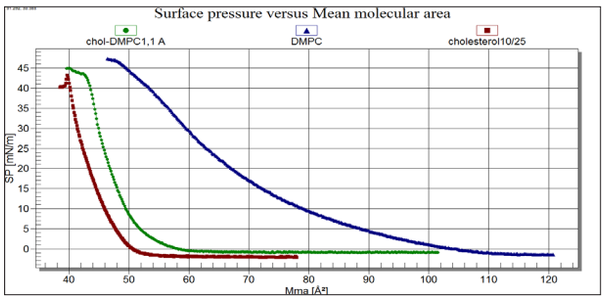

Figure 10. Surface pressure (π, mN/m) vs. area per molecule (MMA) isotherm of DMPC:Cholesterol (1:1) monolayer on water obtained in our lab.

than DPPC's chain. Thus, between DPPC and DMPC, effective packing of cholesterol is more likely in DMPC.

Additionally, it can be observed from the results obtained that the collapse pressure for pure cholesterol lies around 40 mN/m, while pure DMPC lies at 48 mN/m, and DMPC:Cholesterol lies at 45 mN/m. Pure DMPC creates the most stable monolayer since it is able to withstand the highest surface pressure, as indicated by the higher collapse pressure value. Like DPPC:Cholesterol, DMPC:Cholesterol withstood pressure at a value that lies slightly lower than pure DMPC, due to destabilization of the film via cholesterol.

Cholesterol-DPPG (1:1) Monolayer

Figure 11 displays the surface pressure-area (π-A) isotherm obtained for a DPPG:Cholesterol (1:1) monolayer (green curve) relative to pure cholesterol (blue curve) and pure DPPG (red curve) monolayer isotherms. DPPG and cholesterol in the 1:1 mixture are repelling one another, as the DPPG:Cholesterol occupies more area per molecule, thereby displaying higher surface area values than those expected by the rule of additivity. This repulsion is perhaps due to the negative charge present on DPPG and due to its longer acyl chain (Figure 3E).

Furthermore, it can be observed from the results obtained

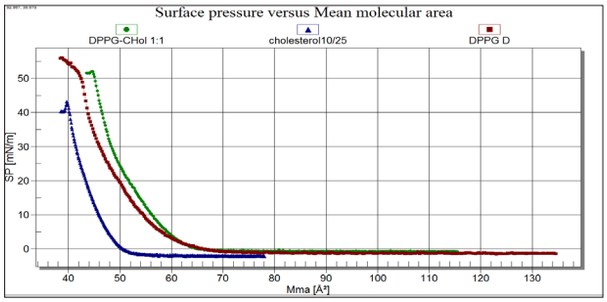

Figure 11. Surface pressure (π, mN/m) vs. area per molecule (MMA) isotherm of DPPG:Cholesterol (1:1) monolayer on water obtained in our lab.

that the collapse pressure for pure cholesterol lies at around 40 mN/m, while pure DPPG lies at 55 mN/m, and DPPG:Cholesterol lies at 52 mN/m. Pure DPPG creates the most stable monolayer since it is able to withstand the highest surface pressure, as indicated by the higher collapse pressure value. Like DPPC:Cholesterol and DMPC:Cholesterol, DPPG:Cholesterol withstood pressure at a value slightly lower than pure DPPG, due to destabilization of the film via cholesterol.

Lastly, the phase change observed in the DPPG:Cholesterol monolayer at 45-58 Å2/molecule, has a more broad range than the phase change observed in the pure DPPG monolayer, occurring at 48-56 Å2/molecule. This is because cholesterol serves as a contaminant or impurity, which broadens the phase change upon compression.

DMPG Monolayer Injected with Daptomycin

Daptomycin was put to the test, as portrayed in Figure 12. For the experiment, DMPG was injected at that start with a steady surface pressure achieved. At around 400 seconds, daptomycin was injected and surface pressure increased to 12 mN/m until levelled out. At 835 seconds, calcium solution was injected and surface pressure increased to 20 mN/m.

Based on our results, it appears that daptomycin penetrated the DMPG at 400 seconds, indicated by an increase in surface pressure, even without the presence of calcium. As discussed, daptomycin is calcium dependent. We would need to continue exploring this

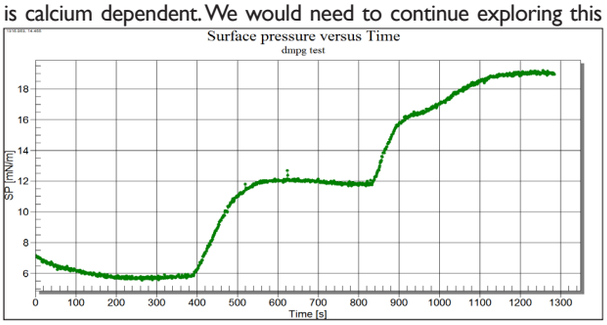

Figure 12. Results of surface pressure (π, mN/m) vs. time (s) interaction of daptomycin with DMPG on subphase of water obtained in our lab. DMPG applied, then daptomycin injected, followed by insertion of calcium solution.

wondrous enigma as to why or how daptomycin inserted into the membrane even when calcium was not yet present.

Conclusion and Future Studies:

The extent to which daptomycin inserts itself into the membrane was observed. How this correlates to effectiveness may be the object of further research subsequent to the findings of the experiments performed. For example, if daptomycin does not penetrate the membrane at all, then a new experiment may be suggested to show that the lack of insertion may have been the cause of ineptitude. On the other hand, if the drug inserts well, then an experiment may be pursued to determine

if successful insertion may be the cause of effectiveness.

Moreover, using the Gibbs Adsorption Isotherm for daptomycin in water, we can determine the concentration of daptomycin in contact with the surface, which in turn alters the surface tension. Additional experiments would have to be conducted to determine such a phenomenon.

Lastly, in keeping with daptomycin's ability to treat MRSA, some mutant strains of MRSA were found to be resistant to daptomycin. As such, analogs of daptomycin may be studied to determine if they might find a way to insert into MRSA with altered surface proteins, since those would be considered mutant.

Acknowledgments

The completion of this study could not have been possible without the mentorship and expertise of Dr. Evan Mintzer. A debt of gratitude is owed for allowing us to use his laboratory in order to conduct the research.

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Is a Whole Food, Plant-Based Diet the Most Effective Treatment of Arthritis?

Elisheva Liberman

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Abstract

Arthritis is a debilitating disease where joint inflammation occurs. There are different types of arthritis, such as Osteoarthritis and Rheumatoid Arthritis. Osteoarthritis is the destruction of the articular cartilage that usually comes with age, whereas Rheumatoid Arthritis is a chronic autoimmune disease. They both have symptoms which include reduced range of motion, pain and swelling. There are various treatments to alleviate the pain and reduce the inflammation of arthritis. Among them are medication, physical therapy, surgery and a whole food, plant-based diet. The whole food, plant-based diet yields the best results and is the most effective and practical.

Introduction

Arthritis is a disease characterized by joint inflammation that causes discomfort and eventually intense pain along with stiffness of the joints. Other symptoms include reduced range of motion, muscle weakness, redness, and swelling. These symptoms can come and go at different intensities. Arthritis is also characterized as a musculoskeletal disorder (Carter & Rizzo 2007). Arthritis is twice as common in women than in men; however, men and children of all ages can experience it as well (Lourdudoss et al. 2018). Though there are various kinds of treatments that can alleviate the pain of arthritis, many say that arthritis cannot be cured. It is a chronic disease that causes pain, but that one can live a lifetime with. Between the years 2013 and 2015, approximately 54.4 million adults had arthritis, of which 23.7 million adults were limited in activity. Arthritis affects approximately a sixth of the American population today and is predicted "to affect 78.4 million people by 2040" (Barbour et al. 2017) (Juyoung et al. 2018).

There are many types of arthritis, some that occur in many joints at once or other types that are isolated in one joint. Arthritis can be found in the ankles, fingers, wrists, back, jaw, and neck. Different causes give rise to different types of arthritis. Osteoarthritis and Rheumatoid Arthritis are two of the most common types. The most rampant musculoskeletal disease amongst the elderly is Osteoarthritis (Hinman et al. 2007).

Osteoarthritis

Osteoarthritis is when the articular cartilage begins to wither away and eventually disintegrates. The articular cartilage allows for painless joint movement through load distribution and reduces friction between the bones. In Osteoarthritis, the bones rub against each other which can cause extreme pain. For example, a person with Osteoarthritis of the knee will have difficulty walking and climbing the stairs. Researchers believe that the cartilage destruction has a chemical and mechanical cause; however, the direct mechanism is unknown. Mechanical factors that can increase the risk of Osteoarthritis are obesity, heavy lifting, abnormal loading of joints from trauma, and joint instability (Beasley 2012). Osteoarthritis is asymmetrical, meaning that if one experiences pain in a joint on one side of the body, this does not mean that they experience it in the same joint on the other side. The pain is most intense in the evening and night hours and is a cool pain rather than hot. Along with Osteoarthritis comes crepitus which is a grinding, clicking, or popping movement of the joint accompanied by a sound (Witulski et al. 2014). Osteoarthritis is most commonly found in elderly people, due to the wear and tear of many years of living. It has a relatively slow onset that worsens with age (Azumasa & Misako 2017).

Rheumatoid Arthritis

Rheumatoid Arthritis is different in that it's a chronic autoimmune disease. The body attacks itself causing inflammation of the joints, which overtime erodes the bones. Normally, joints have synovial fluid which provides lubrication. In Rheumatoid Arthritis the synovial fluid becomes less viscous, causing further cartilage damage because of the reduced joint lubrication (Beasley 2012). In contrast to Osteoarthritis, Rheumatoid Arthritis most often occurs symmetrically, meaning that if one has arthritis in the left shoulder, they usually also have it in the right shoulder as well. Further, the sites of Rheumatoid Arthritis are warm, and sufferers experience the most pain during the morning hours. The flare ups of Rheumatoid Arthritis are random unlike the constant pain of Osteoarthritis. The symptoms of Rheumatoid Arthritis can get better, only to worsen again. Rheumatoid arthritis can be found in people of all different ages.

Methods

The Touro College Online Library allowed access to Ebsco and Proquest which were the two databases used to find the proper scholarly articles needed. Analysis of these studies helped develop the answer to whether or not a whole food, plant-based diet is the most effective treatment of arthritis.

Discussion

Treatments of Arthritis

Popular treatments of arthritis include medication, physical therapy and sometimes surgery. Another less popular but interesting approach is a whole food, plant-based diet. Studies have shown that a whole food, plant-based diet can alleviate the pain and possibly cure arthritis. This paper will try to answer the question: is a whole food, plant-based diet actually the most effective treatment of arthritis?

