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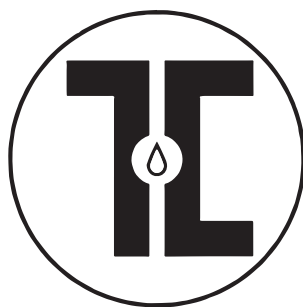
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# The Science Journal

Lander College of  
Arts and Sciences-Flatbush  
A Division of Touro College





# **The Science Journal**

*Lander College of Arts and Sciences-Flatbush*  
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# Table of Contents

<b>Treating Acute Migraines: Triptans vs. Antiemetics</b> <i>Samuel Reisman</i> . . . . .	7
<b>Vigorous Exercise Effect on Cardiovascular Health</b> <i>Aryeh Mahana</i> . . . . .	12
<b>Short-term Effects on the Fetus and Long-term Outcome on Children Exposed to Maternal Chemotherapy</b> <i>Yocheved Kramer</i> . . . . .	19
<b>Is Proton Beam Therapy More Effective than Intensity-Modulated Radiotherapy in Prostate Cancer Treatment?</b> <i>Daniel Zelefsky</i> . . . . .	26
<b>Mechanisms, Potential Therapies, and the Role of TGF-<math>\beta</math> in the Formation of Scars</b> <i>Isaac Traube</i> . . . . .	32
<b>Is There a Link between Saturated Fat Intake and Alzheimer's disease?</b> <i>Justin Konig</i> . . . . .	40
<b>Regulation of Ghrelin A Possible Treatment Option for Obesity and Diabetes</b> <i>Sarah Picciotto</i> . . . . .	48
<b>Transposon Based Gene Therapy as a Treatment for Cancer</b> <i>Jacob Stauber</i> . . . . .	55
<b>Understanding the Hygiene Hypothesis and its Mechanisms</b> <i>Yaakov Tropper</i> . . . . .	62

**Creutzfeldt - Jakob Disease and Alzheimer’s Disease: Does Overlap of Mechanism Mean Overlap of Treatment Methods?**  
*David Rotblat* . . . . . 67

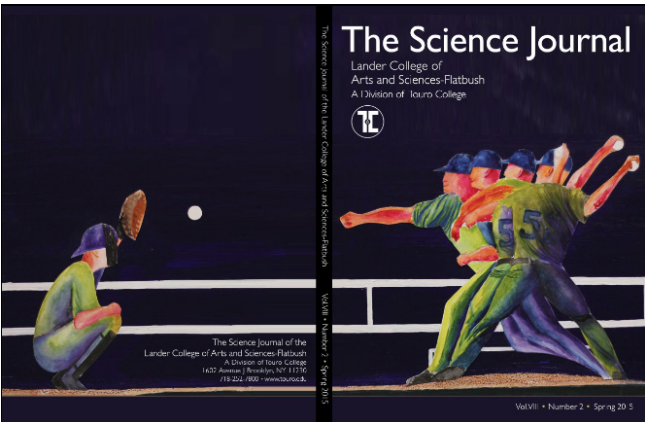
**Pitch-count and its Effects on Shoulder Injuries. How to keep a Pitcher Healthy**  
*Alexander Malek* . . . . . 79

**Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy (SMA):A Genetic Linkage?**  
*Michael Groden* . . . . . 83

**Nanotechnology**  
*Zisi Hursarsky* . . . . . 89

**Should Sports Drinks Be Given To Children And Adolescents Engaged In Athletics As An Effective Source Of Hydration?**  
*Zeev W. Rubinson.* . . . . 96

**Viability of SiRNA as a Clinical Treatment**  
*Zev Blumenkranz* . . . . . 101



The Cover Illustrations are Original Paintings by Karen Bleich They are representative of the article “Pitch-count and it’s Effects on Shoulder Injuries. How to Keep a Pitcher Healthy”

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## **The Lander College of Arts and Sciences at Touro College of Flatbush**

Throughout its 38-year history, Touro's Lander College of Arts and Sciences of Flatbush (with separate men's and women's schools) has provided cohorts of aspiring high school graduates from well-regarded yeshivas and seminaries with a foundation of academic excellence for professional career growth, in an environment that is supportive of the religious values of its students. Graduates have assumed leadership roles and continue to strengthen Jewish communities throughout the world.

Lander College of Arts and Sciences—Flatbush offers more than 25 majors and preprofessional options, and three joint undergraduate/graduate degree programs in occupational therapy, physical therapy and physician assistant studies with the School of Health Sciences. Honors tracks in biology, the health sciences, political science and psychology are currently offered.

Students are also required to complete a carefully designed core curriculum that emphasizes the development of communications skills, critical thinking and analytical competencies, computer literacy and quantitative reasoning. Enrollment in science courses, notably biology and chemistry, continues to increase, reflecting the career interests of premedical and health science students.

Faculty members continue to earn recognition for outstanding achievements, including Joshua November, Assistant Professor of Languages and Literature, who was selected as a finalist for the Los Angeles Times Poetry Book of the Year Prize in 2011; Karen Sutton, Assistant Professor of History, whose significant Holocaust analysis, *The Massacre of the Jews of Lithuania, 1941-44*, was published in 2008; and Atara Grenadir, Assistant Professor of Art, whose works were displayed at the Art Expo 2011 show in New York City.

Notable alumni distinctions of Touro's Lander College of Arts and Sciences of Flatbush include: David Greenfield (JD, Georgetown), elected to the New York City Council (44th Council District) in 2010; Dr. Israel Deutsch (MD, Einstein), appointed as Director of Brachytherapy at New York-Presbyterian Hospital/Columbia University; Yossi N. Heber (MBA, Wharton), President, Oxford Hill Partners; Dr. Haim Mozes (PhD, NYU), Associate Professor, Graduate School of Business, Fordham University; Vivian Schneck-Last, Managing Director, Goldman Sachs; and Sara Grossman Wiederblank, who published her fourth novel, *Pass or Fail*, in 2010. Alumni have published articles in the New York Law Journal, Bloomberg Law Reports, Institutional Investors Journal and other peer-reviewed journals.





# Treating Acute Migraines: Triptans vs. Antiemetics

Samuel Reisman

Samuel will graduate in June 2015 with a B.S. degree in Biology.

## Abstract

Influential American medical organizations and publications have published guidelines for the treatment of acute migraine headaches that omit antiemetics, usually suggesting triptans as the first line of treatment. A review of the few comparative studies directly contrasting clinical outcomes of triptans and antiemetics in the treatment of acute migraines suggest that both treatment options are relatively equal in efficaciousness. The added burden of triptan usage, including an added risk of adverse effects and a high cost per dose, would seem to warrant an antiemetic-first approach to migraine treatment, as recommended by several international health communities. Possible reasons for the prominent omission of antiemetics from leading publications may include medical parochialism and pharmaceutical funding of medical research.

## Introduction

In January 2002, the New England Journal of Medicine published a paper entitled "Migraine-Current Understanding and Treatment," written by several leading neurologists. The paper reviewed all the current treatment options for migraine headaches, and strongly emphasized triptans, a relatively new class of drugs developed by drug companies specifically to address migraines (Goadsby et. al., 2002). One of the most notable aspects of the paper was that it completely omitted the drug class antiemetics, which is increasingly used nationally and internationally, mostly among emergency medicine practitioners, to treat acute migraine headaches (Seguil & Lax, 2014). One factor for this omission may have been the varied perspectives which can often develop between members of different specialties of medicine, with neurologists recommending one therapy and emergency medicine doctors recommending another (Newman, 2009). Nevertheless, it is likely that the authors were aware of the therapeutic history of antiemetics for migraine headaches, and they chose to omit it. In fact, the paper's recommendations are completely consistent with the guidelines published by the American Academy of Neurology (n.d.). The purpose of this review is to assess whether these influential omissions are in fact warranted by clinical observation and meta-analysis, or if antiemetics should be considered an efficacious treatment for acute migraines with the right clinical indications. Possible biases that could have caused conscious or subconscious influences on the recommendations of different groups will also be analyzed as a method of understanding them.

## Methods

In order to assess antiemetics as an efficacious treatment option for acute migraines, a meta-analysis of the published literature was undertaken. Comparative studies between the effectiveness of triptans and antiemetics is the main focus. In assessing clinical value, both primary effectiveness and secondary side-effect prevalence were surveyed to accurately portray an overall picture of patient outcomes. Clinical trials were obtained using the National Institute of Health's PubMed search engine, and only studies published in reputable academic journals were included.

## Migraine Headaches

A migraine headache is defined as a headache that usually affects

one specific area or side of the head and is frequently accompanied by nausea, sensory sensitivity, and possible neuralgia (Ferrari, 2013). Headaches accompanied by neuralgia have been recorded since ancient times, as far back as the ancient Egyptians (Miller, et. al. 2005). The difference between a normal headache and a migraine is often one of degree and thus cannot always be definitively assessed; however, chronicity can be an important indicator of migraines. The first modern treatment for migraines was ergotamine (Woakes, 1868), which was originally hypothesized to slow the stimulation of sympathetic nervous pathways (although its mechanism is now contested). The pathogenesis of migraines was illuminated in the 1940s, when serotonin was isolated as a potent cause of migraines (Wolff, 1948). This discovery led to the serotonin-inhibiting class of migraine treatments, starting with methysergide, which was first used in the middle of the 20th century (Sicuteri, 1959).

This paper focuses on two modern therapies for migraine headaches: triptans, of which the prototype drug is sumatriptan, discovered in 1988, and antiemetics, which are primarily anti-nausea medications, including domperidone, metoclopramide, and prochlorperazine. These drugs are typically given together with an analgesic, usually aspirin. Other commonly used pain-relieving drugs, such as NSAIDs, caffeine, and codeine should be noted, but are not of specific interest to this discussion.

## Triptans

Triptans were first used in the treatment of migraines during the 1980's, when interest surged in examining the role of serotonin (5-HT) in the pathogenesis and pathophysiology of migraines (Bateman, 2000). Triptans are a class of drugs that affect serotonin receptors, commonly called 5-HT receptors, of which there are many subtypes. Triptans are 5-HT agonists, binding with high affinity to many 5-HT subtypes that cause potent vasoconstriction of many intracranial blood vessels. They also affect various neurotransmitters and chemical mediators, but no specific effect has been conclusively tied to their anti-neuralgic properties. Because of a variety of concerns regarding the effectiveness of the original triptans, such as variable bioavailability, variable absorption, and significant adverse effects, new classes of triptans have been continuously developed by drug companies. Some of

the most recent triptans typically prescribed for migraine headaches include almotriptan, frovatriptan, and avitriptan (Loder, 2010). Although the mechanism of the therapy remains unclear, it is generally recognized in the United States as a first-line therapy for patients unresponsive to analgesics. Triptans provide relief of symptoms within the first 10-60 minutes of use, depending on route of entry (Loder).

### **Antiemetics**

Antiemetics are drugs that relieve symptoms of vomiting and nausea. They are usually used to treat motion sickness and to relieve the side effects of nausea-causing therapies. The use of antiemetics as a direct therapy for migraine headaches was a serendipitous discovery. Originally, antiemetics were used to allow sufferers of migraines to ingest drugs given to relieve the headaches. However, physicians soon began to notice that the symptoms of the migraine headaches were relieved before the primary therapy could be given. Thus antiemetics soon became the drug of choice, especially among emergency medicine practitioners, to treat analgesic-resistant headaches (Newman, 2009).

### **Comparative Studies**

Unfortunately, and for possible reasons that will be addressed further, there are very few studies that directly compare the efficaciousness of triptans and antiemetics in the treatment of migraine headaches (Gupta et. al., 2002). However, a number of studies have been completed globally that directly contrast these two treatment options.

The first comparative study was published in 1995, comparing oral sumatriptan (a triptan) with lysine acetylsalicylate plus metopramide (an aspirin plus an antiemetic) in their effectiveness in treating migraines (Tfelt-Hanson et. al., 1995). This study was conducted between October 26, 1993 and July 18, 1994 at over 68 medical centers in Belgium, Denmark, the Netherlands, and France, and included only patients with significant histories of migraine headaches. It was a randomized, double-blind study, which included follow-up for up to as eight weeks, as needed. Four hundred twenty-one patients participated in the study. The study showed that in numerous benchmarks for effectiveness, the two treatment options were virtually identical, including improvement in immediate headache severity, control of adverse effects, headache recurrence, and patient satisfaction. The authors concluded that “there is no difference in primary or secondary efficacy between LAS+MTC and oral sumatriptan...because of its high price physicians should consider whether the routine use of sumatriptan as the initial treatment of a migraine attack really is preferable to the use of cheaper drugs such as analgesics combined with an antiemetic.” Indeed, in Europe and in many other countries, these recommendations are generally considered best practice (Newman, 2009).

A subsequent study was performed in three medical centers in France, with a total of 666 participating patients (Geraud et. al., 2002). It was a multicenter, double-blind, randomized study, and follow up was performed until fifteen days after the last migraine attack took place. Each patient was given one of the following: acetylsalicylic acid plus metoclopramide, zolmitriptan, or a placebo. The patients were then requested to keep an hourly diary to record headache relief, overall pain relief, nausea levels, any adverse effects, and overall satisfaction with the therapy. The study results seemed to be inconclusive initially, as the authors wrote: “Both treatments reduced migraine-associated nausea, vomiting, phonophobia and photophobia. There were no important inter-group differences with respect to the onset of meaningful migraine relief, the frequency of headache recurrence, the usage or efficacy of a second dose of medication or the use of escape medicine.” However, the authors preceded to perform what they called a “post hoc analysis,” in which they found certain benefits to triptan use, including a greater overall patient satisfaction, overall pain-free reporting (as opposed to headache pain), a greater efficacy in patients with “migraine associated with menses,” and the fact that triptan use was “unaffected by age, weight, or gender.” They thus concluded that “Although evaluation using the primary end point in this study was inconclusive, other end points such as freedom from pain, now identified as more clinically relevant end points, showed zolmitriptan 2.5 mg to be significantly better than the standard analgesic-anti-emetic combination of acetylsalicylic acid and metoclopramide.” In summary, this study found slight benefits to triptan use, although it is important to note that for all the primary end points designated before the study was completed, the therapies were identical. Only after the data was collected did the authors find certain benchmarks that could be identified as benefits to triptan use. This is generally considered a far less objective method of gathering data, as it allows the investigator considerable latitude in actively picking specific data sets. In what may be an important note, the study concludes with an acknowledgement that “this study was supported by AstraZeneca Pharmaceuticals.”

A third study, performed in New York City, compared aggressive metoclopramide treatment, consisting of four infusions within the first two hours, to subcutaneous sumatriptan treatment of migraine headaches (Friedman et. al., 2005). Two hundred two patients participated in the trial. Patients were not followed after the initial twenty-four-hour period, which can be considered a weakness in their overall assessment of the therapies; in fact, only 37 of the patients completed the twenty four-hour follow-up protocol. In addition, the patient population studied was almost completely comprised of individuals of Latino origin, making extrapolations to general populations uncertain. Another concern is that the study excluded those suffering chronic migraines, which may be a population which reacts differently to specific therapies.

The study concluded that there were no significant differences between the two therapy options in reaching the primary end points of the study, including headache relief, nausea relief, and overall well-being. However, in their own post hoc analysis, the authors find certain benefits to metoclopramide use, including twenty-four-hour symptom relief.

A fourth comparative evaluation was performed in Iran at the Isfahan University of Medical Sciences, comparing metoclopramide to sumatriptan for migraine headache treatment (Talabi et. al., 2013). This study was performed on emergency room patients. One hundred twenty-one subjects were included in this randomized, double-blind study. Several introductory notes should be mentioned about this study. First, the command of the English language displayed by the authors is competent overall but nevertheless displays signs of possible grammatical and idiomatic peculiarities which may or may not result in important, altered connotations (for example, the authors wrote that their study included a “controlled study design and patient blindness”). Second, the study noted that “it is surprising that no subjects in both groups complained of adverse effects.” This is a significant deviation from other comparable studies, which may be a cause for concern. The authors attempt to explain this discrepancy as “a result of slow metoclopramide injection and the way the question about these effects were phrased.” It also may be a reflection of cultural differences in the way side effects are described, or how often, or upon what level of acuteness, they are remarked upon. The patients were all observed during the initial hour after they were treated. The results of this trial were that metoclopramide was superior to sumatriptan in headache relief (Talabi).