Medication

Although various medications are anti-rheumatic, they come along with many possible negative side effects as well. Names of different arthritis medications are Leflunomide, Sulfasalazine, Methotrexate, Etanercept, Adalimumab, Anakinra, Canakinumab, Abatacept, Infliximab, Rituximab, and Tocilizumab. Leflunomide and Sulfasalazine can be taken orally. Methotrexate can be taken orally or given as an injection. Etanercept, Adalimumab, Anakinra, and Canakinumab all only come in an injection form. Abatacept, Infliximab, Rituximab, and Tocilizumab can only be given intravenously. Some of the common side effects are included below (Table 1). While medication may alleviate the pain of arthritis, the patient may have to deal with other annoying and sometimes painful side effects (Brinkman et al. 2017).

Another downside of taking medication is the cost, which can be very high without insurance. For example, Etanercept costs around one thousand dollars per weekly injection (Brinkman et al. 2017).

Common Medications Used to Treat Arthritis and Their Side Effects

Medication	Common Side Effects
Leflunomide	Headache, diarrhea, upset stomach, cold symptoms
Sulfasalazine	Headache, diarrhea, upset stomach, cold symptoms
Methotrexate	Headache, upset stomach, mouth sores, nausea
Etanercept	Headache, upset stomach, sinus infection, common cold, itchy or allergic rash, redness or soreness where needle enters skin
Anakinra	Headache, upset stomach, sinus infection, common cold, itchy or allergic rash, redness or soreness where needle enters skin
Adalimumab	Headache, upset stomach, sinus infection, common cold, itchy or allergic rash, redness or soreness where needle enters skin
Canakinumab	Headache, upset stomach, sinus infection, common cold, itchy or allergic rash, redness or soreness where needle enters skin
Abatacept	Headache, upset stomach, common cold, sinus or throat infection
Rituximab	Headache, upset stomach, common cold, sinus or throat infection
Infliximab	Headache, upset stomach, common cold, sinus or throat infection
Tocilizumab	Headache, upset stomach, common cold, sinus or throat infection

Table 1. Medications retrieved from www.cincinnatichildrens.org *Arthritis Medication Choice Cards*

Non-Steroidal Anti-Inflammatory Drugs (NSAIDS)

These drugs are preferred to treat arthritis over acetaminophen, aspirin and mild opiates. Many people often take aspirin for arthritic pain and although it mitigates the pain, they get gastrointestinal toxicity from it. This gastrointestinal toxicity inhibits NSAIDs and thus the preferred medical treatment cannot be used (Lisse et al. 2003).

Disease Modifying Anti Rheumatic Drug

Another approach to healing Rheumatoid Arthritis is a modern type of drug known as a disease modifying anti rheumatic drug. This type of drug suppresses the immune system to inhibit the body from attacking itself and causing joint inflammation. It was designed to get rid of pain and tenderness of the joints and improve joint function long term. Yet, this was true for only 10-50% of patients treated with this drug. Many Rheumatoid Arthritis patients still suffer intense pain (Winkvist et al. 2018). Because of this result, disease modifying anti rheumatic drugs do not sound promising.

Physical Therapy

A study was done where the physical therapists did exercises with the arthritis patients. At the end of the treatment period they saw improvement in grip strength. Physical activity improved the functional status of arthritis patients. Some therapists use a paraffin wax bath for distal joints to reduce pain. The moist heat of the wax soothes the joints and makes the exercise of physical therapy less painful (Shinde & Varadharajulu 2017).

This study is not an accurate assessment of a good treatment for arthritis. The study was flawed in that their subjects ranged from ages twenty to fifty-five. While the physical activity may improve the symptoms of younger and middle-aged people, it probably would not have the same effect on the elderly. Elderly bodies are often fragile and worn out. It is harder for old people to recover from diseases. This study does not effectively prove that physical therapy is the most effective treatment for arthritis across the board.

Hydrotherapy

In addition to traditional physical therapy, hydrotherapy, physical therapy done in an aquatic environment, is another treatment for arthritis. During hydrotherapy, physical therapists treat patients through exercises in heated pools. There are multiple benefits of hydrotherapy that standard physical therapy does not have. Firstly, "buoyancy reduces loading across joints affected by pain and allows the performance of functional closed-chain exercises that otherwise may be too difficult on land." Another benefit is that the water movement and pressure can be used as resistance which makes the exercises more effective. Furthermore, pain relief, swelling reduction, and ease of movement increase due to the warmth and pressure of the water. The warm water leads to muscle relaxation, enhancing movement (Hinman et al. 2007).

Electrotherapy

Electrotherapy is another effective form of physical therapy: "Electrotherapy consists of the application of electrotherapeutic procedures (ionization and ultrasound) to the affected regions, with an analgesic, anti-inflammatory and decontracturing indication" (Tosa et al. 2016). It uses electrical energy as a medical treatment and reduces inflammation and decreases pain. Electrotherapy is amongst the most frequently used physical therapy treatments of Rheumatoid Arthritis.

Analysis of Physical Therapy

Different kinds of physical therapy can be helpful in treating arthritis; however, physical therapy is often not so practical. Physical therapy is costly and Medicare may limit access or coverage of services like these. Thus, elderly patients with arthritis are less likely to receive physical therapy (Carter & Rizzo 2007).

Hydrotherapy is costly and pools are not always accessible in every community. Hence, hydrotherapy is not available for all arthritic patients. Additionally, although hydrotherapy somewhat reduced the pain in Osteoarthritis patients, studies have shown that after six weeks of treatment the pain relief was minimal. Hydrotherapy only reduced pain, stiffness and hip strength slightly. Therefore, it appears that hydrotherapy is not the most effective treatment of arthritis (Hinman et al. 2007).

Joint Replacement Surgery

Joint replacement is another approach to healing arthritis, yet this option is a last resort for most people. People get joint replacements once the articular cartilage is almost completely destroyed and they are in a debilitating state. A preventative cure is more preferred (Clinton et al. 2015).

Whole Food, Plant-Based Diet

Whereas all the treatments mentioned thus far were costly, an effective approach called the whole food, plant-based diet is inexpensive. Besides for being affordable, this method of treating arthritis is also doable, practical, and easy. Anyone can do it; it does not require higher education. A study was done where patients with arthritis kept a whole food, plant-based diet and eliminated meat and dairy foods for six weeks. Researchers observed that the patients' Rheumatoid Arthritis symptoms decreased while the patients were off of meats and dairies. The whole food, plant-based diet has been shown to improve the pain and other symptoms of Osteoarthritis patients as well. After only the first two weeks of this six-week study researchers saw clinical results. This demonstrates the diet's effectiveness in lessening the pain, and the pain got less and less as time went on. After being on a whole food, plant-based diet, people had increased levels of alpha and beta carotene, lycopene, lutein, vitamin C and vitamin E in their sera. These are all beneficial and healthy antioxidants or vitamins. According to the USDA pyramid, a human should ingest solid and liquid animal protein daily, such as meats and dairies. This is not in accordance with the whole food, plant-based diet (Clinton et al. 2015).

Arachidonic Acids and Omega-3 Fatty Acids

Arachidonic acids are precursors to proinflammatory eicosanoids and prostaglandins and are found in cell phospholipids. Prostaglandins are hormones that are created during chemical reactions due to an injury or disease and they can increase inflammation. An eicosanoid is a signaling molecule that can increase inflammation as well. More than 80% of ingested arachidonic acids are used to produce eicosanoids (Adam et al. 2003). Animal products are high in arachidonic acids whereas a whole food, plant-based diet is low in arachidonic acids. By keeping to a diet low in arachidonic acids, inflammation in patients with Rheumatoid Arthritis can decrease (Clinton et al. 2015).

Studies show that Omega-3 fatty acids reduce arthritic pain. Omega-3 fatty acids from fish oil stop the formation of cytokines and eicosanoids. This is because they compete with the Omega-6 fatty acids for a spot in the cell phospholipids and for the binding sites of cyclooxygenase and lipoxygenase. Since the eicosanoids cause inflammation, eating Omega-3 fatty acids reduces inflammation, reducing arthritic pain (Adam et al. 2003).

In a study, Omega-3 fatty acid levels were highest in the serum of whole food, plant-based dieters. Omnivores and fish eaters had less Omega-3 fatty acids. Alpha-linoleic acid is found in legumes, vegetables and soy. When alpha-linoleic acid is metabolized, it produces anti-inflammatory prostaglandins. These anti-inflammatory prostaglandins help reduce arthritic symptoms (Clinton et al. 2015).

There was a study done over the span of eight months, in which the subjects were divided into two groups. One group was put on an anti-inflammatory diet and the other on a western diet. There was a positive correlation between arachidonic acid consumption and the percent of arachidonic acids in erythrocyte lipids. Those on an anti-inflammatory diet (AID), where they ate a minimal amount of animal products, had a lower percent of arachidonic acids in their blood than those on a western diet (WD) (Adam et al. 2003).

Populations with high fish consumption include the Alaskan Natives, Greenland Eskimos, and Japanese living in villages where fishing is constant (Zheng et al. 2012). In these areas, the percent of arachidonic acids in plasma phospholipids was approximately 2%, whereas in industrialized countries it was approximately 16% (Adam et al. 2003). Rheumatoid Arthritis is not nearly as prevalent in populations with high fish consumption as it is in industrialized countries due to the Omega-3 fatty acids in fish.

In the eight-month study mentioned above, in addition to the diet they were instructed to follow, some subjects in each group received placebo or verum pills and some fish oil capsules.

Those in the anti-inflammatory diet group who received the placebo had a 14% decrease in tender and swollen joints collectively. Those in the anti-inflammatory diet group who received fish oil had a 28% decrease in tender joints and a 34% decrease in swollen joints. Those in the western diet group who received fish oil had an 11% decrease in tender joints and a 22% decrease in swollen joints. Overall, the anti-inflammatory diet with a fish oil capsule decreased the tenderness and swelling of joints more than the western diet with a fish oil capsule. It is also clear that the fish oil which is rich in Omega-3, had an effect and decreased the tenderness and swelling of the joints more than the placebo did (Adam et al. 2003).

Two different capsules were administered to patients that were not real fish oil. The placebo was corn oil and the verum was menhaden oil. Even without fish oil, which has a greater effect than the other oils, the anti-inflammatory diet is significantly more effective in reducing symptoms of arthritis than the western diet is. This is a good study, adequately proving how a diet low in arachidonic acids and including fish oil supplements can greatly alleviate pain, improve arthritic symptoms and reduce production of eicosanoids and cytokines (Adam et al. 2003).

Another study compared two groups. One group kept to an ordinary, omnivorous diet, while the other kept to a whole food, plant-based diet. The results were that the whole food, plant-based diet caused the swelling of the joints to decrease, less pain and increased functional status. Compared to the omnivorous diet, the whole food, plant-based diet was more effective in pain reduction (Clinton et al. 2015).