In summary, there are very few studies that directly compare triptans to antiemetics for acute migraine headache relief. The few that have been performed suggest that the therapies are relatively similar in effectivity for all primary end points.

### **Triptans vs Antiemetics: Other Differences**

The fact that triptans and antiemetics have been shown to have similar outcomes in treating migraines does not necessarily mean that they are equally sound treatment options. In fact, there are several reasons why triptans may be a less advisable treatment option. The first is adverse effects. Triptans are known to cause several negative effects in patients. The most common set of adverse side effects, affecting almost half of all triptan users, is often referred to as triptan sensations, and includes upper chest pressure or pain and epithelial flushing. Rare cardiovascular events have also been reported and triptans are thus contraindicated in those with possible cardiac disease. This stands in contrast to antiemetics, and specifically to metoclopramide, which have minimal reported adverse effects. The second shortcoming of triptans is their often high price, with the average cost of a single triptan pill

typically exceeding ten dollars, while a single dose of antiemetics can cost less than ten cents (Adelman et. al., 2004). The benefits of antiemetics are thus both in terms of adverse effects, which are minimal, and cost, as they are extremely cheap therapy to provide to patients. Therefore, if antiemetics can be shown to be comparably effective to triptans in specific clinical settings, which seems to be the case, they can plausibly be considered a superior therapy overall under those conditions. Thus it remains puzzling how the 2002 review article in the *New England Journal of Medicine* completely omitted antiemetics in its review of migraine relief protocols, and why it is omitted from the recommendations of the American Academy of Neurology on the treatment of migraines.

### **Medical Parochialism**

Parochialism in research has been a phenomenon long noted and lamented by meta-researchers (March, 2005). It is often based on nationality, with different countries' research communities favoring different approaches. These differences can have cultural, ideological, or experiential origins. Parochialism can also be of disciplinary origin. In the medical field, this has often been the case; for example, medical doctors and nurse practitioners often find themselves at odds over a variety of disciplinary differences (Phillips et. al., 2002). In the specific case of the triptan vs. antiemetic debate, the difference in recommendations may have arisen from the different perspectives that neurologists and emergency medicine doctors have of migraine sufferers. Emergency medicine practitioners generally see patients who are in the midst of acute migraine attacks. Therefore, their perspective is geared toward therapies that are most efficacious at immediate migraine relief, and this may factor into their preference of antiemetics to triptans (Friedman et. al., 2014). Neurologists, on the other hand, see mostly patients who are chronic migraine sufferers, and may therefore strongly favor treatments that provide longer-lasting relief. Nevertheless, it must be emphasized that the review in the *New England Journal of Medicine* was a complete review of both acute and chronic migraine treatments, and thus explicitly included the medical protocols in which emergency medicine practitioners are most experienced. It is therefore quite possible that parochialism is at fault for the varying guidelines proposed to treat migraines, where one field's inherent biases led it to ignore or be unfamiliar with the practices of other fields. (It should be noted, however, that the 2005 study that found that antiemetics were comparable to triptans was authored by emergency room doctors and was published in the journal *Neurology*.)

### **Pharmaceutical Funding**

Surveying the comparative studies of triptans and antiemetics brings to the fore the often uncomfortable question of relationships between for-profit companies and medical institutions and research facilities (Smith, 2003). Although many regulations have

been passed over the last few decades, which have helped prevent the more egregious practices of pharmaceutical companies, many interactions remain that might possibly compromise clinical objectivity (Brody, 2005). It is certain that drug companies would favor the use of expensive, patented triptans over the cheap antiemetic drugs, and it is thus distressing that a drug company was the primary funder of the single comparative study that found, in post hoc data examination, that triptans were a superior treatment option. The other studies were free of any reportable conflict of interest, and came to different conclusions. In addition, the review article from the *New England Journal of Medicine*, which strongly focused on and recommended triptan use, closes with a fine-print disclosure that all the authors have been recipients of grant funding or have acted as consultants for many different drug companies, including all those that currently manufacture triptan medications (Newman, 2009). This fact may explain why the authors, consciously or not, were especially focused on triptan therapy. Of course, this does not mean to slander the authors in any way or to impugn their professional reputation, but rather to bring into focus the problems associated with industry funding of scientific enterprises. Certainly, the fact that such funding is indispensable to many research projects cannot be ignored, but perhaps other remedies, such as the mandatory authorship of one author without reportable conflicts of interest, can be advanced to protect the integrity of these studies.

Another important question regarding pharmaceutical company funding of medical research is the types of studies performed. For example, if a pharmaceutical company deems a therapy to be dangerous toward its bottom line, it may simply withdraw all funding for studies pursuing that therapy, leaving little incentive for researchers to pursue it. This may explain why so few studies have actually been performed comparing triptans directly with almost any other therapy, including antiemetics, and instead most research in the field consists of large studies, including thousands of patients (Ferrari et al., 2002), which look solely at the benefits of triptans.

### Conclusion

A survey of comparative studies available shows that antiemetics are as suitable for treating acute migraines as triptans. The omission of this documented treatment from prominent guidelines and publications is disconcerting, and may point to several fundamental weaknesses in the current research and application model. Medical parochialism and pharmaceutical funding likely amplified these flaws, and must be addressed as part of the solution going forward.

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## Treating Acute Migraines: Triptans vs. Antiemetics

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# Vigorous Exercise Effect on Cardiovascular Health

Aryeh Mahana

Aryeh will graduate in June 2015 with a B.S. degree in Biology and will begin his training in the Rutgers School of Dental Medicine in September.

## Abstract

Many studies examine the effects of vigorous cardiovascular exercise on the heart. Intense exercise causes frequent muscle contractions in the heart and specific biomarkers that usually signify a myocardial infarction are released into the bloodstream. However, studies indicate that there might not be a correlation between the release of biomarkers and cardiac function. Another study shows that long term vigorous exercise negatively affects the heart by dramatically increasing the mass and volume of the right and left ventricles, thereby resulting in hypertrophy. The cardiac hypertrophy is still evident even after the subjects have stopped exercising. Cardiac hypertrophy results in myocardial fibrosis and scarring. The percent of myocardial fibrosis in those engaging in long term vigorous exercise was significantly higher than that of the control group. After intense training, tests found a reduced right ventricle ejection fraction due to dilation of the right ventricle while there was no dilation in the left ventricle. In addition, a correlation was found between reduced right ventricle ejection fraction and ventricle arrhythmias. The mortality rate of vigorous exercisers was slightly higher in comparison to those who moderately exercised. Animal studies showed that rats who exercised developed left ventricle hypertrophy, impaired diastolic function, and right ventricle dilation, the symptoms found in humans with an “athlete’s heart.” Additional research on the effect of vigorous exercise on the heart needs to be conducted to verify these findings.

## Introduction

In 490 BCE Pheidippides, a 40 year old courier ran 24.8 miles from Marathon to Athens to announce the Spartan victory over the Persians, and then dropped dead from what is believed to be cardiac arrest. This is the earliest mention of death due to aerobic activity (Trivax & McCullough, 2012). It was not until the 1970s that jogging would become a popular form of aerobic exercise. The many physical and physiological benefits of aerobic exercise caused this phenomenon to grow. In 2010, statistics indicated around 36 million people jog daily. In 2013, 541,000 people completed a marathon (which is an intense aerobic event of 26.2 miles) in the United States alone (Schnohr, et.al. 2013 & Martin, 2014). It has been hypothesized that a person capable of completing a marathon is immune to coronary heart disease (Bassler, 1974). This hypothesis is not fully supported since a six year study from 1975-1980 showed that eleven men died in Rhode Island while running; the cause of death being coronary heart disease (Thompson et. al. 1982). In addition, a study conducted from 2000-2010, shows that out of 10.9 million people who have completed marathons or half-marathons, only 59 have suffered cardiac arrest, majority due to coronary heart disease. (Kim, et.al. 2012).

The World Health Organization endorses at least 150 minutes a week of moderate demanding aerobic activity (such as walking, water aerobics, and bicycling) or 75 minutes a week of vigorous-intensity aerobic physical activity (such as jogging, running, and swimming laps) for significant health benefits (Prevention and Health Promotion, 2014). People may think that exercising vigorously for longer periods of time would increase the health benefits. However, it is unclear whether more intense exercise improves the efficiency of the cardiovascular health or if it over-exerts the heart and thereby puts the individual’s health at risk. By examining the data and studying trends associated with extreme exercise and productivity of the heart, this review will try

to determine if prolonged vigorous exercise is detrimental to a person’s cardiovascular health.

## Methods

This comprehensive review was written through the critical analysis of clinical research papers and peer reviewed journal articles. The necessary material was found using Touro College’s online databases, such as Pubmed, Medline and EBSCO. Google and Google Scholar were used to search specific keywords related to the topic.

## Discussion

### *The Mechanism of Cardiac Contraction*

Cardiac muscle contraction and relaxation has been studied on the cellular and molecular level. The overall mechanism for muscle contraction is known as excitation–contraction coupling. In excitation–contraction coupling, an electrical stimulus (action potential) transforms into a mechanical response (muscle contraction). In cardiac muscle, excitation–contraction coupling occurs by a more specific mechanism of calcium-induced calcium release, a positive-feedback system where calcium induces the release of more calcium from intracellular  $\text{Ca}^{2+}$  supplies. The internal conduction system initiates an action potential that progresses along the t-tubules of the cardiomyocytes, resulting in the opening of voltage-gated L-type calcium channels. This allows extracellular calcium to flow into the sarcoplasmic reticulum causing a stimulation of the ryanodine receptors (RyR) of the sarcoplasmic reticulum which results in a bulk release of calcium into the cytoplasm. Binding of the cytoplasm calcium to troponin C (a subunit of the troponin complex), results in conformational changes of the tropomyosin complex allowing for cross bridging to occur between the actin and myosin (excitation–contraction coupling mechanism). Contraction occurs as the actin filaments are pulled toward each other. All this transpires through the hydrolysis of ATP in the mitochondria mainly found around the



ryanodine receptors. The cardiac muscle cell relaxes/repolarizes once there is not any calcium left in the cytosol; this is achieved through the different ion channels that sequester the calcium back into the sarcoplasmic reticulum, an ATP dependent action. The more intense the exercise, the more frequently this process happens and a variety of toxic molecules are produced (Bers, 2001).

### Biomarker Release

The troponin complex comprises of three subunits of proteins: troponin I (TnI), troponin T (TnT), and troponin C (TnC). Each of the three subunit proteins has specific interactions with the actin filaments that help regulate muscle contraction. Necrosis of myocytes results in loss of membrane integrity, resulting in the release of cardiac troponin T and cardiac troponin I into the bloodstream. Troponin T and troponin I are considered leakage markers since cardiac muscle has a different isoform than skeletal muscle does for each of these respective troponins. Usually, an increase in troponin T and troponin I in the blood is an early warning sign of a myocardial infarction. It has been hypothesized that the elevated cardiac troponin markers found in the bloodstream after vigorous exercise is from the cytosolic pool rather than from physically bound cardiac troponin (the breakdown of the myocyte). This means that cardiac muscle contraction is not affected by the increase in troponin levels in the bloodstream due to vigorous exercise since the damage to the membrane is reversible (Wells & Sleeper, 2008). A study examined the relationship between cardiac troponin T and left ventricle function in 52 runners who just completed a marathon. The 52 runners were screened with an echocardiogram to determine left ventricle function and a blood sample assessing for cardiac troponin T before and after the race. The 99th percentile for cardiac troponin T in the serum assay of normal subjects is 0.01 micrograms per liter. The cardiac troponin T was not detected in all the participants before the race. However, significant increases of cardiac troponin T levels were observed after the race in all the marathon runners. Twenty runners had values above the acute myocardial infarction cut off of 0.05 micrograms per liter. The study did not find a link between these elevated levels of cardiac troponin T and left ventricle function. This supports the notion that the cardiac troponin T released into the bloodstream during extreme exercise is from the cytosolic pool (Whyte, et.al. 2005).

A study of fourteen amateur runners who completed a marathon were tested pre and post marathon for myoglobin, creatine kinase (both also biomarkers for detecting a myocardial infarction) and cardiac troponin T along with a cardiac magnetic resonance to detect myocardial necrosis. The study was performed to determine whether elevated biomarkers due to vigorous exercise had a relation to myocardial necrosis. Before the race, myoglobin, creatine kinase, and cardiac troponin T serum levels were normal.

Post marathon there was a significant increase in the assay serum levels of all the three biomarkers. If these results were found in a person who did not partake in vigorous exercise it would be cause for alarm. When cardiac magnetic resonance imaging was done there was no evidence of myocardial edema on T2 imaging or delayed enhancement of the left ventricle myocardium. Within one week of the race, all the biomarker levels returned to normal. Some might say vigorous exercise is fine since there is not an immediate relationship found between elevated biomarker levels and cardiac function. However, constant vigorous exercise might affect cardiac function in the future depending on whether the increased biomarker levels are from the cytosolic pool or from physically bound cardiac troponin (Mousavi, et.al. 2009). These two studies provide some evidence to the above hypothesis however further studies must be conducted to understand the exact reason for the increase in biomarker levels.

### The Heart Rate Effect

The body's cardiac output increases during aerobic activity, due to the greater demand for oxygenated blood that the body has during exercise. The two defining qualities of cardiac output is stroke volume (the amount of blood forced out of a ventricle during one contraction [mm/Hg]) and heart rate (the amount of times the heart contracts in one minute [beats/min]). The stroke volume is determined by three factors: the blood that fills the heart during diastole (preload), the force with which the blood leaves the heart during systole (afterload) and, the resistance of the aorta or pulmonary arteries. Cardiovascular exercise causes more blood to be pumped out of a ventricle, resulting in an increased stroke volume. There is a maximum capacity that the stroke volume can increase to before it levels off. At this point, the heart rate responsible for the cardiac output pumps blood to the rest of the body (McArdle, et. al. 2006).

In a recent study, fifty healthy females ages 18 to 24 were randomly selected to examine the benefits that moderate and vigorous exercise training has on a person's heart rate and blood pressure (systolic and diastolic). Participant's heart rate and blood pressure were measured before and after actively engaging in an exercise regimen. The exercise regimen included a thirty minute combination of walking (moderate exercise) and running (vigorous exercise) over a three month period. The heart rate and blood pressure was considerably lower after the three months of aerobic training (see Figure 1 on next page) (Munisekhar, et. al. 2014). This data shows that moderate exercise training is beneficial for improved cardiovascular health due to a decreased resting heart rate and blood pressure. In addition, there is reduced stress on the heart and artery walls. A slower heart rate also gives the coronary arteries more time to fill with blood and oxygenate all the heart cells (Harvard, 2008).



**Figure 1**

	HEART RATE (BEATS/MIN)		DIASTOLIC BLOOD PRESSURE (mm/Hg)		SYSTOLIC BLOOD PRESSURE (mm/Hg)	
	Before Training	After Training	Before Training	After Training	Before Training	After Training
	Mean		Mean		Mean	
Resting	78.1	68.1	66.2	62.15	99.6	95.1
Walking	126.2	106.58	76.65	67.75	115.88	102.5
Running	169.25	127.93	77.7	72.3	144.85	124.35

Mean heart rate, blood pressure (diastolic & systolic) of the 40 participants before and after their training: Modified from (Munisekhar, et. al. 2014).

### Long Term Exercise Conditioning

To attain the physiological condition of “athlete’s heart” there must be a decrease in heart rate and an increase in hemodynamic demands, which modifies the stroke volume loading. As a result of increased hemodynamic demand there is a change in the left and right ventricle mass and volume; the result of which is cardiac hypertrophy. Due to the limitation of imaging tools, this has been shown in the past to be predominately on the left ventricle. Echocardiography and MRI were used in order to study the effect that prolonged vigorous exercise has on both the right and left ventricles’ mass and volume. Twenty-one healthy male endurance athletes, with a history of exercise and a control group of twenty one untrained males were used in the study. In order to meet the criteria of an “athlete’s heart”, the heart volume of the twenty one endurance athletes had to be at least 13 ml/kg. Results showed that in the hearts of endurance athletes, the mass of the left ventricle was 200 grams while in the control group the mass of the left ventricle was 148 grams. In the hearts of endurance athletes, the mass of the right ventricle was 77 grams while in the control group the mass of the right ventricle was 56 grams. This is a significant increase in the mass of the left and right ventricles and reaches the criteria for hypertrophy. The left ventricle and right ventricle end-diastolic volumes were also substantially increased in the endurance athletes compared to the control group (Figure 2) (Scharhag, et.al. 2002). This study shows that an “athlete’s heart” has a balanced enlargement of the left ventricle and right ventricle, which in turn indicates a benign balanced cardiac hypertrophy.

In another study echocardiography and electrocardiography was used to look at left ventricle hypertrophy remodeling in forty elite endurance male athletes after termination of systematic training (deconditioning). Of the forty endurance athletes, fifteen stopped all physical activity and the other twenty five changed from doing intense exercise to doing moderate exercise. The forty athletes had left ventricle cavity enlargement of more than 60mm or wall thickness of more than 13mm at the beginning of the study. After

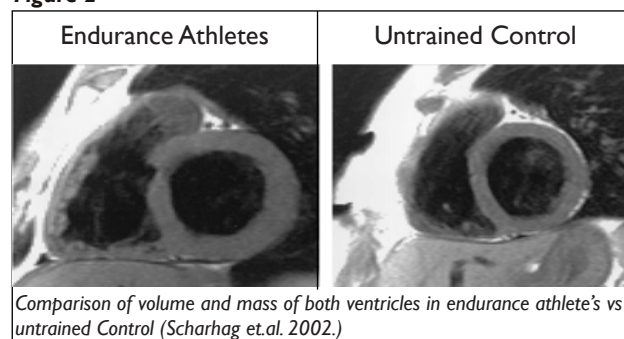
the average deconditioning period of six years, the dimensions of the left ventricle during end-diastolic shrunk by seven percent. The left ventricle cavity size still remained enlarged; more than 55mm in 85 percent of the athletes and more than 60mm in 22 percent of them. The average wall thickness decreased by fifteen percent from 12mm at peak training to 10.1mm after deconditioning. There was not any difference between the fifteen that stopped all physical activity and the other twenty five that changed from intense to moderate exercise. The study showed that once there is substantial left ventricle dilation (hypertrophy), there is only a slight reversal in left ventricle hypertrophy, even

after ceasing to do exercise for a long period of time (Pelliccia, et.al. 2006).

### Myocardial Fibrosis

La Gerche et al. (2012) found that five of the participants (13%) who had greater prior exposure to vigorous exercise displayed delayed gadolinium enhancement (DGE) in their cardiac magnetic resonance imaging baseline test. More specifically, they showed fibrosis and scarring in the interventricular septum. Another study which contained 102 healthy male runners between 50 –72 years were matched with 102 control subjects who were non-runners. In order to be included in the experiment the runners had to have completed at least five marathons in the past three years. The results showed that 12% of marathon runners had myocardial fibrosis and patchy scarring, as demonstrated by delayed gadolinium enhancement with cardiac magnetic resonance imaging. This rate was three times higher than in the control group, who had a 4% occurrence of myocardial scarring. The study also did a 21 month follow up on all the participants and found the rate of coronary heart disease was higher in the runners with myocardial scarring and fibrosis then in the control group. Even though the percentage of marathon runners with scarring or fibrosis is low, compared to the control group, the incidence of scarring is greatly higher. This author proposes that the development of myocardial scarring may be correlated

**Figure 2**



to prolonged engagement in vigorous exercise; in this case, the marathon running (Breuckmann, et.al. 2009).

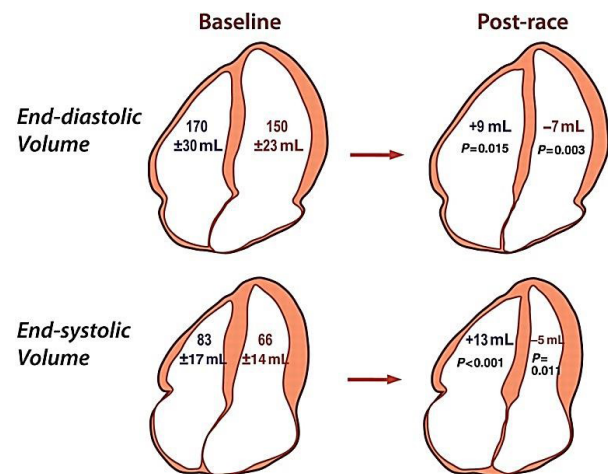
A study on myocardial fibrosis resulting from extreme training was performed on twelve lifelong veteran male endurance athletes, twenty age-matched controls and seventeen younger male endurance athletes. Veteran male athletes had to have 35–52 years of continuous intense training and younger male athletes had to have 11–31 years of intense continuous training. Just as in the previous study, delayed gadolinium enhancement with cardiac magnetic resonance imagining was performed to evaluate myocardial fibrosis. Results showed that in six of the twelve veteran athletes (50%), the existence of myocardial fibrosis and scarring was present. There was no sign of myocardial fibrosis found in the age-matched veteran controls or the younger athletes. The extensiveness of myocardial fibrosis in veteran athletes was not correlated to the age of the athlete, but rather to the amount of years spent training and to the amount of marathons completed. Even though the previous two studies were a small sample size, they still add validity to the correlation between extreme exercise and myocardial fibrosis (Wilson, et.al. 2011).

### Right Ventricle Dysfunction Immediately After Exercise

Vigorous aerobic activity increases the average cardiac output of 5 liters per minute to a maximum of 35 liters per minute (McArdle, et. al. 2006). This increase puts a strain on the heart causing the right atrium (RA) and right ventricle (RV) to dilate. A one-time event will not have a negative impact since the heart will return to its normal size. However more consistent vigorous exercise can cause myocardial scarring due to the volume load and cardiac strain because of the chronic dilation of the right atrium and right ventricle. In fact, a study using cardiovascular magnetic resonance (CMR) showed that running a marathon causes acute right atrium and right ventricle dilation and reduces the right ventricle ejection fraction (RVEF). The participants in the study were randomly selected; 25 healthy participants from a pool of 425 volunteers. Cardiovascular magnetic resonance imaging was conducted four weeks before and after the race. The mean age of the participants was 38 years and the mean time for completing a marathon was 256 minutes. “Cardiovascular magnetic resonance showed before and after marathon left ventricular ejection fractions were comparable,  $57.7 \pm 4.1\%$  and  $58.7 \pm 4.3\%$ . Right atrial volume index increased from  $46.7 \pm 14.4$  to  $57.0 \pm 14.5$  ml/m<sup>2</sup>. Also, right ventricular end-systolic volume index increased from  $47.4 \pm 11.2$  to  $57.0 \pm 14.6$  ml/m<sup>2</sup> whereas the right ventricular ejection fraction dropped from  $53.6 \pm 7.1$  to  $45.5 \pm 8.5\%$ ”. This data shows that marathon running causes a decrease in right ventricular ejection fraction due to a dilation of the right atrium and right ventricle. However, according to the study it does not appear to result in ischemic injury to any of the chambers (Trivax, et.al. 2010).

Forty healthy endurance athletes were studied following a vigorous event (duration of between 3–11 hours) to assess cardiac function at baseline, immediately post-race, and 1 week post-race. The authors found a reduced right ventricular ejection fraction after the race, which was connected to the dilation of the right ventricle after the event. This follows the Frank–Starling mechanism, which states that an increase in the volume of blood stretches the ventricle causing the heart to contract more forcefully. Thus, in these extreme conditions it causes a dysfunctional heart which is evident by the right ventricular ejection fraction. A greater dilation of the right ventricle caused a slight decrease in left ventricle volume (figure 3) (La Gerche, et.al. 2012). This may be explained because the increase in ventricular load due to intense exercise is greater for the right ventricle than the left ventricle (La Gerche, et.al. 2010). The study also showed that the extent of right ventricle deformation is directly correlated to the event period. A week after the event, all of the cardiac dysfunction returned to their baseline readings (La Gerche, et.al. 2012).

**Figure 3**



Intense exercise effect on right and left ventricle. The change in volume from baseline is showed on the left (La Gerche, et.al. 2012).

There was a correlation found between a decrease in right ventricular ejection fraction and ventricular arrhythmias (VA) in endurance athletes. This was a result of right ventricle dysfunction in 82 percent of the cases. The study performed right ventricle angiography on three groups: 22 athletes with ventricular arrhythmias, 15 athletes without ventricular arrhythmias and 10 control non-athletes. The study defines an athlete as one who has 6 hours a week of endurance exercise (running or cycling) for more than 5 years. Results showed that those athletes with ventricular arrhythmias showed a substantial decrease in right ventricular ejection fraction versus athlete without ventricular arrhythmias ( $49.1 \pm 10.4$  vs.  $63.7 \pm 6.4\%$ ). It was also revealed that the athletes who participated in vigorous exercise have

enlarged right ventricles while the control group right ventricles were normal, these findings are consistent with previous studies (Ector, et.al. 2007).

### Mortality Rate

The positive effects of exercise on health are numerous. The greatest benefit is the overall decrease of the mortality rate due to physical activity. A 21 year study observed 55,157 adults from the age of 18 to 100 (mean age 44) to determine the correlation of running with all-cause and cardiovascular mortality risk. Individuals with any health issues prior or present were excluded from the experiment. There was a questionnaire that was distributed to participants which asked specific questions regarding a person's exercise training (figure 4). Of the 55,157 participants 24% of them were involved in weekly running of some sort. The study found that runners had a 30% lower all-cause and 45% lower cardiovascular mortality rate then compared with non-runners. The cardiovascular mortality life expectance decreased in non-runners by 4.1 years. These results were similar, as long as the weekly running time was kept under 175 min/week (less than 1840 MET -min/week). Exercising more than 175 min/week slightly increased the mortality rate but was still less than the non-runners mortality rate. This was not only the case for time spent running but also for speed, frequency, and distance (Figure 3) below for the given data (Lee, et.al. 2014).

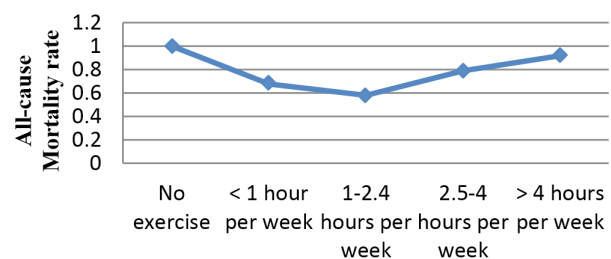
In the Copenhagen heart study, similar results were found after it was adjusted to a comparable set of confounders found in the previous study. The authors reported with less than 150 minutes of exercise a week there was a decrease in all-cause mortality. However, exercise time greater than 150 min/week did not make a difference in all-cause mortality between runners and non-runners. These results produced a U-curve with regard to jogging time versus mortality rate (figure 5) (Schnohr, et.al. 2013). One of the reasons may be because in this study there were only 1,878 joggers which caused wide confidence intervals since there were not enough runners to equally distribute in all the different exercise categories. On the other hand in the previous study by Lee et.al. there were approximately 13,000 joggers which allowed an equal distribution in the different dose running times.

Another observational study was done by the British Regional Heart Study in 1978 on the decreased risk of coronary heart disease due to physical activity. There were a total of 7,735 male participants aged between 40 and 59. The male participants were randomly chosen from general practices in 24 British towns without distinguishing whether they had pre-existing coronary heart disease. A typical questionnaire was conducted which asked questions on exercise and leisure activities (such as walking, weight lifting, and jogging). After eight years, results showed that the male participants without any pre-existing coronary heart disease who

partook in moderate exercise (such as walking at 4 miles per hour) had decreased the risk of coronary heart disease by 50 percent, compared to those who were inactive. The participants with coronary heart disease conditions in the past showed a comparable inverse relationship: decreased risk of coronary heart disease with increased moderate exercise. However, those partaking in vigorous aerobic activity (such as jogging or running) had a higher rate of a heart attack than those participating in moderate exercise (Shaper, et.al. 1991). These three studies show that those who participate in weekly moderate exercise versus those who do not do any exercise have less of a risk of developing coronary heart disease, and a lower all-cause and cardiovascular mortality rate. However these studies also show that there is an upper limit to these benefits and over exercising slightly reverses these positive effects and the mortality rate is similar to those who do not exercise.

Figure 4

### Mortality rate vs exercise time



### Hours of exercise per week

Hazard ratio of all-cause mortality versus the amount of time spent exercising per week. Graph produces a U shaped curve with an optimal exercise time between 1-2.4 hours per week (Schnohr, et.al. 2013).

### Animal Studies

In order to achieve a post-mortem look at the effect intense exercise training has on cardiovascular health, it is necessary to perform studies using animals. Rats were used to examine the long term intense exercise effect on cardiac function; more precisely fibrosis, which can induce cardiac arrhythmias. The Wister rats were separated for 16 weeks (this is equivalent to around 10 years in humans) into exercise and sedentary control groups. After the 16 week experiment, some of the rats from the exercise group were held for another eight weeks without exercise. This was done in order to see if the effect that intense exercise has on the heart is reversible. At the end of the experiment, all of the rats were euthanized and their hearts were removed for dissection. The exercise rats were put on a treadmill for five days a week, at 60 centimeters per second. In order to keep the rats running, a small shock was provided however, it was extremely low so that a stress response would not be elicited. Results showed that after 16 weeks of training, the exercise rats developed left ventricle hypertrophy, and impaired diastolic function; which is related to the left atrium dilatation. There was also right ventricle dilatation,

impaired diastolic and systolic dysfunction. All these symptoms are also found in humans with “athlete’s heart”. Right ventricle fibrosis was found in the histological sections. This can be due to the higher loading conditions on the right ventricle versus the left which is also consistent in humans. TGF- $\beta$ 1, a fibrotic marker, was found in high levels in both atrium and right ventricle. TGF- $\beta$ 1 is a collagen stimulator that leads to development of fibrosis. Another component that was observed was the surge in collagen-I [determines stiffness of cardiac muscle] protein expression in the right atria and ventricle, collagen-III [distensible] remained unchanged. This shows that the diastolic dysfunction of the right atria and ventricle could be due to the increased cardiac stiffness. Intense long term exercise effect on cardiac arrhythmias was evaluated in vivo, by attaching a modified catheter to the right ventricle apex. Inducible ventricle tachyarrhythmia was found in 42% of the exercise rats compared to 6% of the control rats. A possible explanation for the higher prevalence of arrhythmias in exercise rats could be linked back to cardiac fibrosis. After the eight week deconditioning period, all the exercise rats had almost all the cardiac remodeling mentioned above revert to the control rats levels. (Benito, et.al. 2011) There have been other animal studies conducted that show similar results to this one. Even though there are some similarities between cardiac remodeling in animals and humans, researchers cannot be certain that cardiac remodeling in both is exactly the same.

### Conclusion

After intense exercise immediate testing resulted in dilatation of the right ventricle and atrium, reduced right ventricular ejection fraction, and elevated levels of biomarkers found in the blood. Even though there was a correlation found between reduced right ventricular ejection fraction and ventricle arrhythmias, the sample size was too small to fully determine the validity of the correlation. Further research needs to be conducted on the effect and mechanism of elevated biomarkers found in the bloodstream after vigorous exercise. Testing after many years of intense aerobic activity show that balanced cardiac hypertrophy is normally found in a person with the condition known as “athlete’s heart.” Even after many years of deconditioning, the heart does not return to its normal size. A result of cardiac hypertrophy is myocardial fibrosis and scarring. The percent of myocardial fibrosis in those participating in prolonged vigorous activity is low; however, compared to the control group, the percent is significantly greater. Studies also show that while moderate exercise is beneficial to lowering a person’s all-cause mortality rate and cardiovascular mortality rate, intense exercise reverses this positive effect slightly. Humans share similar results found in dissections of rats that underwent exercise training. Though intense exercise does not have a positive effect on cardiovascular health, more studies involving a greater sample size have to be conducted to determine the extent to which vigorous exercise damages the heart.