As an added bonus, a whole food, plant-based diet can result in weight loss and blood pressure reduction (Silberman et al. 2010). Excess weight can add abnormal mechanical stress on the knee joint; obesity makes arthritis worse (Cianflocco 2011). As a result of weight loss, the mechanical load on arthritic joints decreases, alleviating the pain caused by arthritis (Messier et al. 2005). Knee Osteoarthritis symptoms can greatly improve even from small weight losses (Bartlett et al. 2004). For someone with arthritis in the knee, "for every pound of weight lost, there is a four-pound reduction in mechanical load exerted on the knee during daily activities" (Clinton et al. 2015).

Due to articular cartilage disintegration there is a loss of space between the femur and tibia in this Osteoarthritis patient. The bone rubbing against bone can cause extreme pain to this patient. The more weight the patient has to carry around on that knee, the more the bones will rub and the more pain there will be (Cianflocco 2011). For this reason, it is worth attempting to lose weight through a whole food, plant-based diet.

It is a common misconception that a diet without animal protein causes malnutrition. A whole food, plant-based diet provides enough protein, fats, vitamins, minerals and calories (Clinton et al. 2015).

Conclusion

After analyzing the different treatment options to alleviate the pain of and possibly cure arthritis, it is apparent that the whole food, plant-based diet has the most promising results. One on this diet must not eat any animal proteins in order to maintain a low level of arachidonic acids to prevent or reduce inflammation. Along with this diet, dieters should take fish oil capsules for better results. Fish oil contains Omega-3 fatty acids which further reduce inflammation and prove beneficial to those with arthritis.

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Is There A Correlation Between Epstein - Barr Virus (Mononucleosis) and Hodgkin's Lymphoma?

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Abstract

Symptoms of acute Infectious Mononucleosis (IM), which develops after Epstein-Barr virus (EBV) in half the cases, is strikingly like those of Hodgkin's Lymphoma (HL). This, combined with the findings that many patients with HL had a history of EBV and/or IM, triggered an interest in scientists to learn if the two were biochemically related. Many studies in this field unanimously concluded that the development of IM after EBV infection presented a higher risk of developing HL. However, whether this relationship is coincidental or pathological remains a matter of controversy until this day. In the last decade, there has been a lot of research and advances in this field. Various studies have looked at the correlation of EBV and HL considering age factors, genetic predispositions, and immunity and susceptibility dynamics. Researchers discovered that development of HL after EBV was most prevalent in young adults and old patients, pointing to immune system function, or lack thereof. It was also found to be more common in men, suggesting that this may be a sex-related disease. Specific genes activated or mutated in both EBV and HL have proposed that the development of HL after EBV/IM may be genetically regulated. By tracing geminal centre B cell replication patterns, EBV gene expression, and lytic cycle proteins, different mechanisms for the pathogenesis of HL from EBV were proposed. Various studies made use of statistics and patients' medical records to make assumptions. Other methods employed in these studies included probe hybridization to extract DNA and RNA samples from tumor specimens, flow cytometry and polymerase chain reactions (PCR) to identify genetic sequences, and immunohistochemistry to study antigens specific to EBV and related diseases.

Introduction

Is there a correlation between Epstein - Barr virus and Hodgkin's Lymphoma? If so, what is the nature of this relationship? The objective of this paper is to briefly discuss some studies which initially led health professionals to believe that Hodgkin's lymphoma (HL) is caused by EBV. Then, possible causes and mechanisms which may lead the viral disease to transform infected cells to malignant tumors will be expounded upon.

The lymphatic system is an important regulatory mechanism involved in the immune response of the body. It is comprised of lymphatic channels which conduct the lymphatic fluid throughout the body and converge regionally to form discreet lymph nodes (Freeman and Matto, 2018). The function of the lymph node is to evaluate and initiate an immune response when necessary. It functions as an antigen filter consisting of a multi-layered sinus that successively exposes B-cell lymphocytes and T-cell lymphocytes to an afferent extracellular fluid (Evans, 1974). B and T lymphocytes are subtypes of white blood cells that contribute to the immune system. For example, by secreting antibodies, the B cells thus play a central role in immunity. In addition, T cells exhibit cell-mediated immunity. In the course of an immune response, where foreign proteins are recognized and attacked, the immune cell line undergoes multiplication, thereby causing the lymph node to increase in size. Generalized or localized lymphadenopathy, an abnormality in the size or consistency of lymph nodes, is the most common feature in lymphoproliferative disorders. These disorders refer to potentially fatal conditions in which lymphocytes are produced in excessive quantities. Included in this broad category of disorders, are Epstein-Barr Virus, Infectious Mononucleosis, and Hodgkin's Lymphoma.

EBV is a tumorigenic double stranded DNA herpesvirus that commonly develops in the first decade of life of an individual. It infects more than 90% of the human population worldwide (Yang, et. al., 2015). EBV consists of the EBV-1 and EBV-2 subtypes, with EBV-1 being more prevalent in western societies. An immune response consisting of natural killer (NK) cells and EBV-specific cytotoxic CD8+ T lymphocytes controls the primary infection and reactivation that occurs in all EBV-positive individuals. While EBV is often found in B cells in its latent episomal form, lifelong persistence of EBV in infected individuals involves occasional reactivation to the lytic state, resulting in lyses of infected cells and the release of virus particles which triggers acute infection (Babcock, et. al., 1998).

Primary EBV infections in children are usually asymptomatic yet can result in infectious mononucleosis (IM) development in 50% of adolescent patients (Jenson, 1996). It was not until 1968 that EBV was first associated with IM, followed by further research which ultimately confirmed that EBV was a causative agent of IM. Most of the symptoms of infectious mononucleosis are attributed to the proliferation and activation of T cells in response to infection. It is characterized by abnormally high numbers of circulating CD8+ T cells and B cells. Among its many symptoms, lymphadenopathy and lymphocytosis – abnormal numbers of lymphocytes in the circulating blood (Sawari, et. al., 2016) - acutely resemble HL. In fact, IM is occasionally misdiagnosed as Hodgkin's Lymphoma due to the similarity in their symptoms and small biopsy specimens in the Waldeyer's ring (WR), which may be difficult to differentiate (Delecluse, et. al., 2007). This similarity, among other factors had led researchers to suspect that one disease may derive from the alteration of the other.

According to the World Health Organization (WHO), Hodgkin's lymphoma is a disorder characterized by an abnormality in the lymph node's architecture (Tzankov and Dirnhofer, 2006). HL, also commonly known as Hodgkin Disease (HD), encompasses four histological subtypes, with 40% of sclero-nodular type and 80% of mixed cellularity type carrying the EBV virus (Delecluse, et. al., 2007). It is comprised of characteristic

neoplastic cells known as Hodgkin's Reed-Sternberg (HRS) cells. These interspersed cells only constitute approximately 2% of the total tumor mass while the rest of the tumor consists of T cells, B cells, eosinophils, macrophages, granulocytes, and others (Yang, et. al., 2015). The classic Hodgkin Reed–Sternberg cells are large cells with two or more round nuclei. The mononuclear form is referred to as the Hodgkin cell (Ok, et. al., 2015). HRS cells communicate with other cells via cell contact dependent interactions, including proliferative and anti-apoptotic signals favoring tumor cell survival and expansion. EBV is most commonly associated mixed-cellularity subtype, the most common type of classical Hodgkin disease.

Using data from discharge records of patients hospitalized for Hodgkin's Lymphoma and/or Infectious Mononucleosis, studies were performed by compiling the prevalence of the disorder relating to age and gender. Tracking the medical history of these patients allowed for the determination of how many patients with HL have a past history of EBV/IM. B and T lymphocytes in blood of patients with Hodgkin's disease, infectious mononucleosis, and normal controls were collected and compared. Due to the rarity of EBV-infected B cells in healthy virus carriers, it is complicated to analyze them directly (Caldwell, et. al., 1998). To resolve this issue, Thorley-Lawson developed a method in which human B cells are separated via flow cytometry, which works by suspending cells in a fluid and passing them through an electronic detector. Then, using polymerase chain reaction (PCR), cells are analyzed for the presence of EBV infection. Additionally, using, viral DNA and RNA segments from tumor specimens were extracted and studied via southern blot hybridization with probes specific for parts of the EBV genome. Reverse transcriptase of RNA can also be used to identify the genetic sequence of specific genes (Khan, et. al., 1996). Epstein-Barr virus' early RNA transcripts were detected in tumor tissues, via fluorescein-labeled oligonucleotides complementary to the RNA sequence. The fragments were then visualized under a microscope by tetramethyl rhodamine isothiocyanate (TRITC), a bright orange-fluorescent dye. Other studies have applied immunohistochemistry techniques, the detection of antigens in cells of a tissue by introducing antibodies binding specifically to these proteins. Finally, experiments with transgenic mice were useful for studying genes. Control and experimental groups of mice were infected with either wild type (WT) or knockout (KO) genes to see how they affect the course of the disease.

Discussion

It is generally held that a lymph node is considered enlarged when it is larger than 1 cm, however, this can vary depending on the location of the node and the age of the patient (Cheng and O'Connor, 2017). Patients with a diagnosis of EBV, IM, and HD presenting with lymphadenopathy, were studied. The goal was to determine if and how these diseases are correlated. Different propositions as to the nature of their relationship have been reviewed.

Using data from the Oxford Record Linkage Study (ORLS), a cohort of 2797 patients admitted to the hospital with an International Classification of Diseases (ICD) code for infectious mononucleosis on the discharge record were studied. Patients studied were mostly ages <20-30 at the time of admission. Admission was substantially more common in males than in females. Those with a diagnosis of cancer either before or at the same time as admission for IM were excluded. Tracking the medical records of these patients showed an increased risk for the development of HD with a relatively short follow up period ranging from 1-7 years post discharge (Goldacre, et. al., 2008). A similar study looked at 233 cases of HL in children recorded between 1957 and 2001. Reliable data concerning the onset of symptoms was available in 172 cases, and in 31 of these patients, EBV status was unknown. Of the 141 tumors, 69 (48.9%) were EBV-positive (Reiman, et. al., 2003). In another unrelated cohort study on young adults, it was indicated that individuals who had a history of IM were three times more likely to develop HL (Jarrett, 2003). The incubation period of infectious mononucleosis after EBV was observed to be between 32 and 49 days. (Balfour, et. al., 2015). The follow up period between EBV and HL varied with different genders, ethnic groups, and age.

Both IM and HL typically develops in adolescence and early adult life. In fact, the development of IM after EBV is now considered to be a risk factor for Hodgkin's disease. However, the nature of this association remains unclear. Examining blood samples from patients with HD, showed EBV latency in B cells. However, it must be noted that not all subtypes of HL harbor EBV to the same degree. Some data suggests that the incidence of EBV-positive HL may be age-related. The observed age-incidence pattern suggests that risk of HD is increased among younger people, with a peak in incidence at 25 to 30 years and a second rise after the age of 45 (Khanna, et. al. 1995). An increase in the severity of infection in young patients leads to the development of cancer. In one study, a similar pattern was recorded among aging patients suggesting that the immunologic status of the patient plays a crucial role in the subsequent development of pathologies (Mueller, 1987). Dr. Evans proposed that perhaps HD represent the host's response to an EBV infection that is delayed until adolescence/early adulthood (1974). Another plausible explanation would be that HD develops as a secondary response in patients with genetic susceptibility, low immunity, or a combination of both.