In addition to the negative effects that intense exercise has on the heart one has to consider the adverse effects that it has on the body and more specifically the skeletomuscular system.

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# Short-term Effects on the Fetus and Long-term Outcome on Children Exposed to Maternal Chemotherapy

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## Abstract

Ethical questions with regard to treatment arise when pregnant women are diagnosed with cancer. Does the health of the mother or the health of the fetus take priority? However, research suggests that concern over transplacental transfer should not deter those seeking treatments since avoidance of favorable transfer drugs can allow chemotherapy to be a viable option if properly administered. Doctors highly contraindicate the use of chemotherapeutic drugs in the first trimester due to high risks such as teratogenesis and fetal death. However, they reassure that treatment can be given in the final two trimesters. It presents minimal consequences as long as the drug is not favorably transferred, treatment is not given after thirty five weeks, and delivery is not less than three weeks after chemotherapy administration. Iatrogenic prematurity should also be avoided. Additionally, long term risks are minimal, but further research needs to be performed with longer follow ups and larger sample sizes in studies in order to obtain more conclusive data.

## Introduction

There has been an increase in treating pregnant women with cancer and scientists suggest that the change in trend is due to older women conceiving. Reports indicate that cancer is diagnosed in approximately 1 in 1000-1500 pregnancies (Voulgaris, et al., 2011). If cancer treatment cannot be postponed until after birth, ethical concerns arise with regard to the health of the mother and fetus, and so the benefits and side-effects need to be discussed by patient, oncologist, and obstetrician. Factors that impact treatment include cancer type and stage, gestational age and consequences of treatment on the mother and fetus. In addition, risks, treatments and, if necessary, the option of abortion must be determined. The goal is to determine if and at what gestational age treatment should be given, whether cytotoxic drugs administered to the mother impacts the development of the fetus and results in long-term risks for the child.

## Methods

Information and research was obtained through Ebsco, Proquest and Pubmed. Access was provided by Touro College library. Main key words included pregnancy, short-term, long-term, transplacental, in utero, fetus, and chemotherapy.

## Discussion

### *Transplacental Transfer*

To first address the impact of transplacental transfer, it is essential to determine if there is drug transfer between the mother and the fetus, and if so, to what extent. A mouse model using 40 pregnant mice was studied to determine the amount of transplacental transfer of chemotherapeutic agents, doxorubicin, epirubicin, cytarabine, paclitaxel, carboplatin and vinblastine. Due to the difference of drug metabolism in mice, the drugs were given at proportionately higher dosages than when given to humans. Fetal and maternal blood was collected on day eighteen and a half, during the phase of fetal development. The mice were delivered by caesarean section ninety minutes (an arbitrary amount of time) after chemotherapeutic drugs were intravenously given to the mother. At this point in time, an increase in plasma levels

ensured drug detection and was the first phase of distribution after the drugs were intravenously administered. The drug levels in the maternal and fetal plasma were detected using two methods. High performance liquid chromatography determined the amount of the five chemotherapy drugs and Atomic absorption spectrometry determined carboplatin levels, based on the amount of platinum in the blood.

### **Plasma Drug Analysis**

Paclitaxel was found in maternal plasma, but was not detected in fetal plasma. It has a high molecular weight, is highly protein bound and is a substrate of P-glycoprotein (ATP-binding cassette transporters) and multidrug resistance protein. P-glycoprotein is found in fetal-derived epithelial cells which forms the maternal-fetal blood exchange border and protects the fetus.

Vinblastine and anthracyclines, which includes epirubicin, a 4'-epimer of doxorubicin, and doxorubicin, only presents as a small transfer ratio due to being P-glycoprotein substrate, which protects the fetus against xenobiotics' harmful effects. Another factor is that it possesses pharmacokinetic properties that contrast to the substances that cross the placenta. Thus, one can imply that vinblastine, anthracyclines and paclitaxel present a safe option for treatment when given in small dosages during the last two trimesters.

However, both carboplatin and cytarabine have high transfer rates and can penetrate the membranes barriers since they are slightly bound and have a low molecular weight. Therefore, researchers recommended, if possible, avoiding these drugs (Calsteren, et al., 2010; Van Calsteren et al., 2010).

The transplacental transfer is generally through passive diffusion and is typically penetrable. However, there are three factors that influence the maternal-fetal transfer. They are: (1) maternal and fetal circulation concentration gradient, (2) the placental blood flow, and (3) chemotherapy's drug properties. The pharmacokinetics of chemotherapy that favor transplacental transfer are

uncharged, low molecular weight, lipid soluble, and unbound or low protein bound compounds (Calsteren, et al., 2011).

### Maternal- Fetal Transfer in a Baboon Model

The data obtained from the previous experiment, however, is limited in its clinical application due to the differences of human and mouse metabolism and permeability and formation of the placenta (Van Calsteren, et al., 2011). This elicited further investigation of the effects of maternal-fetal transfer of chemotherapeutic agents given in second and third trimesters. Two studies, using the same subjects, examined the results of different chemotherapeutic drugs on a group of nine pregnant baboons. Data more relevant to humans can be obtained from baboon models as compared to mice models due to the greater similarity of human and baboon embryological development, placental structure and function, reproductive physiology and endocrinology of pregnancy and drug metabolism. Based on the outcome, since only low levels were detected, the authors were reassured that the use of doxorubicin, epirubicin, vinblastine, taxanes (docetaxel and paclitaxel) and active metabolite of cyclophosphamide (4-OHPC) were not damaging to the fetus. However, they were concerned with the results of trastuzumab and carboplatin since they were transferred in large or complete amounts. The study was limited since there was no record of long term results, and it was suggested that further research needed to be performed (Calsteren, et al., 2010). Furthermore, other medical professionals contraindicate trastuzumab since it has been correlated with oligohydramnios, reduced production of amniotic fluid resulting from the drug binding to fetal renal receptors (Gziri, et al., 2012).

### Placental Perfusion Model

As it would be unethical to perform human testing on pregnant women to determine whether there is maternal-fetal transfer due to fetal risk of fetal blood sampling from the umbilical cord known as cordocentesis, Van Calsteren et al. (2011) suggested that they can be applied to a single cotyledon models that have separate fetal and maternal circulations. Unfortunately, some complications with this proposal included difficulties obtaining absolute physiological conditions in a perfusion system, temporary status of tissue viability, and the differences in data between *in vivo* and placenta perfusion model with some drugs (Myllynen, Vhakangas, 2002; Van Calsteren, et al., 2011).

An attempt was made, however, to examine the effects of maternal-fetal transfer of taxanes (paclitaxel and docetaxel) to determine whether it can be a viable treatment option. The single cotyledons were taken from twelve placentas from uneventful pregnancies with each test group containing six models. The test groups were further divided into two groups of three, with one group containing high concentration of albumin (30 g/L) and the other group with low concentration of albumin (2 g/L). In the

intervillous space two catheters were inserted to begin the perfusion and the twelve placentas were perfused for ninety minutes. The results indicated that taxanes have a low transplacental transfer due to their chemical properties and added that there was no difference in amount of drug transfer between paclitaxel and docetaxel. Furthermore, the outcome between the groups of high and low levels of albumin reinforced the theory that protein levels affect the maternal-fetal transfer. This was supported with the observation of less transfer of both docetaxel and paclitaxel with high levels of albumin. Although this reinforced previous studies of placental transfer, sample size was too small and further investigation should be performed (Berveiller, et al., 2012).

### Factors and Effects of Chemotherapeutic Drugs

Based on research that positively indicated transplacental transfers of chemotherapeutic drugs, studies were initiated to determine the drug effect on the developing fetus. There are four main drugs that are typically administered to pregnant cancer patients. They are: anthracyclines (e.g., doxorubicin, epirubicin), platinum-based antineoplastics (e.g., cisplatin, carboplatin), cyclophosphamide and taxanes (e.g., paclitaxel, docetaxel). Anthracyclines result in cardiotoxicity and inhibit topoisomerase, interfering with DNA replication. Platinum based antineoplastics result in neurotoxicity and ototoxicity if given at high doses. It also allows for apoptosis to occur since it binds to and causes crosslinking of DNA. The effect of cyclophosphamide is permanent infertility if given at high dosage. Lastly, taxanes disrupt the microtubule function in cell division thereby inhibiting mitosis (Vandenbroucke, et al., 2014). The overall similarity between them is that they halt normal cell proliferation cycles which results in interrupting crucial cell processes. Studies on somatic cell mutations report that some adverse effects of chemotherapy are gene mutations, chromosomal breaks and rearrangements, and aneuploidy (Arnon, et al., 2001).

The impact of chemotherapy on the fetus can vary due to physiologic changes in the pregnant patient. Some examples that may occur include an increase in glomerular filtration, which increases the amount of elimination or drug excreted by the kidney, and an increase of drug metabolization in the liver, which might impact the presence of active drugs. Additionally, there is an increase in entero-hepatic circulation in pregnant women. This causes active metabolites to return to the liver and then colon after being secreted from bile into the colon and reabsorbed by enterocytes. The circulation prolongs the drug exposure since it lengthens the time needed to eliminate the drug from the body because it returns to the blood stream. The amount of active drugs available can decrease due to an increase in protein binding which results in a greater impact of cytotoxic drugs. However, plasma levels of cytotoxic drugs may be reduced as a result of an increase in plasma concentration (Amant, et al., 2008). The third space that



develops in pregnant women resulting from an increase of amniotic fluid can also impact the distribution volume of chemotherapy, causing a slow release of drug which results in an increase of fetal chemotherapy exposure (Williams, Schilsky, 2000). Other factors that impact the maternal chemotherapy on the fetus are metabolic activity and excretion of the placenta, increase of body fat of the pregnant patient and pH difference between maternal and fetal fluids (Gedeon, Koren, 2006). When creating a treatment plan, these physiologic changes must be considered.

Various other factors can also result in teratogenic risks to the fetus. Although the placenta can excrete fetal waste products and assist in fetal drug elimination, some antineoplastic agents may not be eliminated from the fetus if drugs are administered close to delivery time. Additionally, due to the fetus's immature liver and kidney, the fetal metabolism and excretion of the chemotherapeutic drugs may be abnormal. This can result in an increase of chemotherapy exposure and acute toxicity (Williams, Schilsky, 2000).

### **Administration Variables**

The effect on the fetus is not only dependent on drug type and performance, but is also based on dose, route and gestational age. Studies have proven higher toxicity can develop from short infusions, topically applied cytotoxic agents and intraperitoneal administration. Drugs given orally may be absorbed less due to a decrease of elimination from the stomach and intravenous administration can result in fetal risks. As mentioned previously, prolonged drug elimination from the mother also impacts the toxicity on the fetus (Wiebe, et al., 1994).

### **Chemotherapy Exposure- Fertilization/ Implantation**

To determine the effect of chemotherapy on the developing embryo and fetus, observations were performed during the three stages of pregnancy. The three phases of fetal development are fertilization/implantation, organogenesis, and the fetal phase. Chemotherapy is cytotoxic, thereby inhibiting cell growth. Therefore, exposure during the first ten days of pregnancy, the stage of fertilization/implantation, results in an all-or-nothing phenomenon. Cells are omnipotent and may develop into the three different embryological layers, ectoderm, mesoderm and endoderm. However, their ability to develop normally is dependent on the impact of maternal chemotherapy. If there is a sufficient amount of cells, the embryo will develop normally, but a miscarriage can occur if too many cells are destroyed by the chemotherapeutic drug (Reed, et al., 2010).

### **Chemotherapy Exposure- Organogenesis**

During the first trimester, it is highly inadvisable to treat the mother with cytotoxic drugs. Voulgaris et al. reports (2011) that

there is a 10-20% chance of teratogenicity, fetal malformation, occurring if agents are administered in the first trimester. An increase of embryonic death can occur if there is damage to the embryo in the first month of gestation resulting from chemotherapy exposure (Voulgaris, et al., 2011). Spontaneous abortion and major malformations are also possible risks that can occur. The fetus is more susceptible to malformations two to eight weeks after conception, the period of organogenesis. Specifically, the heart, neural tube, and limbs are more vulnerable, preceding the palate and ears of the fetus. If chemotherapy exposure continues after eight weeks, the eyes, genitalia, hematopoietic and central nervous system still remain at risk (Cardonick, Iacobucci, 2004). This strongly reinforces that maternal chemotherapy should be avoided in the first trimester.

### **Chemotherapy Exposure- Fetal Phase of Development**

Based on observations of the fetus exposed to maternal chemotherapy during the final two trimesters, researchers have suggested that these exposed fetuses did not exhibit more congenital malformations than fetuses from untreated mothers. Nevertheless, intrauterine growth restriction, low birth weight and neonatal myelosuppression have been detected in neonates exposed to chemotherapy in the second and third trimester (Cardonick, Iacobucci, 2004; Van Calsteren, et al., 2011). From week eleven until delivery, functional damage will frequently occur as a result of cell death. In addition, some organs may later develop structural anomalies. For instance, the fetus can develop neuropsychological impairment since the central nervous system is progressing throughout gestation (Vandenbroucke, et al., 2014).

### **Short-term Effects -Obstetric and Neonatal Outcomes**

One study reported on 62 patients who were treated predominantly in the last two trimesters with chemotherapy alone or combined with other treatments, such as radiotherapy. These patients were part of a sample of 180 pregnant women who received various treatments for cancer. The results were analyzed to determine the fetal risks of treated and untreated mothers diagnosed with cancer.

Regarding patients treated with chemotherapy, there was a significant increase in preterm labor, but not in the rate of preterm premature rupture of membranes (PPROM) when compared to neonates not exposed to maternal chemotherapy. However, the only incidents of preterm labor and PPRM that occurred in the 180 cases analyzed involved patients treated with chemotherapy. Preterm labor can occur from the activation of hypothalamic-pituitary-adrenal axis of either the mother or fetus resulting from physical or psychological stress. Scientists propose that PPRM can result from apoptosis in amnion epithelial and chorion

trophoblast layers of the membranes after maternal chemotherapy exposure (Van Calsteren, et al., 2010).

The risks of preterm labor include intraventricular hemorrhage, bradycardia, apnea, respiratory insufficiency, necrotizing enterocolitis, sepsis, seizures, hypoglycemia, and feeding problems (Van Calsteren, et al., 2010). Additionally, Johnson (2007) reported that preterm births are associated with a decrease in IQ scores, cognitive delay, behavioral problems and higher risk for psychiatric disorders, primarily ADHD. Preterm labor in this research was preventable since several of the neonatal issues were iatrogenic. Therefore, researchers suggested that patients that can delay treatment until after delivery should do so to avoid prematurity (Van Calsteren, et al., 2010).

Data of small-for-gestational-age children was available for only 175 neonates. Regarding those treated with cytotoxic treatments (chemotherapy and/or radiotherapy), 16 of 66 children were small-for-gestational age as opposed to 10 of 109 children who were not exposed to maternal chemotherapy/radiotherapy.

The malformation outcome in comparison to normal pregnancies reveals that there was no significant increase in rate. The range of physical abnormalities of neonates exposed to maternal chemotherapy was still within normal range, with 2 of 66 (3.0%) inflicted with major malformations and 5 of 66 (7.5%) affected with minor malformations. The average rate of major and minor malformations was 4.1% to 6.9% and 6.5% to 35.8%, respectively. This suggests that the concern of malformation defects due to chemotherapy is not applicable and should not deter patients from avoiding treatment (Van Calsteren, et al., 2010).

Another fetal risk that may occur is transient neonatal myelosuppression (TNM). Elburg et al. (2008) defined TNM as "leukopenia and/or neutropenia combined with anemia and/or thrombocytopenia, during the first weeks of life in newborns exposed to maternal chemotherapy during pregnancy." The disease is rare, potentially life-threatening and may not necessarily develop right after birth. Additionally, it may pose a risk for developing infections. The development of TNM may result from a short gap between chemotherapy administration and birth, maternal neutropenia at delivery, type of chemotherapy and maternal disease and dosage given. Treatments for TNM include thrombocyte and/or erythrocyte transfusions, bedside isolation, erythropoietin, and recombinant human granulocyte colony stimulating factors (G-CSF). In a study of fifteen neonates diagnosed with TNM, only one died due to infection resulting from TNM. However, the other fourteen neonates' physical and neurological developments were normal by the age of one and their TNM was resolved between two to ten weeks after birth. Generally, there is no difference detected in short term outcomes and survival

rates between neonates exposed to chemotherapy that develop TNM and those not diagnosed with TNM. Researchers stated that this is true as long as the disease is recognized and aggressive treatment is given. However, the long-term consequences are still unknown (Udink ten Cate, et al., 2009).

Another study performed by Aviles et al. (1991) consisted of 43 infants exposed to maternal chemotherapy. The infants were examined from age three through nineteen years old and it was observed that only eight were pancytopenic at birth, which characterizes them as being deficient in red and white blood cells and platelets. However, the mothers of these neonates were treated with chemotherapy in the last three weeks prior to delivery which disputes the suggestion that a short gap between chemotherapy administration and delivery results in TNM. There were no congenital anomalies and neurological abnormalities reported and like the findings of the Udink ten Cate et al. (2009) study, the TNM resolved 2-10 weeks after birth. In addition, in a study of 62 patients, two children suffered postnatal hematologic toxicity (leukopenia and pancytopenia) when born ten days after maternal chemotherapy administration for acute lymphatic leukemia and required hematologic growth factors as treatment (Calsteren, et al., 2010). This study supports the theory, unlike Aviles et al. (1991) that a short interval between the chemotherapy administration and delivery can result in hematologic toxicity. As seen with these results, hematologic toxicity can present a slight concern for pregnant women with cancer, yet the risks are minimal if proper treatment is given.

Cardonick and Iacobucci (2004) have studied the impact of chemotherapy given after the first trimester on 376 fetuses. Of the 376, nineteen of the fetuses and four of the neonates died, 28 neonates were born with intrauterine growth restriction and 18 were born prematurely (iatrogenic cases excluded). Additionally, two were diagnosed with transient neonatal cardiomyopathy due to maternal idarubicin treatment, fifteen neonates developed transient neonatal myelosuppression. Eleven malformations were also reported with the majority resulting from chemotherapy exposure in the first trimester.

Another study was performed which was composed of 61 patients receiving chemotherapy during the second and third trimesters. Of the 61 patients, 32 were treated with neoadjuvant chemotherapy and 2 received adjuvant chemotherapy. The 61 neonates that were exposed to maternal chemotherapy were examined and compared to 60 neonates who were born prematurely and were not exposed to maternal chemotherapy. Results suggested that there were no differences in birth weight, admissions to the NICU, and neonatal survival. No malformations were reported in either group. Nonetheless, one contrast between the groups was the considerable difference in Apgar score, which

tests the future neurobehavioral development first at one minute after birth, then five minutes after and after ten minutes after birth. The results favored the group of children not exposed to chemotherapy at one and five minutes, but there were no significant difference at ten minutes. Although it reflects positive short-term outcomes, it's limited in long-term follow up on these children and researchers suggest further studies need to be performed (Abdel-Hady, et al., 2012).

Fetal cardiotoxicity was also researched to determine whether it can develop from maternal chemotherapy and pose a threat to neonates. One study consisted of ten pregnant patients treated with anthracyclines in the last two trimesters to investigate whether these drugs, which are thought to cause fetal cardiotoxicity, can result in fetal cardiac abnormality. The results indicated that one fetus was diagnosed with fetal supraventricular tachycardia, but researchers believed it was unrelated to maternal chemotherapy. Additionally, differences in myocardial performance index and in the tricuspid inflow pattern were detected, but were considered clinically insignificant. The neurological development and weight, height, and clinical examination of the fetuses in the study group were within normal limits. Significant cardiac abnormality and intrauterine growth restriction were not detected, although low birth weight was observed. Although the researchers gathered data systematically, the study was small and therefore limited (Gziri, et al., 2012).

### Long Term Effects

A concern of maternal chemotherapy is the long-term cardiotoxic effects on the fetus. A study used tissue Doppler and strain imaging to identify any specific changes in cardiac performances that might result from chemotherapeutic drug. The study focused on children exposed to anthracyclines, a drug shown to cause cardiomyopathy and cardiac dysfunction. The children's ages ranged from 1-9 years old, with the mean age being 1.7 years old. Those exposed to anthracyclines were observed to have minor changes in left ventricular (LV) wall thickness that might have occurred from the effect of anthracycline's toxicity. Anthracycline is known to lead to loss of cardiac muscle. There was also indication that there might be a lower, but normal ejection fraction and left ventricular ejectional fractional shortening, which is not clinically important since there is no change in functionality. Since there were no differences in tissue Doppler and strain imaging between the control group and patients, researchers indicated that there was no change in myocardial function. They stated that changes recorded could have resulted from the fact that the majority of the neonates born were preterm. Overall, their observation showed that there was no significant change between the 62 patients and 62 age gender-matched controls. Researchers recommended further research to observe any future irregularities and suggested performing further studies with larger patient groups with proper follow up (Gziri, et. al., 2013).

Further research was performed to assess the neurodevelopment, cardiac function, and general health of 70 children who were exposed to chemotherapy in utero. Seventy children from 68 pregnancies (two twin pregnancies) were assessed at age 18 months and at ages 5-6, 8-9, 11-12, 14-15, or 18 years. The median gestational age at birth was 35.7 weeks, with a median follow-up period of 22.3 months. Most of the children had normal cognitive development. However, children who were born prematurely had lower cognitive development scores albeit within normal range of cognition. Moreover, for each additional month of gestation, the IQ score increased by an average 11.6 points. Therefore, iatrogenic preterm delivery is not suggested since it significantly impacts the cognitive development.

Additionally, because the CNS continues to develop after the first trimester, the possibility that maternal chemotherapy may adversely affect neurological development is a serious concern (Gziri, et al., 2012). However, only one set of twins of 70 children observed experienced severe neurodevelopmental delay. This cannot be completely attributed to the exposure to a chemotherapeutic drug (Amant, et al., 2012). The cortex of the fetal brain develops surface sulci and gyri at 14 weeks gestational age and doesn't remain the original smooth cerebral surface. The formation of polymicrogyria, in which the cerebral cortex develops abnormally before birth in the deeper layers and excessive gyri form, removes some of the probability that chemotherapy exposure after 15 weeks instigated the developmental delay observed in the twins (Amant, et al., 2012; Barkovich, et al., 2010). Yet, the researchers did acknowledge that although the neurodevelopment was normal in the overall researched group, subtle changes could occur and further research on longer follow-ups should be conducted.

Nonetheless, the average results of the children's behavior, general health, hearing, and growth were within the normal range, along with their cardiac dimensions and functioning. No increase in CNS morbidity was recorded. Although there were no congenital cardiac abnormalities detected, further study was recommended on the cardiac function since there were clinically small, but statistically significant changes in ejection fraction, fractional shortening and diastolic variables. They did detect, however, a higher incidence of disharmonic intelligence profiles. The results of the Wechsler test depicted a discrepancy between verbal and performance IQ values. This was seen in 39% of patients in comparison to 15% in the normal population. The discrepancy has been associated with several neurological disorders, learning problems and behavioral issues.

The study was limited due to the small sample size, short follow-up periods, and lack of children born at the same gestational age and similarly assessed, who were not prenatally exposed to

chemotherapy. Additionally, researchers could not predict if secondary malignancy and infertility would have developed due to time constraint of the experiment. Only one twin, who also had congenital malformation, was diagnosed with secondary malignancy and suffered from papillary thyroid cancer at age eleven and neuroblastoma at age fourteen.

Nonetheless, the researchers suggested that chemotherapy not be administered after 35 weeks since this can trigger spontaneous labor. Delivery, they stated, should be arranged to be no less than three weeks after the last chemotherapy cycle. This ensures that the bone marrow recuperates properly, prevents the development of fetal and maternal sepsis and hemorrhage and hematological toxicity. As mentioned, preterm delivery should be avoided due to the impact on cognitive development. Preterm neonates are limited in their capacity to metabolize and eliminate drugs since they have immature liver and kidneys. Therefore, if delivery can be postponed, the fetus can excrete the drug through the placenta instead.

The positive outcome led the researchers to conclude that there are three factors that may reduce the risks associated with maternal chemotherapy. First, is that maternal chemotherapy administration should be given after the first trimester, as this avoids the period of increased susceptibility of the fetus to toxicity. Secondly, they believe that the fetal blood brain barrier protects the brain from drug diffusion with tight junctions and decreases rates of transcytosis and expression of specialized influx and efflux transporters, such as P-glycoprotein. Furthermore, vascular permeability and CNS immune cell infiltration is decreased through the inhibition of pericytes. Lastly, they suggest that the fetus is protected since the plasma filters the chemotherapeutic drugs (Amant, et al., 2012).

The most reassuring study which demonstrated the safe use of maternal chemotherapy was the observation on the long-term effect on 84 children/adults with a follow-up period of 6-29 years. The mothers of these children were diagnosed with hematological malignancies, and were treated with intense chemotherapeutic agents given in adequate doses. The treatments, however, varied in which trimester the dosage was administered. Observations of the children/adults reported that the height, weight, birth and cytogenetic material were normal and that there was no evidence of cellular damage. Additionally, educational performance and neurological and psychological evaluations were on par with the general public. Furthermore, sixteen of patients were married, with twelve children born in total, who also did not suffer from any abnormalities. Although many doctors disagree, researchers in this study suggest that treatment can be given in the first trimester due to their positive results (Aviles, Neri, 2001). This gives positive encouragement to pregnant cancer patients since

it provides concrete evidence that the fetus can escape harm and develop normally into a healthy accomplishing adult.

### Conclusion

Overall, transplacental transfer of cytotoxic drug is not detected in all of the treatments and its impact varies based on the physiologic changes in pregnant women and the pharmacokinetics of drugs administered. Many emphasize that chemotherapeutic agents should certainly be delayed and only be given after the first trimester since the fetus is highly vulnerable to teratogenicity. When given in the final two trimesters, risks can still persist. However, this should not prevent the mother from seeking treatments due to insignificant short-term and long-term effects.

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# Is Proton Beam Therapy more Effective than Intensity-Modulated Radiotherapy in Prostate Cancer Treatment?

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## Abstract

Prostate cancer is the most common form of cancer found in American males. Breaking technological advances in prostate cancer treatment continue to develop to help fight this disease, one such is proton beam therapy. Proton beam therapy is theorized to spare even more healthy tissue than photon radiotherapy because it delivers a majority of its radiation during the Bragg peak. Since this technology is substantially costlier than any other form of radiation therapy, physicians are assessing its effectiveness and determining if it is worth the cost. Currently, there is no significant difference seen in patient quality of life between recipients of proton or photon therapy. This can possibly be because of secondary neutrons that are generated when protons exit the nozzle. Pencil beam scanning, a recent advancement in proton therapy delivery, is theorized to make protons have much better treatment outcomes than photons and would eliminate the issue of secondary neutrons. More studies need to be conducted to determine if pencil beam scanning ensure better quality of life over photon therapy.

## Introduction

Carcinoma of the prostate, more commonly known as prostate cancer, is a malignant tumor that develops on the prostate—a male organ that wraps around the urethra. Prostate cancer can occur in many different forms, such as carcinoid, small-cell tumors, and ductal carcinoma, however, these types are extremely rare and little is known about which treatment most effectively eliminates them. The vast majority of cases are adenocarcinoma, a tumor that originates in the gland cells. Prostatic adenocarcinoma is observed in over 95 percent of patients (National Cancer Institute 2014) and affects 15 percent of men predominantly above the age of 66, making it the most common cancer among American males (SEER 2014). Fortunately, prostate cancer tends to grow extremely slowly, and early treatment can often yield high survival rates. The most common treatment interventions for prostate cancer include surgery or radiation therapy. Within radiation therapy there are two methods, external beam radiation therapy (EBRT) and internal radiation therapy. The latter, also referred to as brachytherapy, involves the placement of radioactive pellets within the prostate gland that release a highly concentrated dose of radiation to eliminate the cancerous growth. External beam radiation therapy is employed by using a linear accelerator to accelerate electrons close to the speed of light and strike them against a metal plate. On impact, high-energy, ionizing photons are produced, which travel towards the targeted cancerous cells. The high amount of energy in the photons can irreparably damage vital structures in the cell such as DNA, RNA, or protein. Photons are able to directly harm the cancerous cells' DNA by energizing its electrons and pulling them out of their orbits. This produces free radicals, causing irreversible damage by altering the chemical structure of the nucleotides. Ionizing radiation can also delete, fragment, and translocate the nucleotides. Radiation can also indirectly harm DNA when photons collide with a cell's water molecules and energize its electrons. When a water molecule's excited electrons bounce out of their orbits it produces a hydroxyl ion (OH<sup>-</sup>). The hydroxyl ion is then able to extract a hydrogen atom from the deoxyribose part of the

DNA, ruining the nucleotide's chemical configuration. Direct or indirect radiation damage to a cancerous cell's DNA is usually lethal. This is because the uncontrollable growth of the prostate cancer involves the mutation of the tumor suppressor gene TP53 (Wang et al. 1997). The protein that TP53 codes for is not only responsible for regulating the cell cycle, it is also responsible for initiating DNA repair. As a result, cancerous cells are ineffective at repairing radiated DNA, and the cell's ability to proliferate is practically destroyed. Damaging 1000 nucleotides in DNA only takes one cobalt grey equivalent—the unit measure of radiation used during treatment.

Although external beam is an effective form of treatment, it also delivers radiation to surrounding healthy tissue. This is because before the advent of image guided treatment, physicians had to target the entire prostate to ensure that the entire tumor was radiated. Additionally, since the dose of radiation is gradually reduced as the beam passes through the patient, tissue directly in front of the tumor must be radiated to the prescribed dose. This leaves a track of radiation damage as the photons enter the body until it reaches the tumor. Furthermore, photons are massless particles and are not stopped or slowed down when they impact body tissue. This causes healthy tissue behind the tumor to receive radiation when photons travel past the tumor. Radiation to nearby healthy tissue often causes gastrointestinal and genitourinary toxicity, which can impair the patient's quality of life.

Advances in radiotherapy have greatly reduced these problems. The advent of three-dimensional conformal radiotherapy (3D-CRT) helps radiation oncologists treat prostate cancer more effectively. This sophisticated computer program creates a three-dimensional map to conform the radiation beam to the shape of the cancer and its surrounding normal tissues. Concentrating the beam more accurately to the cancerous area reduces the exposure of normal tissue to radiation, further reducing side effects after treatment. More recent advances in high precision targeting have led to intensity-modulated radiotherapy (IMRT), where

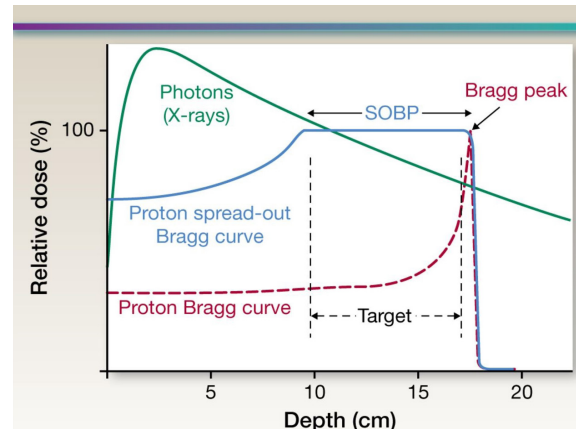
there is an even more effective manipulation of the radiation beam. The beam in IMRT is divided into multiple 'beamlets', aimed at the target from different directions. The 'beamlets' shape and intensity are adjusted to avoid radiating nearby healthy tissue. By taking advantage of intensity-modulated 'beamlets', IMRT is able to deliver higher doses of radiation to the prostate. This arguably ensures greater eradication of cancerous cells while sparing even more healthy tissue than 3D-CRT. Additional advances include image-guided radiotherapy, which provides more accuracy to the targeted area in the event that patients or the organs inside them move during treatment.