A study was done in which a samples of B and T lymphocytes in patients with HD, IM, and a normal control were analyzed. They then compared the results of each group to the others. In overall counts, the number of T lymphocytes significantly exceeded that of B cells. However, while in infectious mononucleosis the count of T-lymphocytes was elevated, there was a slight reduction in Hodgkin's Disease. It was also observed that in patients with

HD there was an increase in the number of DNA synthesizing lymphocytes was found when compared to normal controls. The ability of the disease to persist despite potential immune responses against it indicates that the virus has invented pathways to evade the immune system. Also, untreated patients differed from their treated peers in their absolute lymphocyte counts; treated patients presented with a lower number of DNA-synthesizing blood lymphocytes. Cells observed in the blood of patients with Hodgkin's disease and infectious mononucleosis were indistinguishable. Based on these findings, it was inferred that transformed lymphocytes represent normal lymphocytes that have undergone a proliferative response signaled by tumor surface antigens or viral infections (Huber et. al.1975).

EBV-associated lymphoproliferative disorders (LPDs) are life-threatening diseases commonly found in those with a compromised immune system, especially children. T-cell dysfunction can cause development of mature B-cell lymphoma by impairing immune function. Additionally, a partial lytic infection plays a role in EBV-induced B-cell tumors. Although the main targets of EBV are B cells, EBV may also infect other cells, such as T cells and epithelial cells (Rickinson and Kieff, 2001).

Once infected, EBV persists in rare peripheral blood lymphocytes for the rest of the host's life, usually as a harmless passenger residing in B cells (Ohashi, et. al., 2012). Since there is limited viral protein expression in the latent form, EBV reduces the number of viral proteins that permit the recognition of infected cells by cytotoxic T cells. During its reactivation, which takes place during the lytic life cycle, memory B cells are activated and differentiate into plasma cells. People who have EBV can transmit the disease through oral secretions because the virus replicates in cells in the oropharynx. Generally, the number of infected lymphocytes is stable over time. However, in rare instances, infection of epithelial cells by EBV in vitro results in active replication, and consequently, lysis of the cell which spreads the virus to other cells (Cohen, 2000). Although the primary site of virus replication is in the pharyngeal epithelium, circulating B lymphocytes can become infected too, thereby generalizing the infection. In B cells, persistent infection can transform the cell, which may result in the development of malignant lymphomas, especially Hodgkin lymphoma. Like all herpes viruses, EBV has latent and productive (lytic) phases in its life cycle (Niedobitek, et. al., 2001). Therefore, EBV establishes a life-long infection in individuals which is generally harmless unless the host–virus balance is upset. The latent genes which keep the virus in the host's B lymphocytes induce immortalization and oncogenic potential too. EBV differs from other herpes viruses though in its capacity to transform B cells. Latent infection of B cells by EBV in vitro can result in a transformation to lymphoblastoid cells, thereby leading to immortalization of the cells. In fact, the International Agency for Research on Cancer classified EBV as a group 1 carcinogen.

In more recent years, studies of tissue cultures of Epstein-Barr virus growth in peripheral blood lymphocytes has shed some light on the molecular events associated with B cell growth transformation and virus replication (Ohashi, et. al., 2012). Studies have shown that the main type of EBV-associated B-cell lymphoma — that is, Hodgkin lymphoma— seems to derive from germinal-Centre B cells, a structure found in lymphoid tissues that is composed of proliferating B cells. This indicates that EBV-infected germinal-Centre B cells are at the greatest risk for malignant transformation (Küppers, 2003). After B cells are infected, virus–receptor interaction induces activation of the infected cells which enter the mitotic cycle, leading to lymphoblastoid cell lines (LCLs). LCL has the phenotype of an activated B-blast, expressing activation, adhesion, and anti-apoptosis proteins. Since lymphoblasts resemble activated B cells both in their phenotype and molecular features, the virus is thought to push infected naive B cells into the memory state by switching the cell from the growth program to the default program. Normally, the latently infected memory cells shut down the viral expression entering the latent state. However, when the latently infected memory cells divide, they express the EBNA-1 protein causing the viral DNA to replicate. Ultimately, the latent virus is reactivated and proliferates, spreading to other hosts. It is therefore derived that the virus can remain latent in the memory cells for long periods of time without causing any harm to the host. A risk arises if memory cells are accidentally triggered into expressing the growth program (Thorley-Lawson and Gross, 2004).

In germinal centre B cells, a more restricted EBV gene expression pattern is detected in which only EBNA1, LMP1 and LMP2A proteins are expressed (Bancock, et. al., 2000). Also, in the germinal centre expansion causes the number of EBV-infected B cells to increase. These germinal centre B cells then differentiate into memory B cells, which are the long-term latent form of EBV (Babcock, et. al., 1998). In EBV-associated HL, viral genomes were found in their monoclonal form, indicating that infection of the tumor cells occurred prior to their unrestrained replication (Kapatai and Murray, 2007). Therefore, it can be concluded that EBV infects mostly germinal-centre and/or memory B cells, and some naive B cells before they begin to replicate and proliferate (Kurth, 2000). HRS cells are believed to derive from germinal-centre B cells that evaded apoptosis by restricting their own gene expression and by receiving survival signals through stimulation of CD40 (Kanzler, et. al., 1996). EBV-positive malignant cells provide proliferative and anti-apoptotic signals, assisting in survival and growth of the tumor (Kapatai and Murray, 2007). It remains unclear however, under which conditions EBV+ B cells enter germinal centres and how the EBV-gene expression in these cells is regulated, leading to the development of Hodgkin's Lymphoma.

The EBV genome consists of a linear DNA molecule that encodes nearly 100 viral proteins. During the lytic state, these proteins regulate the expression of viral genes causing viral DNA to

replicate. After infecting B cells, the linear EBV genome becomes circular, forming an episome. In these cells, the episome can replicate independently of the host cell, regulated by the cell as part of the normal mechanism of memory B-cell homeostasis, thereby establishing a latent infection (Sarwari, et. al., 2016). The EBV viral genome is encased within a nucleocapsid surrounded by the viral envelope. Entry of EBV into B cells is initiated when the envelope glycoprotein, gp350, binds to the viral receptor on the surface of the B-cell called the CD21 molecule (Kojima, et. al., 2010). Infection of B lymphocytes with EBV results in persistent latent infection and immortalization of the cells to perpetual proliferation.

Studies done using flow cytometry and PCR in biopsy specimens demonstrated reactive B lymphocytes (Kojima, et. al., 2010). Based on these studies, it was observed that EBV infects naive B cells. This was derived from the fact that these are the only cells that were observed expressing all the latent EBV genes. The naïve EBV-infected B cells then undergo an expansion phase, thereby making use of normal B-cell differentiation pathways to establish a lifelong persistence in the B-cells. When tracking the primary infection of naive or memory B cells, Latent Membrane Protein 1 (LMP1) and Latent Membrane Protein 2A (LMP2A) were both detected which suggests that they provide the signals for antigen-independent replication in the germinal centre (Macsween and Crawford, 2003). In most HD cases the neoplastic cells are derived from B-cells as indicated by a sequence analysis of the immunoglobulin V region genes revealing mutations. In some cases, nonfunctional genes have been detected by stop codons in their sequence. Another study concluded that the site of persistence of EBV within the body must be the resting memory B cells based on the observation that "shedding of EBV from the oropharynx is abolished in patients treated with acyclovir whereas the number of EBV infected B cells in the circulation remains the same as before treatment" (Cohen, 2000) .

In one important study, biopsy specimens of Hodgkin's tumors revealed a 40-50% proportion of EBV positive tumors (Hjalgrim, et. al., 2003). Using southern blot hybridization viral RNAs (referred to as EBERs) and DNA fragments of EBV were detected in 20-25% of specimens. Likewise, patients with Hodgkin's disease were often found to have higher titers of antibody to EBV proteins before or with the onset of lymphoma, suggesting a correlation between the two. Immunohistochemistry was performed on biopsy specimens from patients with HD. Using the immunoperoxidase technique, LMP-1-antibody was applied to the tumor section. Epstein-Barr virus early RNA transcripts (EBERs) were detected in tumor tissues via fluorescein-labeled oligonucleotides complementary to EBER (Alexander, et. al., 2000). The fragment was visualized under a microscope by tetramethyl rhodamine isothiocyanate (TRITC), a bright orange-fluorescent dye.

One study went so far as to suggest that EBV infection is actually the beginning of tumor development. EBV infection permanently induces B-cell activation, ultimately leading to uncontrollable cell division. EBV-positive HRS cells exhibit a type of virus latency and express different combinations of EBV nuclear and latent membrane proteins. In the first type, only EBNA-1 and EBER are expressed, while in the second, EBNA-1, LMP-1, LMP-2, and EBER are expressed. In the third pattern, all the latency genes are expressed. An additional pattern of latency was found in B cells obtained from the peripheral blood of patients with a past infection of EBV, in which only EBER and LMP-2, and in some cases, EBNA-1 have been detected (Mandage, et. al., 2017). Epstein-Barr virus nuclear antigen 1 (EBNA1) is a protein-encoding gene that is expressed in all EBV malignancies. EBV latent membrane protein 1 (LMP1) is a gene which induces cellular activation and proliferation while inhibiting apoptosis through expression of the B-cell activation markers, CD23 and CD40 (Kapatai and Murray, 2007). EBV latent membrane protein 2 (LMP2A) expression enhances cell survival and inhibits normal B cell transduction. LMP2A does this by mimicking an activated B cell receptor (BCR), replacing the signals that are normally supplied to the B cells and suppresses cell immunity (Caldwell, et. al., 1998). Expression of LMP-2 in transgenic mice allowed B cells to survive even in the absence of normal B-cell–receptor signaling. It was also noticed that during primary infection, many EBV-positive cells appear to express all latent genes associated with viral-driven lymphoproliferative diseases. (Steven, 1997).

BLF1 is a lytic cycle protein found to be involved at both initial and late stages of viral infection. It is involved in DNA replication, repair, and ultimately, immune avoidance. A recent study on EBV BPLF1-knockout mice demonstrated that the BPLF1-knockout mice were approximately 90% less likely to be infectious than wild-type (WT) mice. Without the BPLF1 there was a reduction in transformed human B cells. Overall, humanized mice infected with BPLF1-knockout virus survived longer than mice infected with the WT virus. Additionally, tumors were formed in 100% of mice infected with WT EBV but in only 25% of mice infected with BPLF1-KO virus (Whitehurst, et. al., 2015). These findings suggest that BRLF1 is required for activation of lytic replication and expression. Additionally, ten million cells were injected with either WT or BPLF1-knockout virus and after labeling with B- cell antibody, the total percentage of B-cells in each group was determined using flow cytometry. There was rarely B-cell outgrowth in cells infected with delta BPLF1 virus, suggesting that BPLF1 is necessary for immortalization of B-cells. This provides evidence that BPLF1 plays a role in B-cell transformation, and therefore contributes to EBV's oncogenic role in cells. As of now, the mechanism by which BPLF1 inhibits these processes is still unknown.