Despite all the improvements in radiotherapy healthy tissue behind the tumor is still getting bombarded with radiation. Proton therapy is able to stop this. Proton therapy involves the therapeutic use of protons to radiate the DNA of cancerous cells. Protons are positively charged heavy particles, approximately 1800 times the mass of an electron. Therefore, protons need a particle accelerator to strip hydrogen atoms of their electrons, accelerate them to nearly the speed of light, and shoot them into tumors. Particle accelerators, either cyclotrons or synchrotrons, require an enormous amount of space, which makes the cost of a particle accelerator exceed 100 million dollars (National Association for Proton Therapy 2010). Upon entering the body, protons slow down due to their heavy weight and their attraction to the electrons in the body's cells. Consequently, physicians must program the particle accelerators to provide the protons with enough energy to reach the cancerous cells. After traveling the specified distance protons stop abruptly, depositing most of their energy at the targeted cells, sparing harmful radiation to the healthy cells behind the tumor. This biologic phenomenon, of depositing high dose and energy of the beam within the tumor while having minimal energy deposited beyond the tumor is known as the Bragg Peak Effect. During radiation treatment, physicians modify the Bragg peak and extend its distance to cover the entire depth of the tumor, ensuring that the entire tumor is radiated. Extending the distance of the Bragg Peak is accomplished by treating the tumor to different energies, which sends multiple sprays of protons to different depths of the tumor. This manipulation of the proton beam is called the Spread-Out Bragg Peak. Additionally, protons do not deposit much radiation when they enter the body. Taking advantage of the Bragg Peak Effect and the low entrance dose, proton therapy should theoretically achieve less exposure of radiation to normal tissue. This is believed to lead to higher prescribed doses, reduced complications of therapy, and greater efficacy in eradication of the tumor than any other form of radiotherapy.

## Methods

Literature for this article was obtained using the Touro College Online library, in particular PubMed.

**Figure 1**



The graph compares the Bragg peaks of IMRT vs. Proton beam therapy. Protons, unlike photons, are clearly seen to deliver most of the radiation at the targeted cell.

(Mohan et al. 2013)

## Discussion

Over the past decade, intensity-modulated radiotherapy has become the standard form of radiation treatment for prostate cancer, accounting for more than 80% of all radiotherapy (Nguyen 2011). Although IMRT is the most common method of radiation treatment, new forms of treatment are still emerging, most notably proton beam therapy. Proton therapy is currently being implemented at various cancer centers across America because of the excitement over the radiological advantages protons seem to offer. Even though there is a lot of enthusiasm over PBT, no factual evidence to prove protons are better than photons has been found. The lack of evidence in conjunction with the enormous cost of delivering the treatment is compelling physicians to determine if protons are more beneficial. To date there has been no clinical study that establishes that protons have a major advantage in cancer cure rates. This is because it is difficult to conduct cure rate trials, since such differences take many years to manifest. Therefore, the initial studies on proton therapy focus on treatment related toxicity and quality of life (Pearson et al. 2007). The organs most frequently affected by radiation toxicity after treatments are the bladder and rectum.

## Dosimetric Analysis

A dosimetric analysis is the estimated measurement of ionizing radiation from the dose that is absorbed by nearby organs and is projected before treatment is delivered. This analysis helps physicians determine if there will be toxicity to nearby healthy tissue, which can downgrade the patient's quality of life. A study comparing the dosimetry of intensity-modulated radiotherapy and proton therapy was conducted to discover if protons spare more healthy tissue than photons on the bladder and rectum. Ten



patients were planned with both three dimensional conformal proton therapy and intensity-modulated radiotherapy. The prescribed dose for both treatments was 79.2 cobalt gray-equivalent (CGE) to the prostate gland. The study concluded that protons reduce the area of nearby healthy tissue that inadvertently receives low to medium, but not high doses of radiation. In contrast, intensity-modulated radiotherapy spreads more low and medium doses of radiation over a larger area of the pelvis (Table 1) (Trofimov et al. 2007). IMRT likely 'bathes' the pelvis with low to medium doses of radiation because it uses multiple 'beamlets' to target the tumor from different directions. As a result, there are more entry points into the patient's body as radiation is shot from the linear accelerator to the tumor.

More significant results were reported by a study from the University of Florida. The study also used 10 patients to compare proton beam therapy treatment with intensity-modulated radiotherapy, but with a prescribed dose of 78 CGE (Table 1) (Vargas et al. 2008). The difference in results between the study by Trofimov et al, in comparison to Vargas et al, can most probably be attributed to the difference in beam margins and arrangement. To be certain the cancerous growth was completely radiated, Trofimov et al, added a 10 mm margin around the clinical target volume, Vargas et al, only added 5 mm by 8 mm. By minimizing the margins, Vargas was able to better conform the beam to the tumor. When IMRT was delivered both studies used 7 beams, 52° apart from each other. However, for proton therapy, only Vargas et al optimized the beams. Beam angle optimization means physicians determine the 'beamlet' angles and intensities, so that the beams strike the tumor while sparing as much normal tissue as possible. Two steps are taken when determining the correct beam angle

and intensity. "First, the physicians through their experience and intuition decide the arrangement of the beams. Secondly, based on the orientation of the beams and the shape of the cancer, computer software optimizes the beam intensities." (Bertsimas et al. 2013).

A clinical investigation conducted by Chera et al, did not use beam angle optimization, but used the same prescribed dose and similar margins to Vargas et al (Table 1). Chera et al, concluded that the, "use of PBT significantly reduced the dose to normal tissues in the pelvis while maintaining adequate target coverage compared with IMRT" (Chera et al. 2009). Although the absolute difference between the two treatments was greater in the study by Chera et al, Vargas et al, managed to spare slightly more healthy tissue. One can conclude that beam margins play a more important role in limiting radiation from healthy tissue than beam arrangement.

In a recent study about the dosimetric impact on anatomical movements, Zhang et al, noted that the proton beam is affected by compensators that shape the beam at the end of the nozzle. As a result, protons experience more scatter than photons and minimizing the margins is extremely crucial. Although a dosimetric analysis was not the main focus of their study, Zhang et al reported that, "The proton therapy plan was better at sparing the rectum at doses of less than 50 Gy. However, above 50 Gy, IMRT was better at sparing the rectum" (Zhang et al. 2007). Clearly, all studies confirm that proton therapy reduces the amount of low to medium radiation unintentionally delivered to nearby healthy tissue. Yet, IMRT can sometimes yield better conformity to the target area in cases where high doses are delivered.

**Table 1**

Author	Dosimetric	Value Bladder	Rectum	Absolute Difference Bladder / Rectum
Trofimov	30% CGE	44.5%(IMRT) / 32.8%(PBT)	65.3%(IMRT) / 43.8%(PBT)	11.7% / 21.5%
	70% CGE	11.4%(IMRT) / 17.3%(PBT)	9.7%(IMRT) / 10.3%(PBT)	5.9% / 0.6%
Vargas	30% CGE	42.8%(IMRT) / 27.7%(PBT)	55.4%(IMRT) / 20.7%(PBT)	15.1% / 34.7%
	78% CGE	—	5%(IMRT) / 2.9%(PBT)	- / 2.1%
Chera	30% CGE	73.1%(IMRT) / 29.3%(PBT)	71.8%(IMRT) / 22.5%(PBT)	43.8% / 49.3%
	70% CGE	9.7%(IMRT) / 9.8%(PBT)	11.5%(IMRT) / 7.9%(PBT)	0.1% / 3.6%

*Dosimetric Analysis Comparing IMRT Versus PBT (Pearlstein et. al 2013)*

### Toxicity and Quality of Life

Radiation poisoning of healthy tissue caused by protons can irritate the bladder, cause frequent urination, rectal bleeding, and sexual dysfunction. The question is, does toxicity to surrounding normal tissue result from the combined low and medium dose areas or the high dose area? A clinical investigation compared the rate of second cancers between IMRT and 3D-CRT. The study found that IMRT almost doubles the amount of second malignancies from 1% to 1.75%. This is because IMRT increases the amount of low dose radiation absorbed by surrounding healthy tissue (Hall et al. 2003). With this information it should follow that proton therapy, which substantially reduces low to medium unintentionally absorbed radiation should have significantly lower toxicity rates than IMRT.

A study was launched to discover if proton therapy causes less toxicity to nearby organs than intensity-modulated and three-dimensional conformal radiotherapy. There were 95 proton therapy patients, 153 IMRT patients, and 123 3D-CRT patients. Every patient's bowel and urinary functions were assessed 3, 12, and 24 months after treatment. The Expanded Prostate Cancer Index Composite (EPIC) was used to grade patients that received IMRT. Patients that received PBT or 3D-CRT were evaluated with the Prostate Cancer Symptom Indices, a similar scale to EPIC. In both tests scoring lower signifies lower quality of life. At three months following treatment, patients who received 3D-CRT or IMRT scored lower on the indices. They reported symptoms of urinary irritation, obstruction, incontinence, and a decrease in bowel quality of life. Proton therapy patients only reported minimal bowel and urinary morbidity. At 12 months all groups were equal on the indices when they reported decreased bowel quality of life. Only proton therapy patients reported a slight decrease in quality of urinary functions, such as irritation and obstruction, in comparison to the other groups. By 24 months all groups reported minor but clinically meaningful decrements in bowel and urinary functions quality of life. Proton therapy had a slight advantage for toxicities after 3 months and IMRT and 3D-CRT only had a slight advantage for urinary toxicities at 12 months. All groups had the same level of toxicity 24 months later. However, there are problems with this study. Firstly, each group received a slightly different dose to the prostate: 75.6 to 79.2 CGE for the IMRT patients, and 74.0 to 82.0 CGE for PBT patients. Different doses of radiation could have caused the different patterns of toxicity reported by the patients. Also, treatment was delivered according to each center's preferred practice and planning target volume margins were not explicitly mandated (Gray et al. 2013).

Another study on whether PBT can control the incidence of rectal toxicity only comprised of patients receiving the same dose, 74 CGE. Patients were followed up with after treatment to collect data on the toxicities at 1 month and once every 3 months

for the first two years and once every 6 months thereafter. The rectal toxicities observed included anal pain at defecation and rectal bleeding. The bladder toxicities were urinary frequency, painful urination, and urinary retention. The patients were graded with the Common Toxicity Criteria 4.0, a scale created by the National Cancer Institute. Grade 1 toxicity usually means minor toxicity. Grade 2 means symptoms requiring medications and grade 3 means symptoms requiring minor corrective surgery. The results revealed that PBT can achieve a low occurrence of grade 2 rectal toxicities and no major late toxicity was seen. (Nihei et al. 2011). The study by Nihei et al, takes care of some of the issues of the trial by Gray et al, that were mentioned earlier. Each patient had the same dose of radiation. In both studies a low amount of rectal toxicity is seen early on.

More significant results were found by a study conducted by Mendenhall et al. The authors used image-guided proton therapy to target the cancerous tissue. They discovered low amounts of grade 2 genitourinary and gastrointestinal toxicities (Mendenhall et al. 2012). These toxicity results do appear more favorable than results commonly reported with IMRT. There was a high incidence of grade 1 and 2, and some patients experienced grade 3 toxicity. (Valeriani et al. 2014). Even more surprising was the fact that Valeriani et al, used image-guided IMRT and the prescribed dose was much lower, 68 CGE. The image guided radiation and the low amount of radiation should have curtailed the amount of toxicity that was reported.

Only one study reported overall worse gastrointestinal toxicity rates for protons than photons. The study used data from Surveillance, Epidemiology, and End Results (SEER), a Medicare linked database. Sheets et al, analyzed patient reported outcomes based on billing claims for diagnoses and procedures from sixteen cancer registries. There were 684 patients treated with proton therapy and 6666 with IMRT. The authors observed that, "proton therapy-treated patients were more likely to receive a diagnosis of gastrointestinal morbidity and undergo gastrointestinal procedures" (Sheets et al. 2012). However, this investigation did not report information on the prescribed dose and target margins. If the dose and margins differed between the two groups it could have caused a difference in toxicity rates. Another issue with this study is that Sheets et al, used claims for colonoscopy to measure gastrointestinal toxicity rates. This would be an imprecise surrogate for any population. This is the same population that might also be more concerned about colonoscopy screening, and therefore would receive more gastrointestinal procedures. This study should not be used to relay any important morbidity information to inquiring patients. The few studies on quality of life have only shown modest advantages associated with proton therapy in comparison with other forms of radiotherapy.

### Problems with Scatter Beam Proton Therapy

Theoretically, protons should be the superior form of radiation treatment because of the Bragg Peak Effect. However, this phenomenon also makes the protons extremely sensitive to uncertainties during treatment. If a physician is unsure how deeply situated the tumor is, protons will not destroy the cancerous cells if it is not given even energy to reach that area. In contrast, photons continue to deposit a substantial amount of radiation as they transverse the entire body. As a result, physicians end up programming the Spread-Out Bragg Peak to extend deeper into the body, irradiating nearby healthy tissue. Additionally, the Spread-Out Bragg Peak requires multiple shots of protons to be passed through the body to completely radiate the entire depth of the tumor. This causes the tissue in the front of the prostate to be exposed to many doses of radiation. Additional concerns have recently been raised that organ toxicity can also be a result of secondary neutrons. Secondary neutrons are created when protons collide with the collimator—a metal piece that shapes the beam. The collimator is made out of brass and when struck by protons it sends off secondary neutrons. Low secondary neutron radiation can cause patients receiving treatment to develop a high chance for contracting second malignancies (Brenner et al. 2008). These neutrons could be stopping PBT from having better toxicity results. Although there are simple fixes to reduce the amount of neutrons, such as using a material with a low mass number, these techniques do not substantially curtail the amount of neutrons (Gould 2009).

### Pencil Beam Scanning

Recently, a newer method of delivery has been added to proton therapy. The older method, Scatter beam uses thick beams of passively scattered protons. The beams are shaped through slits in metal plates that form a relatively large beam as it exits the particle accelerator. The latest improvement to proton beam therapy is pencil beam scanning. Also known as spot scanning, this method is more accurate version of proton therapy. Pencil beam employs an extremely narrow beam that is brushed from side to side, 'painting' the dose on the tumor spot by spot. Because no apertures are needed, the protons do not collide with any metal and secondary neutrons are not produced with the collimator. The beam targets each spot on the tumor with a specific dose of radiation. Pencil beam scanning can even adjust the Spread-out Bragg Peak for every individual cancerous cell. "Scattered beam and pencil beam can be compared to a painter spraying with a spray can versus an airbrush. Instead of needing a stencil to master the shape, the proton beam is made ultra fine to define the contours and landscape of a tumor" (MD Anderson 2009). With pencil beam protons are turned into an intensity-modulated type of treatment. This is projected to give protons more conformation to the cancer and its surrounding healthy tissues. Recently, a small number of cancer centers that deliver proton therapy introduced

this new technology (Weisenbaugh 2014). To date, it is premature to determine if pencil beam is the most optimal method to use in the fight against prostate cancer. Only one study concluded that there is no toxicity or quality of life differences between passively scattered and pencil beam. The clinical investigation was comprised of 226 men who received passively scattered proton therapy and 65 men who received pencil beam proton therapy. Both groups reported similar gastrointestinal toxicity results, grade 2, throughout the 24-month trial. Genitourinary toxicity was also grade 2 and occurred mostly in the first 12 months following treatment. Yet, the authors acknowledged that, "Future comparative analyses between spot-scanning and passively scattered are warranted in a larger cohort" (Pugh et al. 2013).