Another EBV lytic gene expressed during infection of B cells is BALF1. This gene is known for its anti-apoptotic properties leading to B cell transformations. BARF1 is a secreted protein that blocks Colony Stimulating Factor 1 (CSF-1) signaling, functioning as a trap to block the action of the cytokine. This helps

the virus evade the host's immune system during acute EBV infection or reactivation of virus from latently infected cells (Ohashi, et. al., 2012).

Since the Epstein-Barr virus is associated with an increasing number of diseases, including infectious mononucleosis and Hodgkin's disease in both immunocompetent and immunocompromised individuals (Yang, et. al., 2015), some research suggests that this correlation may be due to genetic factors. The SH2D1A gene provides instructions for the synthesis of a protein called signaling lymphocyte activation molecule associated protein (SAP). SAP interacts with other proteins to activate signaling pathways that are involved in the control of immune cells. These cells are important in that they help control immune reactions and signal apoptosis. The SH2D1A gene is expressed primarily in T and NK cells as well as in some B-cells (Parolini, et. al., 2002). Therefore, a defective gene causes an alteration of these lymphocytes which most likely results in the inability to control EBV infected cells. To test the possibility of SH2D1A gene involvement in the pathogenesis of Hodgkin's disease, a SH2D1A gene analysis was studied in patients with a history of EBV and EBV- related diseases like Hodgkin's lymphoma. A group of healthy patients was also analyzed to serve as the control group. Results showed an alteration in the 5′ region of the SH2D1A gene in the majority of patients with a diagnosis of mononucleosis, of which 25% went on to develop malignant B cell lymphomas. This indicates that there may be a genetic correlation between EBV and HL.

The SH2D1A gene is located on the X-chromosome, which may contribute to the fact that males are more prone to develop Hodgkin's Lymphoma after EBV (Robinson, 1976). Unlike females, males do not have another X chromosome to counteract the mutated gene. In one study 23 patients with HD, consisting primarily of males, had their X-chromosomes analyzed. Analysis by reverse transcriptase polymerase chain reaction (RT-PCR) found EBV genome or protein in 16 of 23 tumor samples. The risk of contracting Hodgkin's disease was found to be significantly increased in males who had a positive reaction to the Paul-Bunnell test, a heterophile antibody test that screens for IM. (Rosdahl, et. al., 1974). Notably, anti-EBNA-1 antibody levels showed 43% heritability.

In a study on transgenic mice, EBNA1 and LMP1 proved to play a key role in lymphoma development. EBNA1 is essential during cell division ensuring equal partitioning of a cell, while contributing to immortalization of cells by allowing for the maintenance of newly synthesized plasmids. The central part of EBNA1 consists of Gly-Ala repeats, which are believed to block the processing of proteins by proteasomes. This functions in inhibiting immune recognition and apoptosis of infected cells because viral proteins are normally broken down by proteasomes to peptides for recognition by cytotoxic T cells (Kojima, et. al., 2010). In turn, the infected cells that accumulate have oncogenic potential. During recovery from the acute phase, CD8+ T cells return to normal levels and antibodies develop against EBV nuclear antigen-1 (Balfour, et. al., 2015). LMP-1 mimics CD40 and activates the nuclear factor-kappa B, promoting cellular proliferation (Ok, et. al., 2015). LMP-1 also induces BCL-2 and cyclin-dependent kinase 2, activating cell replication and inhibiting cell death.

Conclusion

Although an association has been identified, the pathogenetic role of EBV in these diseases is still unclear (Flavell, et. al., 2000). Likewise, the exact factors responsible for cancer development remains a matter of debate. Researchers are limited in their investigation of the relationship between viral infection and the development of cancer due to the lack of small animal models that can accurately reproduce the biology of EBV. Yet over the past few years, some important advances have been made in understanding the biology of EBV and its role in the development of EBV-associated lymphomas. The observation that a significant proportion of cases of HL contain the Epstein–Barr virus genome strongly suggests that the virus contributes to the development of the lymphoma. Significant progress has been made in understanding the functions of EBV-encoded genes in B cells (Sarwari, et. al., 2016) and viral protein functions. Several key issues remain to be clarified.

Additionally, whereas EBV infection in tumors has been firmly established by several independent research groups, its association remains controversial. Many still believe that it is by coincidence, mainly due to the fact that the origin of Hodgkin's can be regarded as heterogeneous because only half of the cases are known to be associated with EBV (Tamayose, et. al., 2004). Others maintain that Hodgkin lymphoma is a multi-factorial disease which depends on both biological and environmental factors and therefore, a specific causative agent cannot be pinpointed (Huang, et. al., 2012).

There is no current approved treatment for EBV. Ongoing research is currently trying to develop vaccines to prevent or treat these conditions. This is a difficult task due to a lack of an animal model to study the pathogenesis of the disease. It is further complicated by the fact that the number of virus-infected cells in the body would amplify and establish latency before immune mechanisms develop. A future approach would be to synthetize therapeutic vaccines designed to generate specific immune attacks for the latent virus. Adoptive immunotherapy and EBV-specific pharmacologic therapies offer promise for future treatment.

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The Role of Diet in Inflammatory Bowel Diseases

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Abstract

Inflammatory Bowel Disease encompasses two diseases, Crohn's Disease and Ulcerative Colitis. During the last 70 years the evolving of diet in the industrialized countries has led to the utilization of foods that have been processed rather than those in their more natural original state. This increase in the processing of foods has been correlated with the more recent occurrence of metabolic diseases such as Inflammatory Bowel Disease. Patients suffering from this problem exhibit lesser amounts of anti-inflammatory bacteria such as Roseburia and Faecalibacterium and increased amounts of pro-inflammatory bacteria Escheria and Fusobacterium. Since diet directly alters the composition of the gut biome, this review aims to define the role of diet in the pathogenesis and management of Inflammatory Bowel Disease.

Abbreviations:

IBD – Inflammatory Bowel Disease
CD – Crohn's Disease
UC - Ulcerative Colitis

Introduction

What is Inflammatory Bowel Disease?

Inflammatory Bowel Disease (IBD) encompasses two diseases, Crohn's Disease (CD) and Ulcerative Colitis (UC). CD is characterized by inflammation of the digestive tract anywhere from mouth to anus. In CD inflammation may reach through many layers of the walls of the gastrointestinal tract causing severe tissue damage. However, UC occurs when there is an inflammation of the colon and the rectum. In UC, the inflammation only affects the innermost layer of the lining of the colon. There are many symptoms for CD and UC. Some of the more common ones include diarrhea, abdominal pain, cramping, blood in stool, reduced appetite, unintended weight loss and severe bowel constriction (Conners, et al., 2017). Many times, inflammation is so severe that the intestinal tissue is damaged beyond repair. In the event of such inflammation the damaged tissue is removed via colectomy. IBD tends to affect individuals of all ages. However, IBD is most commonly found in individuals at a young age, interfering with education, work ability, social interaction and basic quality of life. Therefore, researchers feel compelled to find resolutions for this disease (Lovasz, et.al., 2013).

How the Diet has Changed

Over the centuries the western diet has changed incredibly. In the traditional diet, food was produced and consumed shortly after harvest. In contrast, much of today's food supply is processed, modified, and transported over great distances before being ingested. In recent years, the western diet has been dominated by an increased consumption of refined sugars, omega-6 polyunsaturated fats and fast food, in addition to a deficiency in fruits, vegetables and fiber. The western diet is prevalent in industrial countries such as the United States, Canada, Western Europe, Australia, and New Zealand, and has been spreading and reaching newly developing countries as well (Lovasz, et.al., 2013). This change in diet has led to the worldwide development of many metabolic diseases, such as IBD. (Ng, 2013) (Moschen, 2012). Recent studies indicate that the incidence rate of IBD in developing industrial countries has been increasing in the past 50 years. This increase suggests that IBD may be triggered by an environmental factor (Martinez & Chang, 2013). Although many studies have been done, there is still no definite pathogenic pathway nor cure for this disease.

Recent research suggests that diet is the key environmental risk factor in IBD pathogenesis. IBD is an intestinal disease and is associated with a dysbiosis in the gut microbiota, the microbe population found in the intestine (Reddavide, et al., 2018). In general, IBD patients have been found to have lower amounts of anti-inflammatory bacteria such as *Roseburia* and *Faecalibacterium* and have increased amounts of pro-inflammatory bacteria *Escheria* and *Fusobacterium* (Aleksandrova, et.al., 2017). Many studies have shown that one's diet directly alters microbe composition, making diet a pivotal risk factor of IBD. Moreover, changes to the traditional diet of newly industrialized countries can be linked to the increasing incidence of IBD. The purpose of this review is to define the role of diet in the pathogenesis and management of IBD.

Methods

Critical analysis of peer reviewed articles and clinical research papers were used to write this review paper. Articles were obtained from the Touro College online library in order to determine the relationship between diet and IBD.

Discussion:

IBD and Gut Microbes

The gut microbiome is an ecosystem, often referred to as a Super Organ (Dolan, 2017). The microbes live in a symbiont relationship with the host, performing beneficial tasks such as nutrient production, development and maturation, regulation of immune system and preventing the growth of harmful microorganisms (Aleksandrova, et.al. 2017). Dysbiosis, which is an alteration to the gut flora, can cause disruption to these vital tasks. Several studies show that IBD is correlated with altered composition of gut microbes in the intestines.

Interestingly, Studies have shown, that there is an age-related variation of IBD onset with three distinct stages. Early onset is less than 10 years of age, with a peak onset at 15-30 years old, and late onset cases occur at 60 years old. These changes correspond to the change in the microbiome's stability and diversity. Early life is associated with a microbiome of minimal complexity and stability and can be affected by diet change, illness and puberty.

As individuals reach adulthood, the microbiome stabilizes and shows improved resilience, but the composition may be altered. Correspondingly, decreased stability is often observed in the elderly (Kostic, et.al., 2013). Considering this, the microbe composition and stability in the gut is a key factor in IBD progression.

One role of the microbiota is to maintain a healthy mucosa by the production of anti-inflammatory interleukins. This is the production of SCFA- short chain fatty acids such as butyrate, succinate, lactate, phenols, thiols, and indoles (Reddavide, et al., 2018). These SCFA act as energy for colonocytes which protect against inflammatory responses (Frank, 2007). Patients suffering from IBD have reduced levels of beneficial SCFA producers *Bacteroidetes*, *Lachnuspiraceace,* and *Ruminococcaceae* (Martinez & Chang, 2013). An Additional study shows that IBD patients show reduced levels of several other SCFA producing bacteria such as *Calibacterium praunsnizti* and *Roseburia* (Dolan, 2017). This shows a direct link between dysbiosis and IBD.

Another task of the gut microbiome is to maintain the mucus layer of the colon and the small intestine. If the mucus layer is intact the gut flora will not directly interact with the epithelial cells and inflammation will not occur. However, there is an adherent invasive strain of *E. coli* that is found in abundance in IBD patients compared with controls. These invasive bacteria can potentially invade epithelial tissue and induce granuloma formation during inflammation. A second group of invasive bacteria *Fusobacteria* is found in abundance in the colon mucosa of IBD patients. *Fusobacterium varium* have been found to cause colon mucosa erosion, indicating the possibility of the influence of *Fusobacterium varium* on the pathogenesis of IBD (Kostic, et. al. , 2013).

Bile Acid signaling is another point where host, microbe and even dietary factors all come together. Bile acids assist in digestion and emulsification of fats to be absorbed in the intestines. These bile acids are made in the liver from cholesterol. After undergoing conjugation to glycine or taurine, the bile acids are released in to the digestive tract. In the intestines, deconjugation of bile acids occurs through Bile Salt hydroxylase (BSH) enzymes, making the bile acids functional. These BSH enzymes are made exclusively by bacteria, making the bile acid composition in the intestine dependent on the microbiota composition. The two products of bile acid metabolism act as ligands for FXR (Farnesoid X receptor) and TGR5 (G protein-coupled bile acid receptor 1), which act as signals for anti-inflammatory and barrier function. It was found that IBD patients have increased levels of conjugated bile acids compared to the control group, making these patients prone to inflammation. Furthermore, when bile acids are increased in the gut, it can promote growth of pathobionts such as *Bilophila Wadsworth*, which is known to induce inflammation (Dolan, 2017).

On the other hand, many microbes perform protective tasks when enriched in the host. *Bacteroides* and *Clostridium* species have been shown to cause expansion of T-cells and reduce inflammation. Additionally, many bacteria can reduce intestinal inflammation by regulating nuclear factor (NF)κB activation. *Faecalibacterium praunsnizti* has anti-inflammatory properties and is underrepresented in patients with IBD. Many species, such as *Bifidobacterium, Lactobacillus* and *Faecalibacterium,* protect the host from mucosal inflammation by down regulating inflammatory cytokines or stimulating IL-10 anti-inflammatory cytokines (Kostic, et. al., 2013). Lastly, intestines rich in *Bacteroidetes* are shown to initiate host responses that protect the host from lethal infectious colitis (Brown, et. al., 2012).

Due to this connection between IBD and dysbiosis, there are many current treatments for IBD that are directed at the microbiota. Many antibiotics such as ciprofloxacin, metronidazole, the combination of both rifaximin and anti-tuberculous regimens are used to treat IBD (Nitzan, et.al., 2016). Normally antibiotics may cause thinning of the mucus layer, weakening the barrier function, leading to increased gut infections. Additionally, antibiotics tend to cause an increase in E. coli composition in the gut, which is a distinctive feature in intestinal inflammation. However, studies show that brief exposure to antibiotics causes slight changes to the microbiota in IBD patients that provides relief from symptoms. Alternatively, repeated exposure of the same antibiotic causes persistent changes to the microbiota leading to infection (Kostic, et.al., 2013). Due to this controversy of antibiotics, in the last few years methods to repopulate the gut with healthy beneficial microbes has been gaining popularity as a potential treatment for IBD. Evidence shows that Fecal Microbiota Transplantation (FMT) can be effective in replenishing a healthy microbiota. FMT, also known as a stool transplant, is the transplantation of gut microbes from the stool of a healthy individual into the recipient. There has been a high success rate for FMT in many other metabolic diseases such as relapsing *C. difficile* infection. Therefore, researchers are considering FMT for other gastrointestinal illnesses such as IBD. Additional studies are needed to determine the outcome of FMT on IBD patients (Kostic, et.al., 2013).

It is now clear that IBD is inherently linked with dysbiosis. There are many factors that can cause dysbiosis such as genetics, immune function, and diet. Genetic susceptibility is the most obvious factor. IBD patients carrying a mutation in a NOD2 related gene, have increased numbers of mucosa adherent bacteria, as well as significant alterations in bacterial composition (Kostic, et. al., 2013). Studies have shown that CD is common in the Ashkenazi Jewish population. Many studies were done using large families of Ashkenazi Jews to determine a genetic variant cause to CD. One study found the haplotype 268SJW1 on chromosome 16 to contribute to the risk of CD in Ashkenazi Jews (Sugimura, 2003). Although genetic susceptibility cannot be ignored, due to the increasing incidence rates of IBD, researchers believe that environmental factors are the core triggers.

Dietary Factors effecting the Microbiome

Diet is suspected to be the prime contributor to the pathogenesis of IBD. Dietary composition has been proven to be associated with intestinal inflammation by directly or indirectly modifying the gut microbiome (Reddavide, et al., 2018). So, current research is aimed at examining how diet changes the microbiome and how these changes affect the host, in order to determine foods that can cause or prevent inflammation (Kostic, et.al., 2013).

In a study done, subjects were put on a western-style diet for one month and showed a 71% increase in plasma endotoxins. The western diet is characterized by a high fat diet and therefore may cause endotoxemia by causing changes in the intestinal barrier function or microbiota composition (Pendyala, et.al., 2012). Another study showed that mice fed a high fat diet were associated with endotoxemia and systemic inflammation. These mice showed lowered amounts of *Bifidobacterium spp*, indicating that this species of bacteria have anti-inflammatory properties (Cani, 2007).

Variations in long term diet patterns change the ratios of *Bacteroides, Prevetolla,* and *Firmicutes*. However, modifications in short term diets have no significant influence. It has been determined that enriched protein and animal fat diets, common in the western diet, favored *Bacteroides*. Diets rich in carbohydrates, such as in agrarian societies, favored *Prevetolla* (Wu, 2011). Switching to a high fat diet causes a decrease in *Bacteroides* and an increase of *Firmicutes* and *Proteobacteria* (Hildebrandt, 2009). It has been proven that protein rich diets increase enzymes that produce toxic metabolites which trigger inflammatory response (Brown, DeCoffe, et.al., 2012).

Another microbe often associated with IBD inflammation is *Bilophila Wadsworth*, a sulfite producing bacteria. In a recent study when mice were fed a milk fat enriched diet, it led to a series of reactions that ultimately led to an abundance of *Bilophila Wadsworth*. Although there is no precise mechanism for how it causes inflammation, there are many hypotheses (Frank, 2007). Furthermore, an interesting study concluded that refined dietary glucose intake can change microbe composition and increase intestinal inflammation (Whitehead, 2011). A strict Vegan or Vegetarian diet led to a significant reduction in *Bacteroides spp, Bifidobacterium spp,* and the *Enterobactericeae* which helps manage inflammation of IBD (Enck, 2011).

A further connection between diet, microbes, and host inflammation has been discovered through studies of the intestinal function of the aryl hydrocarbon receptor (AhR). AhR is a nuclear receptor that activates certain metabolic genes. AhR also plays a key role in adaptive immunity through the regulation of T-cell activity. IBD patients have a decreased expression of AhR which may cause pro-inflammatory immune activation conditions. It has been discovered that certain gut microbes can produce AhR agonists. IBD patients contain less amounts of the microbes necessary to produce these agonists. An important dietary source of AhR ligands are found in cruciferous vegetables such as cauliflower, broccoli, brussels sprouts and bok choy. This may explain why these vegetables have a protective effect on IBD risk (Dolan, 2017).

One major role of the microbiota is to act as an immune system of the host. The gut flora protects the host from pathogens by maintaining the integrity of the intestinal mucosa. Dysbiosis can cause a disruption in the intestinal mucosa and allow access for pathogens which can lead to reactions which cause inflammation and eventually tissue damage. Many studies were conducted to determine the influence of micro and macro nutrients on immunity. Many micronutrients are essential for immunonutrition such as vitamins A, C, D, E, folic acid, zinc, and iron (Reddavide, et al., 2018). Deficiencies in vitamins A and D may reduce function of natural killer cells, while supplemental zinc and vitamin C can increase their activity. Vitamin D is important in intestinal function for it is known to suppress microbial invasion into the epithelium. 82% of IBD patients have been found to have vitamin D deficiencies which have been linked to a weakened epithelial barrier. Although studies have been done to determine the effects of vitamin D supplements on IBD patients, more research is needed. Furthermore, Iron deficiencies have been associated with faulty T cell response and weakened cytokine production. However, iron supplements have been shown to have a negative effect on IBD patients by increasing an individual's susceptibility to infections. It is evident that micronutrients can affect the immune system thereby causing dysbiosis and the onset of IBD (Aleksandrova, et.al., 2017). More research is needed to recognize optimal nutrient levels and therapeutic options.

These findings support the fact that the microbiota is directly related to food quality. Diet influences the composition of the microbiota and thereby controls IBD pathogenesis. The current diet is significantly different than the diet of previous generations when the prevalence of IBD was not as widespread. Countless experiments were performed to see the effects of diet on IBD. Dietary factors can have a negative effect on IBD and increase inflammation, or they can have a protective or even healing effect on patients with IBD.

Dietary Risk Factors of IBD

Refined and processed carbohydrates are risk factors for IBD, but complex carbohydrates, such as fruits, vegetables, or fibers can help manage IBD. A diet high in animal protein was correlated with a 3.3-fold increased risk in IBD (Reddavide, et al., 2018). Individuals that consumed milk products including milk, yogurt and cheese, had a lower chance of developing IBD. This suggests that dairy intake has no risk effect on IBD and may even have a protective effect (Malavia, 2017). Many studies were done on the effects of fat intake on IBD. Although it is known that a typical high fat western diet has a negative effect on IBD, it is unclear which specific fats cause that. One study showed

that an increase in poly-unsaturated fatty acids was positively associated with IBD (Parekh, 2015). Another study concluded that long term intake of trans-unsaturated fatty acids is associated with a trend towards IBD. Evidence shows that excessive consumption of omega-6 poly-unsaturated fatty acids increases the risk of IBD, while consumption of omega-3 poly unsaturated fatty acids is associated with a decrease in one's risk for the onset of the disease (Brown, et. al., 2012).

Dietary Management in IBD

Interestingly, research has shown that the Mediterranean Diet, due to its unique balance of fat, is directly correlated with decreased inflammation. The Mediterranean Diet (MD) is characterized by fruits and vegetables rich in fiber, vitamins, and antioxidants, whole grains, and nuts, as well as olive oil and oily fish that are high in poly and mono unsaturated fatty acids. Each item in the diet provides benefits to patients with IBD (Serra-Majem, 2009).