### Conclusion

Proton beam therapy can cure prostatic adenocarcinoma as efficiently as IMRT. If proton therapy would not be extremely expensive this therapy would be less controversial. Protons would have been regarded as another type of treatment used to eradicate cancerous cells. However, because proton therapy is considerably more expensive it draws criticism to the fact that it only has a meager benefit over IMRT. PBT is slightly more effective in curtailing the amount of low to medium dose of radiation to the nearby organs. However, this only translates into a modest advantage in early toxicity over intensity-modulated radiotherapy. Currently, there is no clinical evidence that proves scatter beam proton therapy is a significantly better form of treatment than IMRT. However, since there are limited centers that deliver proton therapy and relatively few patients that received it, more studies need to be conducted to solidify this claim. Although scatter beam might not be so advantageous in comparison to intensity-modulated radiotherapy, pencil beam proton therapy is theorized to turn protons into the ultimate form of treatment. More clinical trials need to be conducted to find out if pencil beam technology gives proton therapy the edge in the battle against prostate cancer.

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# Mechanisms, Potential Therapies, and the Role of TGF- $\beta$ in the Formation of Scars

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## Abstract

Scarring is the inevitable outcome of wound healing. This review looks at some of the underlying mechanisms of this complex process with the aim of identifying targets for therapeutic manipulation that could result in reduced scarring or even scarless wound repair. Fetal wounds are shown to heal without scars primarily due to low levels of TGF- $\beta$ 1 and TGF- $\beta$ 2 and high levels of TGF- $\beta$ 3 as compared to adult wounds which heal with scars. Abnormal excessive scarring in keloid and hypertrophic scars are also attributed to TGF- $\beta$ . Clinical manipulation of TGF- $\beta$  ratios showed promise as a therapeutic means of controlling scar formation. The effect of the COX enzyme and PGE2 levels remains controversial and more research is needed to understand the exact roles these molecules play in the wound healing process before they can be exploited in a clinical setting.

## Introduction

Scarring, both as a component and complication of, wound healing affects millions of US citizens every year at a cost of more than 7 billion dollars (Wilgus, et al. 2003). In addition to esthetic concerns, scar formation is also implicated clinically in fibrotic diseases that some reports suggest are responsible for up to 45% of deaths in the industrial world (Wynn, 2008). Despite such a heavy toll on the healthcare system, current treatment options are limited. Further research is needed to understand the underlying causes and mechanisms of scar formation so that effective treatments and even methods of prevention can be developed.

Cellular response to tissue damage and the consequent formation of fibrotic tissue is a complex process that is still not very well understood. Current paradigms include the contribution of such varied cells as epithelial cells, macrophages, T-cells and, of course, fibroblasts. Initial response to tissue wounds involves the rapid recruitment of clotting factors and immune inflammatory moderators to stem blood loss and reduce potential infection by pathogenic microbes. Later, the relatively extended process of wound healing begins. Injured epithelial cells undergo a process of reprogramming, initiation, and propagation of mesenchymal pathways that includes the ubiquitous TGF- $\beta$  related pathways. Macrophages also contribute to the inflammatory response and secrete profibrotic cytokines as well as growth factors, including TGF- $\beta$ , that recruit and activate fibroblasts. Fibroblasts accumulate and ultimately produce and deposit new extracellular matrix and collagen that replaces normal tissue architecture. It is this deposition that characterizes scar formation and clinical fibrosis.

Interestingly, the development of scar tissue differs greatly between adult and embryonic wounds. Contrary to the normal fibrotic result in adult wounds, fetal wounds have been shown to exhibit scarless healing. Many cellular and molecular differences, including differences in key inflammatory mediators and various cytokines and growth factors, have been studied and documented to attempt to explain this phenomenon.

In addition to expected scarring as a result of injury, there exist

two clinical examples of atypical wound healing that result in abnormal scar formation. The etiology of hypertrophic scars and keloids is not well-understood, and although important differences between them have been detailed, both clearly involve the deposition of excessive amounts of collagen even as compared to normal scarring.

Such anomalous results, from the scarless healing of fetal wounds to the excessive scarring of keloid and hypertrophic scars, may serve as paradigms for understanding the mechanisms behind the wound healing and scarring processes and the ability to prevent undesired outcomes.

## Methods

Literature searches were chiefly performed using the PubMed database. After relevant literature was found, related articles were accessed using the Related Citations feature on the PubMed website. Additionally, references in these articles were retrieved and then served as additional points of reference for further research. Review articles relevant to the research topic were helpful in providing references to source material. The Touro College Online Library and Google Scholar proved to be valuable for finding and accessing the necessary relevant articles. Moreover, conversation with plastic surgeons led to additional searches for source material on the website of the Plastic and Reconstructive Surgery journal.

## Discussion

The formation of scar tissue in the process of wound healing is both a positive construct that effectively patches breaks in damaged tissue and a negative consequence that can have dire effects. Scar tissue production is intended to increase the tensile strength of wounds but never surpasses 70% of the strength of the original undamaged tissue (Wilgus, et al. 2003). Scar tissue also can impede tissue function, restricting mobility in joints and impairing normal growth in the case of external injuries involving the skin. The issue is even more problematic in the case of scar deposition as a result of internal organ damage. In such cases, scars can dramatically reduce function of vital organs as with



victims of myocardial infarctions whereby normal heart tissue is replaced with nonfunctional scar tissue. However, the problem of scar formation is perhaps greatest with respect to its role in devastating fibrotic diseases where scar tissue often times fatally overtakes normal tissue function.

Scars are essentially large deposits of extracellular matrix and collagen that are deposited by activated fibroblasts in response to tissue damage. After an injury, inflammatory mediators are immediately recruited and activated. Numerous cell types are involved in this response which includes the production and excretion of various growth factors and cytokines such as the well-studied TGF- $\beta$ , IL-6, IL-8 and others.

Tissue injury involves disruption of capillaries and immediately triggers activation of platelets which begin a clotting cascade. Inflammatory events follow with recruitment of neutrophils which secrete a variety of growth factors and cytokines. Monocytes arrive to facilitate changes in the wound matrix and macrophages release factors that activate fibroblasts to begin adding hyaluronate and glycoproteins such as fibronectin to the extracellular matrix (ECM). Gradually, these fibroblasts shift to producing proteoglycans and collagen which are then deposited at the wound site. Over the course of several days, macrophages and fibroblasts work to remodel the matrix and thus convert the granulation tissue into scar tissue (Harty, 2003).

Studies comparing scarless fetal wounds to adult wounds have found significant differences in the levels of proinflammatory cytokines such as IL-6 and IL-8. Liechty et al. (1998) grafted adult and fetal skin subcutaneously into SCID mice, wounded the site and subsequently excised the wound after varying lengths of time. The presence of IL-8 was confirmed using reverse transcription polymerase chain reaction (RT-PCR). Their results showed that fetal fibroblasts produced significantly less IL-8 at baseline ( $50 \pm 6$  pg/mL versus  $450 \pm 115$  pg/mL,  $P < 0.001$ ). IL-8 mRNA was detected in unstimulated adult fibroblasts but not in fetal fibroblasts and much less IL-8 mRNA was detected in stimulated fetal fibroblasts than in adult fibroblasts. IL-8 mRNA was detected 4 hours after wounding in both fetal and adult wounds but by 12 hours no IL-8 mRNA was detected in fetal wounds, whereas IL-8 mRNA persisted for up to 72 hours in adult wounds.

In a similar experiment, Liechty et al. (2000a) excised incisional wounds and examined them for IL-6 mRNA quantification by RT-PCR. As with IL-8, fetal fibroblasts produced less IL-6 protein and mRNA at all points examined ( $P < 0.01$  vs adult). IL-6 mRNA was detected 4 hours after wounding in both fetal and adult wounds. By 12 hours there was no IL-6 mRNA in the fetal wounds but the adult wounds had IL-6 mRNA persisting to 72 hours. In this experiment, another set of grafts had 5 micrograms of IL-6 injected

at wounding and the wound subsequently harvested at 7 days for analysis. The wounds with administered IL-6 resulted in scar formation. Since these cytokines are responsible for the recruitment and activation of various leukocytes including macrophages, the diminished inflammatory response by fetal tissues may explain the lack of cellular recruitment and inflammation in fetal wound healing and further suggests that the inflammatory response plays a key role in the process of scar formation.

One well studied growth factor, TGF- $\beta$ , exists as three known isoforms and is involved in many aspects of wound healing, from the immediate inflammatory response to matrix deposition (Adzick & Lorenz, 1994). In response to tissue injury, macrophages release TGF- $\beta$  which stimulates the deposition of collagen and other matrix components by fibroblasts, thus implicating it in the etiology of fibrosis. Specifically, the TGF- $\beta$ 1 and TGF- $\beta$ 2 isoforms have been directly implicated in the fibrotic response and formation of scar tissue. Whitby and Ferguson (1991) detected both TGF- $\beta$ 1 and TGF- $\beta$ 2 isoforms with immunolocalization in adult wounds but not in fetal wounds. In a pioneer study, Shah et al. (1992) studied the effect of reduced levels of TGF- $\beta$  in adult rodent wounds by using various neutralizing agents. The results of that experiment indicated inhibited fibrosis and scar formation. In another similar experiment, Shah et al. (1994) injected a polyclonal neutralizing antibody to TGF- $\beta$ 1, 2 into full-thickness cutaneous wounds of adult rodents just prior to wounding or within 24 hours of wounding and repeated daily for two days post-wounding. The effect was successful reduction in scarring. Numerous similar studies have been performed that confirm these results with more or less comparable data (Singer, et al. 2009; Shah, et al. 2000). Conversely, up-regulation of these TGF- $\beta$  isoforms via exogenous application resulted in excessive scar formation in a number of animal models (Lin, et al. 1995; Wang, et al. 1999; Lanning, et al. 1999). Thus direct evidence exists that increased levels of TGF- $\beta$ 1 and TGF- $\beta$ 2 play an important role in the fibrotic response and suggests that manipulation of TGF- $\beta$  levels may provide an efficient strategy of reducing scar formation.

The culpability of TGF- $\beta$  in the formation of scar tissue is further supported by studies that have investigated the cellular and molecular differences abnormal scar formation such as keloid and hypertrophic scars. Experimental evidence suggests that upregulation of TGF- $\beta$  is necessary for the excessive scarring characteristic of these pathologies (Lee, et al. 1999; Campaner, et al. 2006). Wang et al. (2000) compared the levels of TGF- $\beta$ 1 mRNA in hypertrophic scar tissue and normal skin and found that hypertrophic scar tissues expressed five-fold more TGF- $\beta$ 1 mRNA than normal skin per unit of wet weight. TGF- $\beta$ 1 mRNA expression in 5 pairs of fibroblast cultures derived from hypertrophic scar tissue also was found to be significantly elevated as compared to those derived from normal cells ( $116 \pm 6$  vs. 97

+/- 7, p = 0.017). Additionally, Schmid (1998) demonstrated by immunohistochemistry and in situ hybridization, that hypertrophic scars exhibit unresolved persistent overexpression of TGF- $\beta$  receptors by fibroblasts leading to overproduction of matrix protein and subsequent fibrosis.

Table 1

TGF- $\beta$ Isoform	Scarless Fetal Healing	Scar-forming Adult Healing
TGF- $\beta$ 1	low/absent	high
TGF- $\beta$ 2	low/absent	high
TGF- $\beta$ 3	high	low

Summary of relative TGF- $\beta$  isoform differences in scarless fetal wound healing and scar-forming adult wound healing. (Ferguson, O’Kane, 2004).

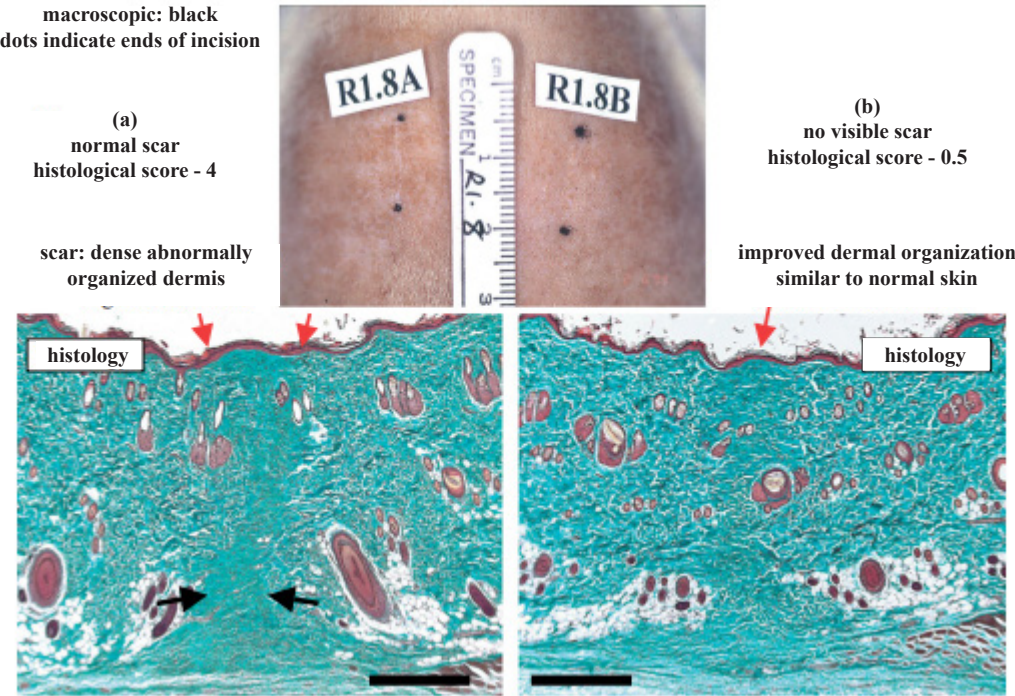
However, although the TGF- $\beta$ 1 and TGF- $\beta$ 2 isoforms have been

implicated in scar formation, a third isoform has been shown to reduce scarring. Whitby and Ferguson (1991) demonstrated that fetal wounds express low levels of TGF- $\beta$ 1 and TGF- $\beta$ 2 but high levels of the third isoform, TGF- $\beta$ 3 (Table 1).

This implied that the ratio of TGF- $\beta$  isoforms in wounded tissue may be the key to understanding the mechanism of wound healing and whether or not a scar forms. Shah et al. (1995) confirmed this hypothesis when they demonstrated that pan-neutralization of all three known TGF- $\beta$  isoforms in linear incisions made on rats did not improve scarring, while exogenous application of TGF- $\beta$ 3 resulted in marked improvement in scarring and even scarless healing. Numerous studies have exploited this therapeutic approach of increasing the relative ratio of TGF- $\beta$ 3 to TGF- $\beta$ 1 and TGF- $\beta$ 2 with novel pharmaceutical agents (Ferguson, O’Kane, 2004) (Figure 1).

The first clinical application of human recombinant TGF- $\beta$ 3, avotermin (Juvista; Renovo, UK) administered as an intradermal injection at the time of surgery, had shown promise in reducing scarring in controlled, double-blind, randomized phase I/II clinical studies (Durani, et al. 2008). So et al. (2011) studied sixty patients (35 men and 25 women; age, 19 to 78 years; 53 Caucasians; scar length, 5 to 21 cm) who received intradermal

Figure 1



(a) Placebo-treated incisional rat wound compared with (b) scarless healing in TGF- $\beta$ 3-treated wound 84 days post-wounding (Ferguson, O’Kane, 2004).



avotermin (200 ng/100  $\mu$ l/linear cm wound margin) and placebo to wounds immediately after scar revision surgery and again 24 hours later. Primary efficacy was measured as a total scar score derived from a visual analogue scale and scored by a lay panel from standardized photographs from 1 to 7 months following treatment. Profilometry showed a greater reduction in scar surface area from baseline with avotermin treatment compared with placebo and histologic analysis showed collagen organization that more closely resembled normal skin. However, an earlier study of dermal ulcers on the ears of female rabbits had demonstrated only that exogenous topical TGF- $\beta$ 3 had accelerated wound healing but with no improvement in scar morphology and prominence. Wu et al. (1997) showed that the use of TGF- $\beta$ 3 (0.3-0.75 microgram per wound) increased granulation tissue formation by more than 100% ( $P < .005$ ) but no significant difference in the hypertrophic index was noted as compared with controls. In fact, phase III trials of avotermin failed to meet primary and secondary endpoints, suggesting that the difference between scarless and scar-forming wound healing is perhaps more complex than simply altering the ratio of TGF- $\beta$  isoforms (Penn, et al. 2012) .