Legumes, a major part of the MD, contain soluble fibers that do not aggravate the gut microbes. Furthermore, legumes tend to have a prebiotic action and promote the growth of beneficial SCFA producing microbes which protect the gut mucosa. Fruits and vegetables have a naturally high fiber content and therefore using a juicer to extract the essential vitamins and minerals showed best results when treating IBD patients. Recent studies claim that olive oil has ani-inflammatory effects. This is due to the synergic action of its oleic acid with other antioxidant molecules. Grains that were proven to have the most beneficial effect were unmodified grains. These grains have low immunogenic impact and do not cause inflammation in the gut mucosa. Bluefish, which is popular in the MD, is shown to have anti-inflammatory effects due to its omega-3 fats EPA and DHA (Reddavide, et al., 2018).

An alternative diet that has shown promise in IBD management is the Specific Carbohydrate Diet (SCD). This diet works with the hypothesis that patients with IBD can not break down disaccharides and amylopectin. Large amounts of disaccharides can lead to an over growth of microbes and cause IBD related symptoms. Therefore, the SCD is a strict monosaccharides diet excluding disaccharides and most polysaccharides. The diet includes vegetables with a high amylose to amylopectin ratio. The real potential for this diet is in the maintenance of IBD patients in remission to help maintain a healthy gut microbiome (Reddavide, et al., 2018). A similar diet the FODMAP – Low Fermentable oligosaccharide disaccharide monosaccharide and polyols diet also showed significant improvement of symptoms in patients with IBD (Dolan, 2017). Although these findings clearly define the effects of diet on IBD, the role of diet does not stop there.

Dietary Factors and Epigenetics

New emerging evidence points to epigenetics as a major component of IBD pathogenesis. Epigenetics is the study of heritable changes in gene expression that do not involve changes in the actual DNA sequence. This leads to a change in phenotype without a change in genotype. Epigenetic modifications, such as histone modifications, influence DNA accessibility and chromatin structure, thereby effecting the regulation of gene expression. The body uses epigenetic modifications as natural ways to control gene expression as needed throughout one's life. However, epigenetic changes can also be induced by external factors including age, the environment, lifestyle, and disease state. Dietary factors have been shown to contribute to IBD in an indirect manner via epigenetics (Reddavide, et al., 2018).

Over time, researchers have discovered new methylation patterns emerging for IBD. Many therapeutic studies were done to try to counteract this methylation using drugs and other processes. However, researchers found it difficult to pinpoint the methylation in specific tissue and to predict drug response in clinical trials so other strategies were considered. Dietary factors have been known to alter epigenetics which can influence an immune response that can potentially offset inflammation. Therefore, scientists looked at dietary components for a solution to IBD methylation patterns. It was discovered that secondary plant metabolites, such as polyphenols, have been demonstrated to inhibit DNA methyltransferase activity. Furthermore, gut microbiome has been shown to alter host histone acetylation and methylation in human colon tissues. Interestingly, SCFA, which is generally produced from microbial fermentation of fibers, is shown to be important in epigenetic regulation of inflammatory responses. It was determined that a lack of SCFA can disturb chromatin effects. This deficiency of SCFA can be a result of a diet low in fiber or a decrease in beneficial SCFA producing bacteria. As mentioned previously, IBD patients have been found to have low amounts of *Bacteroidetes*, *Lachnuspiraceae*, *Ruminococcaceae*, *Calibacterium praunsnizti,* and *Roseburia,* SCFA producing bacteria, making IBD patients susceptible to epigenetic changes which can lead to inflammation. (Reddavide, et al., 2018).

Other Environmental Factors

Although diet has been presumed to be the primary trigger for IBD, there are many other environmental factors that can prompt IBD symptoms. Smoking, and stress are such factors that have also been shown to influence IBD pathogenesis. Interestingly, Smoking has been found to be negatively associated with UC but positively associated with CD. Patients who did smoke only developed UC after they ceased smoking. Additionally, male UC patients who smoked during disease progression, reported reduced symptoms and had lower hospitalization rates compared to non-smokers. Increased colectomy rates were seen in non-smokers and ex-smokers than in current smokers. Also, reduced rates of relapse were seen in patients who began smoking after the onset of UC. On the other hand, many

studies show an increased risk of CD in smokers compared to non-smokers. There is a 34% increased relapse rate for CD in smokers. Furthermore, severe lesions at the anatomic sight have developed more in smokers than non or ex-smokers in CD patients (Thomas, et.al., 2000).

There are many theories for the possible mechanism of the influence smoking has on IBD. Firstly, due to the interrelationship of gut microbes and immunity, researchers studied how smoking influences the immune system. Studies show that changes in T cells, such as increased levels of suppressor OKT8+ cells and a decreased ratio of OKT4+ to OKT8+ cells have been seen in heavy smokers. Remarkably, these changes revert to normal range once a patient terminates smoking. The low levels of IgA's in the saliva and intestinal secretion of smokers dictates an additional immune effect. The IgA antibody is a vital component of secretions that lubricate the mucosal surfaces which act as the first line of defense in the immune system. Therefore, patients who smoke will already have a weakened immune system. Another possible mechanism involves the inflammatory pathway. Nicotine, a substance found in most cigars and cigarettes, is known to effect cytokine production in the body, specifically by reducing pro-inflammatory cytokines. Also, certain eicosanoids, which are correlated with inflammation, are seen in decreased amounts in smokers. This may be the cause to the negative effect of smoking on UC patients. Nevertheless, smokers show lower levels of two interleukins, IL-1β and IL-8, which help regulate inflammatory response. Lastly, smoking may also influence IBD via intestinal mobility. One major symptom of IBD is issues with bowel movement and defecation. It has been found that nicotine can affect the intestinal motility at many sites. Nicotine relaxes the smooth muscles of the intestine and therefore reduces contractile activity during bowel movement (Bhatti, 1997). These mechanisms portray the direct influence that smoking may have on IBD.

Another environmental factor that researchers have discovered to be effective in the pathogenesis of IBD, is stress related influences. In earlier years IBD was classified as a psychosomatic disorder. Although this classification had been long disproved, it does represent the deep relationship between stress and IBD. In general, stress acts as a threat to an organism's homeostasis. Therefore, the body will exert mechanisms to maintain equilibrium in the face of any stress. There are two interconnected pathways in the body that govern stress related responses; the HPA axis - hypothalamic pituitary adrenal axis and the ANS - autonomic nervous system. The pathway of the HPA axis begins when stress stimulates the release of corticotropin releasing hormone from the hypothalamus. This causes the release of adrenocorticotropic hormone from the anterior pituitary gland which then stimulates the secretion of cortisol from the adrenal cortex. Cortisol then travels throughout the blood stream causing changes all around the body to help deal with stress. The pathway of the ANS involves the stress induced activation of pontomedullary nuclei, which control autonomic responses. As a response to stress, adrenaline and noradrenaline are released from the adrenal medulla via the stimulation of the sympathetic nervous system. Furthermore, The HPA axis and ANS are key modulators of the rich nerve supply of the gut, the ENS – enteric nervous system. The ENS is composed of 100 million neurons that regulate functions of the gastrointestinal tract. The term "Brain-Gut Axis" refers to this complex network of neurons of the HPA axis, ANS, and ENS (Mawdsley, 2005).

The actual pathway of the Brain-gut axis response to stress is complex. Various interconnected activities occur in both the gut and around the body in response to stress that can lead to inflammation. Specifically, in the gut, in response to stress, inflammatory cytokines, SCFA, and microbial products can alter the ANS and in turn lead to the secretion of cortisol and adrenaline. Also, under stress, the gut microbiota and mast cells can release various chemicals such as histamine or serotonin which are known to impair the intestinal secretion and intestinal mucosa, which can lead to inflammation. There are many stressors that can affect the intestinal mucosa's permeability, making a host predisposed for infections and inflammation (Brzozowski, 2016).

Due to this intimate correlation between the nervous system and the gut, it is obvious that stress can influence the pathogenesis of IBD. Chronic heightened stress has been determined to have an important role in predicting the relapse of IBD in patients in remission (Mawdsley, 2005). Also, mice that were induced with depression demonstrated an increase in inflammation which was modulated by an increase in pro-inflammatory cytokines secreted by macrophages (Brzozowski, 2016). Although researchers have not determined an absolute direct pathway for the control of stress on inflammation, there are many suggestions. Many propose that patients with IBD have altered HPA axis functioning, which may explain the relation of stress to IBD symptoms. Interestingly, cortisol, which is released under stress, is known to have anti-inflammatory effects. However, in patients with UC, levels of cortisol showed no correlation to the levels of inflammatory cytokines. On the other hand, others hypothesized that due to the high levels of inflammatory cytokines in the blood of active IBD patients, the response of the HPA axis and thereby the response of cortisol may be dampened. Likewise, stress is known to impact gastrointestinal motility, and water and ion secretion. While these are non-inflammatory effects, they may contribute to the non-inflammatory symptoms of IBD patients (Mawdsley, 2005). The above mechanisms demonstrate the correlation between stress and IBD, making psychological factors a key influence in the pathogenesis of IBD.

Conclusion

The intestinal microbiota performs vital jobs in host metabolism making its composition vital to the host's health. Dysbiosis,

which is found in IBD, can be caused by several different factors. While genetics and other environmental factors cannot be ignored, an altered microbiota resulting from diet-induced dysbiosis may also be a factor that contributes to the inappropriate inflammatory responses that occur. As our understanding of the microbiota continues to grow, promoting microbes to prevent or control inflammatory-mediated diseases through diet may represent an exciting therapeutic avenue.

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Cognitive Rigidity in Patients with Anorexia Nervosa

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Abstract

Anorexia Nervosa (AN) is a psychiatric disorder characterized by significantly low body weight and over-evaluation of weight and shape in self-identity. The complex etiology of Anorexia Nervosa renders many current treatments ineffective, thereby prolonging the course of the illness. Among the many psychological and behavioral features of AN, cognitive features like obsessiveness, rigidity, and perfectionism are often described. Patients hold firm beliefs about an ideal body weight and find it difficult to alter their thinking patterns. Recent studies have identified a pathway to understanding this disorder through characterization of it's cognitive features. With this research, advances have been made in identifying cognitive processes that likely mediate symptom expression in AN. This paper will describe the cognitive rigidity observed in patients with AN and will include a discussion of therapeutic approaches to target this feature.

Introduction

Anorexia Nervosa (AN) is an eating disorder characterized by restricted food intake and inability to maintain an appropriate body weight. Individuals with AN engage in extreme weight control behaviors, such as caloric restriction and compulsive exercise. According to the National Institute of Mental Health (2017), AN has an estimated prevalence of 0.6% in adults and has the highest mortality rate of any psychiatric disorder. The onset on AN typically occurs during adolescence and can persist into adulthood, increasing the risk of mortality. Research has demonstrated that risk of mortality is higher for patients with a comorbid psychiatric condition, such as depression and substance abuse disorder (Jordan et al., 2003). One study found that substance use disorders, particularly alcohol use disorder, was associated with increased mortality in individuals with AN (Kask et al., 2016). Furthermore, a meta-analysis of 36 studies showed that the weighted annual mortality for AN was 10 deaths per thousand people (Arcelus et al., 2011). The incidence proportion for AN (10 death per thousand people) is higher than the one for other eating disorders, like Bulimia Nervosa (3 deaths per thousand people).

Diagnostic criteria for AN include: 1. Restriction of energy intake leading to significantly low body weight 2. Intense fear of weight gain or persistent behavior that interferes with weight gain 3. Disturbance in the way in which ones' body weight or shape is perceived (American Psychiatric Association, 2013). The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) includes severity specifiers for AN based on current body mass index (BMI). The level of severity can reflect clinical symptom expression and degree of functional disability in patients with AN.

The etiology of AN is complex; many factors contribute to the development and maintenance of the disorder. Predisposing genetic and personality factors can render an individual susceptible, while environmental and social factors can trigger the onset of the disorder at a vulnerable time. Aside from individual personality factors, previous research has found that cognitive factors play a role in the development and maintenance of the disorder. Specifically, cognitive deficits in working memory, mental flexibility, and set-shifting have been observed in patients with AN.

This paper will examine the neurocognitive deficits found in patients with Anorexia Nervosa, particularly cognitive rigidity and set-shifting. Etiology, maintenance, and therapeutic approaches for the disorder will be discussed, with a focus on specific treatments targeted at decreasing rigidity in patients with AN.

Methods

This review was conducted using databases like PubMed and ProQuest, through the Touro College library system. Peer-reviewed articles were also retrieved using Google Scholar and Semantic Scholar.

Discussion

Much of the research on AN has been conducted with patients undergoing treatment. Study samples often exclude those individuals who are not actively engaged in outpatient or residential treatment. However, many individuals with AN do not seek treatment due to perceived stigma (Dimitropoulos et al., 2016). As a result, they are not included in study samples and the data obtained may not fully represent the general population. Therefore, this literature review is largely limited to studies that have used clinical samples to obtain data.

Neurocognitive Deficits found in AN

Numerous studies have identified neurocognitive impairments in patients with AN (Phillipou et al., 2015, Chan et al., 2013). Neurocognition encompasses processes, such as planning, attention, visuospatial function, working memory, and concept formation. A broad range of neurocognitive functions have been found to be compromised in patients with AN, particularly cognitive flexibility and set-shifting. Cognitive flexibility refers to the capacity to adapt cognitive processing strategies in the face of new and unexpected conditions in the environment. In patients with AN, this ability is compromised and thus contributes to the behavioral symptomatology of the disorder.

Personality Traits in AN

Personality traits associated with AN include perfectionistic tendencies, introversion, and rigidity. These traits impact the way patients with AN perceive and react to people and their environments. Patients with AN have been described as having high harm avoidance and persistence, and low novelty seeking. These dimensions correspond with behaviors exhibited by those with AN, such as avoidance of weight gain and determination to reach a low body weight. Additionally, patients with

AN often endorse a rigid cognitive style and are unable to alter their cognitions related to eating behavior. They exhibit inflexible behaviors such as counting calories, compulsively exercising, and preoccupation with rigid rituals.

Possible Causes of Cognitive Rigidity in AN

A considerable amount of research supports the familial nature of neurocognitive deficits in AN. One study found that when compared to healthy controls, mothers of patients with AN displayed a more rigid thinking style on a neuropsychological measure of cognitive rigidity (Lang et al., 2016). Neurocognitive impairments have also been noted in healthy siblings of patients with AN, suggesting that these disturbances are not solely the result of the illness (Hason et al., 2011). Rather, these individuals may have biologically-based predispositions to respond to their environments in particular ways. Another study found that patients with AN had poor cognitive flexibility irrespective of the duration of their illness, highlighting the trait nature of cognitive rigidity in patients with AN (Tchanturia et al., 2011).

From a neurocognitive perspective, a rigid cognitive style stems from a lack of mental flexibility. Mental flexibility is the ability to think about multiple concepts simultaneously and to transition from thinking about one concept to another. Mental inflexibility can result in rigid thinking patterns, such as dichotomous thinking (black-and-white thinking) and inability to alter cognitions and behaviors. Individuals with rigid cognitive style are unable to change beliefs or behaviors that are ineffective in helping them achieve their goals. There are several processes that are compromised in individuals with cognitive rigidity; one example is set-shifting ability. Individuals with impaired set-shifting ability cannot efficiently adapt to new situations in response to changing environmental demands. Similarly, individuals with AN have been found to perform poorly on tests of mental flexibility in relation to healthy controls. For example, one study found that compared to healthy controls, patients with AN showed fewer fluctuations in their patterns of response in a test of perceptual judgement, even when their answers were incorrect (Tchanturia et al., 2001). In other words, patients with AN did not attempt to change their incorrect response patterns as frequently as healthy controls.

Assessment of Cognitive Rigidity in Patients with AN

One commonly used neuropsychological measure of cognitive rigidity is the Wisconsin Card Sorting Test (WCST). The WCST assesses set-shifting ability which is the ability to move back and forth between tasks, operations, or mental sets in response to changing goals or environmental demands. In this test, an individual must classify items according to one of four rules, while receiving feedback only when the rule that was used is correct. The classification rule changes every ten cards; therefore, once the participant has figured out the rule, the rule may quickly change. This task measures how well individuals can adapt to the changing rules and if they are able to change their behavior accordingly.

In one study, researchers hypothesized that patients with AN will have lower set-shifting scores on the WCST when compared to healthy controls. In their study, the AN group consisted of 24 adolescent females, and the control group consisted of 37 healthy adolescent females with no prior history of eating disorders. The adolescents in the AN group had lower set-shifting scores than the healthy controls (McAnarney et al., 2011). In a similar study, the WCST was used to measure of set-shifting ability in two groups: one had patients with AN and one was healthy controls. All participants were female and ranged in age from 18 and 55 years. Results of the study indicated that patients with AN performed poorly in relation to the healthy controls (Tchanturia et al., 2012). Another study demonstrated that children and adolescents share a similar cognitive profile with adults with AN, providing support to the idea that these cognitive deficits may be underlying traits in all patients with AN (Lang et al., 2015). However, in contrast to this finding, a meta-analysis found that problems with set-shifting that are observed in adults with AN were not as prevalent in children with AN (Lang et al., 2014). This observation suggests that set-shifting inability may directly result from long-term starvation and is related to duration of the illness.

Neural Basis for Cognitive Rigidity in AN

In addition to causes like heritability, research has pointed to neurological differences between patients with AN and healthy controls. These differences in brain structure may be responsible for features of cognitive rigidity. Prior research has established the role of the prefrontal cortex in executive function and cognitive control. Specifically, one prefrontal subregion, the anterior cingulate cortex, mediates complex cognitive functions such as affect regulation, cognitive control, and decision making (Stevens et al., 2011). Cognitive control is required during neuropsychological tests that measure cognitive flexibility, such as the WCST. During this test, individuals must change their behavior if previously successful responses fail to yield reinforcement. Researchers used animal models to determine the role of the anterior cingulate cortex in cognitive control. After creating lesions in the anterior cingulate cortex of rats, they found that the rats had difficulty adjusting cognitive control, demonstrating that hypoactivity in this brain region can interfere with cognitive function (Newman et al., 2015).

Similarly, in a literature review on cognitive rigidity in AN, researchers suggested that the hippocampi and anterior cingulate showed a reduced thickness in gray matter in individuals with AN; therefore, processes associated with these structures, like working memory and set-shifting, have been impaired in AN patients (Kucharska et al., 2019) Likewise, another study found that during a test of set-shifting, patients with AN showed

hypoactivity in the dorsal anterior cingulate cortex (Zastro et al., 2009). This finding suggests that patients with AN have reduced motivation and initiative to complete tasks in a non-routine way.

The Effect of Cognitive Rigidity on Treatment Outcomes

A rigid cognitive style can influence treatment outcomes for patients with AN. Patients commonly have rigid rules about how many calories they can consume and the restricted range of foods they can consume (Tchanturia et al., 2001). This rigid thinking style can prevent behavioral change and can negatively affect treatment outcomes. Behavioral therapies intended to change perceptions of eating behavior can be ineffective for patients with a rigid and inflexible thinking pattern. Therefore, treatments aimed at decreasing cognitive rigidity can help promote new and adaptive thinking patterns in patients with AN. Recent translational research has begun to focus on characterizing the cognitive features of AN and adapting existing treatments accordingly.

Therapeutic Approaches Aimed at Decreasing CognitiveRigidity in AN

Cognitive remediation therapy (CRT) is a method designed to improve neurocognitive abilities, such as cognitive flexibility, set-shifting, and attention. This form of therapy can lead to improved psychosocial functioning in individuals who have cognitive distortions. CRT consists of a set of mental exercises aimed at improving cognitive strategies and increasing holistic thinking. CRT was initially developed for patients with brain injuries and has more recently been adapted for patients with AN. It aims to address two primary features found in patients with AN: a lack of cognitive flexibility, and a preoccupation with details. Unlike traditional treatments for AN, CRT does not directly address behaviors related to eating disorders. Rather, CRT interventions for AN address these deficits by focusing on the patients' *process* of thinking, rather than on the *content* of their thoughts, thus helping patients develop a metacognitive awareness of their own thinking style.

A study on the efficacy of CRT in improving cognitive function has demonstrated that cognitive flexibility improved significantly after a ten-week CRT training (Kucharska et al., 2019). Furthermore, one study noted clinical changes following ten CRT sessions, notably, patients became more aware of their own cognitive deficits and were able to apply alternative strategies that were demonstrated during the CRT sessions (Wood et al., 2011).

Conclusion

Anorexia Nervosa is a complex illness with both behavioral and cognitive features. One of the main cognitive deficits found in AN is cognitive rigidity, or an inability to think about multiple perspectives simultaneously and to change one's view on an issue. Several studies have suggested that this deficit is heritable and is found in healthy siblings and parents of AN patients. Other studies have shown that level of cognitive rigidity is correlated to duration of illness, suggesting that this deficit may be a result of long-term starvation. Cognitive rigidity contributes to the maintenance of AN by making it difficult for patients to alter their cognitive distortions surrounding the disorder. Treatments that address secondary symptoms of the disorder, such as weight loss and food avoidance, can be ineffective if the underlying cognitive features of the disorder are not properly addressed. A considerable body of research has demonstrated that cognitive remediation therapy is effective in increasing cognitive flexibility in patients with AN. This form of treatment can allow patients to see their behaviors as maladaptive and can help promote behavioral change.

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