Interestingly, a number of studies have demonstrated that even in fetal wounds, exogenously applied TGF- $\beta$  can induce fibrosis. McCallion and Ferguson (1996) applied exogenous TGF- $\beta$ 1 to fetal wounds and observed a profound fibrotic response with scar formation. Krummel et al. (1988) showed that the addition of TGF- $\beta$  to polyvinyl alcohol sponges implanted in fetal rabbits produced fibrosis. Sullivan et al. (1995) also examined human scarless fetal wounds transplanted subcutaneously into adult nude mice. Immunohistochemistry performed on the wounds did not show TGF- $\beta$  staining. In a second part of the study, a slow-release disk with varying amounts of TGF- $\beta$ 1 was placed beneath the fetal skin graft at the time of wounding. Marked scarring in these fetal grafts was observed fourteen days post-wounding with the size of the scar proportional to the amount of TGF- $\beta$ 1 applied. These data suggest that the cellular mechanism of scar formation characteristic of adult wound healing is present in fetal wounds. The absence of scar formation in fetal wounds thus implies active suppression of these inflammatory mediators that have been implicated in scar formation.

The concept of immune inflammatory suppression as the mechanism of scarless healing has been investigated further by testing with the known anti-inflammatory cytokine IL-10. IL-10 deactivates macrophages and inhibits expression of both IL-6 and IL-8. Liechty et al. (2000b) hypothesized that the diminished levels of IL-6 and IL-8 they had revealed in a previous study were in fact due to the effects of IL-10. They developed a new syngeneic murine model of fetal wound repair in which 15-day-gestation skin from either normal or transgenic IL-10 knockout mice was grafted to the back of the same strain adult mice. Incisions were made

in the grafts after 5 days, which were then harvested at 1 week and analyzed for inflammatory response and scar formation. The normal fetal skin grafts showed minimal inflammation and were histologically consistent with scarless healing. The IL-10 knockout fetal skin grafts, in contrast, displayed significant inflammation and scar formation. Thus, the anti-inflammatory effects of IL-10 are necessary for active suppression of the inflammatory response leading to the observed paucity of macrophages, less TGF- $\beta$  release, and consequently, less scarring.

The impact of IL-10-induced immune suppression is a key factor in scarless wound healing. Yamamoto et al. (2001) performed Northern blot analysis to show that IL-10 differentially regulates collagen deposition by downregulation of TGF- $\beta$ 1-induced collagen mRNA. In a phase II randomized controlled clinical trial, Kieran et al. (2013) demonstrated that exogenous IL-10 applied to human incisional wounds reduced scarring and generally improved scar appearance.

In a recent study, Wise et al. (2014) studied the effects of a purified IL-10 homolog derived from orf virus (ovIL-10). Orf virus infections induce persistent skin lesions that are reminiscent of sustained wound healing, yet surprisingly resolve with minimal scarring (Gurel 2002), and genetic studies have discovered that orf virus encodes for a variety of factors that allow it to subvert host immune responses, including an IL-10 homolog (Fleming 2007). Wise et al. made excisional wounds in mice and divided them into four groups. The first three groups were treated with either recombinant murine IL-10 (mIL-10) or ovIL-10 in a buffered saline solution (PBS), or PBS alone, while the fourth group received no treatment. The wounds were biopsied at varying times and analyzed. Histological analysis revealed that wounds treated with ovIL-10 exhibited accelerated healing as evident by increased wound reepithelialization as compared to controls. Reduction in visible scarring was also evident and quantitative PCR confirmed decreased levels of key proinflammatory mediators. Thus supporting evidence exists that limiting the inflammatory response in wound healing can improve the quality of wound repair.

TGF- $\beta$  is an upstream regulator of the cyclooxygenase (COX) pathway (Wilgus, et al. 2004). COX enzymes catalyze the conversion of membrane phospholipid-derived arachidonic acid into prostaglandins which are known mediators of the inflammatory response. Western blot analysis and immunohistochemistry of biopsied hypertrophic and keloid scar lesions revealed significant overexpression of COX-1 and COX-2 isoforms, respectively, in comparison to normal dermal tissue which further suggests a significant role these enzymes play in the pathogenesis of these abnormal scarring processes (Rossiello, et al. 2009). Indeed, TGF- $\beta$  was shown to induce prostaglandin production in cultured

fibroblasts via COX-1 overexpression (Diaz, et al. 1998).

The significance of COXs in the etiology of pathological scarring such as keloids and hypertrophic scars has led to the investigation of its role in wound healing in general. Wilgus et al. (2003) examined the role of COX inhibitors in reducing scar formation. Celecoxib belongs to the family of nonsteroidal anti-inflammatory drugs (NSAIDs) which target the COX enzymes. Specifically, celecoxib selectively targets COX-2 and prevents its enzymatic activity and subsequent production of prostaglandins including prostaglandin E2 (PGE2). Wilgus et al. made full-thickness incisions in mice and closed the wounds with stainless steel wound clips to mimic surgical wounds. The staples were removed after 5 days. The wounds were treated topically immediately after wounding and for up to 14 days daily with either 200  $\mu$ l of the vehicle control (K-Y Jelly; Ortho Pharmaceutical Corp., Raritan, NJ) or with 1 mg of celecoxib capsules (Celebrex®, Searle, St. Louis, MO) dissolved in 200  $\mu$ l of vehicle such that 200  $\mu$ l contained 1 mg of the drug.

Skin sections were excised and analyzed. Immunohistochemical analysis using an antibody specific for the neutrophil surface marker Ly-6G, revealed a stark contrast between vehicle-treated and celecoxib-treated wounds. While a major inflammatory response, manifested as a massive infiltration of neutrophils, was observed in the vehicle-treated wounds in comparison to unwounded tissue, wounds treated with celecoxib were virtually devoid of neutrophils.

Additionally, Biotrak enzyme immunoassays (EIA; Amersham-Pharmacia, Piscataway, NJ) were used to quantify the concentration of PGE2. An approximately 50% decrease in wound PGE2 levels was observed in wounds treated with celecoxib ( $p < 0.05$ ). Levels of TGF- $\beta$ 1 were examined by Western blot analysis which revealed significantly lower levels in celecoxib-treated wounds compared to vehicle-treated wounds. These data were substantiated by the observation of reduced scarring later on.

Wilgus et al. (2003) also reported that PGE2 promotes fibroblast proliferation and collagen synthesis consistent with known findings (Lupulescu 1975) as well as their data showing decreased scar tissue deposition in treatment with celecoxib. This seems to be corroborated by the fact that lower levels of the proinflammatory fibrogenic TGF- $\beta$ 1 were detected. A similar result was presented by Miyajima et al. (2001) who also showed the ability of COX-2 inhibitors to reduce TGF- $\beta$  levels and thus fibrosis.

The demonstrated effect of treatment with topical celecoxib in reducing scar formation in adult wound healing led Wilgus et al. (2004) to examine the role of COX-2 in scarless fetal wound repair. Incisions were made in the dorsum of fetal mice at either

15 (E15) or 18 (E18) days after the detection of a vaginal plug using microsurgical scissors. These time points represent the ages at which either scarless or fibrotic healing occurs. Normal E15 and E18 skin were harvested as controls along with the wounded samples at varying times for examination. Immunohistochemical staining at 24 hours post-wounding demonstrated COX-2 protein expression only in wounded E18 skin but not in E15 skin. Western blot was also used to detect COX-2 protein and the levels were analyzed by image analysis software. Levels of COX-2 protein as well as PGE2 were higher in wounds introduced at E18 compared to those at E15, implying that COX-2 is involved in the process of scar tissue formation.

It is thought that one mechanism by which PGE2 contributes to scar deposition is by increasing the rate of fibroblast proliferation. Indeed, Wilgus et al. showed that exogenous PGE2 applied to fetal fibroblast cultures resulted in statistically significant ( $p < 0.05$  compared to untreated cells) dose dependent increased proliferation.

However, conflicting data suggest that fibroblasts from other organs do not respond in the same way to increased PGE2 levels. For example, PGE2 reduces proliferation and collagen synthesis by lung fibroblasts. This suggests that PGE2 can have varied effects perhaps resulting from differences in the expression or activity of various PGE2 receptors (Wilgus 2004).

Thus, the mechanism of PGE2-induced scarring could involve a number of plausible options. PGE2 could be contributing to the inflammatory response which has already been implicated in scar formation. Alternatively, PGE2 could be promoting scar deposition through increases in the profibrotic TGF- $\beta$ . Lastly, PGE2 could be directly encouraging scar formation by stimulating fibroblast proliferation.

Of note, however, is that although keloid and hypertrophic scars were found to express high levels of COX proteins, Yeh et al. (2006) found that fibroblasts derived from keloid patients exhibited diminished capacity to produce PGE2, in line with the studies that identified PGE2 as an antifibrotic agent. In this experiment, fibroblast cultures were stimulated using the proinflammatory cytokine IL-1 which is known to activate fibroblasts and induce the formation/release of COX pathway products such as PGE2 (Elias, et al. 1987). The effects of PGE2 on the synthesis of collagen were determined using enzyme-linked immunosorbent assay (ELISA) kits. According to these data, the excessive deposition of collagen characteristic of keloids may in fact be due to decreased levels of PGE2 rather than increased.

The conflicting and perhaps confounding data surrounding PGE2 and its effect on fibroblast proliferation and collagen synthesis

necessitate further investigation into the exact mechanisms by which this enzyme affects the healing process. Such contradictory data does not now lend to sufficient understanding that can lead to exploitation and development of therapeutic clinical applications in the treatment or prevention of scarring. In fact, some have suggested that the use of NSAIDs and COX-2 inhibitors may even exacerbate wound scarring because of their ability to decrease PGE2 production (Su, et al. 2010).

Until such time as the role of PGE2 and its effect on wound healing becomes better understood, a more promising approach to treatment and/or prevention of scarring would seem to be manipulation of the various TGF- $\beta$  isoforms, which perhaps play a more direct role in the process of scar formation.

Already, clinical trials that manipulated the ratio of TGF- $\beta$  isoforms have proven efficacy in the controlling scarring, although not without some failure. Further investigation into the exact role the various isoforms of TGF- $\beta$  play in the process of wound healing can hopefully lead to the development of better targeted therapies to reduce scarring and perhaps even cure the numerous fibrotic diseases.

## Conclusion

In this review, the underlying mechanisms of scar formation in wound healing were explored. The various TGF- $\beta$  isoforms (and their ratio) seem to play a vital role by regulating the inflammatory response although the process is undoubtedly more complex. The role of COX and PGE2 remains unclear with contradictory evidence pointing either to a reduction in scarring or perhaps exacerbation of the problem. The focus of potential therapeutic agents should for now remain dedicated to TGF- $\beta$  as past studies have already proven potential in reducing scarring, while the other options reviewed here remain clouded in uncertainty. Research has further investigated the effects of interactions between cell adhesion molecules such as integrins and cadherins and the various TGF- $\beta$  isoforms in an effort to explore targeted therapeutic agents (Eslami, et al. 2009; Agarwal, 2014). Additionally, research has explored TGF- $\beta$  as a potential target for anti-scarring gene therapy (Liu, et al. 2004). These and other efforts show promise in the development of successful clinical means of controlling scarring and perhaps even fibrotic disease.

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# Is There a Link between Saturated Fat Intake and Alzheimer's disease?

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## Abstract

Alzheimer's disease is a neurodegenerative disease that causes dementia and ultimately death. Currently, there is no treatment available for this disease. The aging of the population will only increase the incidence of Alzheimer's disease, making it ever more important to find an effective method of prevention. Dietary intervention is a practical and affordable method of intervention. The brain is a fat rich organ, and dietary fats are critical for proper development of the brain. A literature review was conducted to determine whether there is a link between saturated fat intake and Alzheimer's disease. According to the literature reviewed, saturated fat increases the amount of amyloid beta in circulation, and is linked to blood brain barrier dysfunction. Consistent with this, high saturated fat diets lead to cognitive decline in animals. Epidemiologic studies have yielded conflicting results, but most studies show a link between increased intake of saturated fat and increased incidence of Alzheimer's disease. Lipid lowering agents and anti-inflammatory drugs have been found to attenuate the effect of a high saturated fat diet in animals. An examination of medical records has also shown that patients who had been prescribed statins were less likely to be afflicted with Alzheimer's disease. The literature reviewed indicates that lowering intake of saturated fat as part of a healthy diet may reduce the risk of Alzheimer's disease later in life. There is also evidence that lipid lowering agents and anti-inflammatory drugs may slow the progression of Alzheimer's disease.

## Introduction

Alzheimer's disease is a neurodegenerative disease that causes severe memory loss and ultimately death. The first part of the brain affected by Alzheimer's disease is the hippocampus, a region of the brain associated with memory. Memory loss is usually the first symptom of Alzheimer's disease. As the disease progresses the degeneration spreads to other parts of the brain leading to the loss of other cognitive skills such as language skills and the ability to plan. Eventually, damage to the basal nucleus of Meynert leads to a sharp drop in the amount of the neurotransmitter acetylcholine present in the brain, leading to further deficiencies (Peterson, 2002). Approximately ten percent of the population over the age of 65 and fifty percent of those above 85 suffer from Alzheimer's disease (Carlson, 2011). Currently, there is no medication that can cure Alzheimer's disease or stop its progression. Patients suffering from Alzheimer's disease are treated with medications that alleviate the symptoms. Acetylcholinesterase inhibitors are prescribed to alleviate the cognitive symptoms. Anxiolytics and antipsychotics may be prescribed to treat behavioral symptoms. These drugs, however, may worsen cognitive deficiencies. Ultimately, the inexorable progression of Alzheimer's disease leads to death (Peterson, 2002).

Although the cause of Alzheimer's disease has not been definitively determined, two pathological conditions have been linked to Alzheimer's disease, amyloid plaques and neurofibrillary tangles. The amyloid plaques are extracellular deposits of the protein beta amyloid (sometimes referred to as amyloid beta). These plaques are surrounded by degenerating neurons and activated microglia, the phagocytotic cells of the central nervous system. Activated microglia are a sign of inflammation or immune response. The neurofibrillary tangles are intracellular accumulations of hyperphosphorylated tau proteins. These tangles disrupt transport within the neurons eventually killing the cell and leaving behind the tangle of filaments (Carlson, 2011).

Alzheimer's disease is split into two categories: early onset, also called familial, and late onset, also called sporadic. Alzheimer's disease occurring before the age of 65 is considered familial whereas Alzheimer's disease occurring after the age of 65 is considered sporadic. Familial Alzheimer's shows a clear pattern of inheritance. In different families the disease is caused by different genetic mutations. Three genetic mutations have been identified: a mutation in the amyloid precursor protein gene (APP) gene, a mutation in the presenilin 1 (PS1) and a mutation in the presenilin 2 (PS2) gene. All three of these mutations have been shown to increase the relative amount of amyloid beta 42 in the brain. Amyloid beta 42 is "stickier" than the wild type variant, amyloid beta 40. Consequently, amyloid beta 42 is more likely to conglomerate and form plaques. Sporadic or late onset Alzheimer's disease does not show any clear pattern of inheritance. However, a variant of the apolipoprotein E gene, apolipoprotein epsilon 4, has been linked to increased occurrence and earlier age of onset of Alzheimer's disease. Apolipoprotein E is a carrier protein for cholesterol. The epsilon 4 variant of this protein has been linked to an increase in the amount of amyloid beta in the brain. The mechanism behind this relationship is unknown. Researchers suspect that apolipoprotein E is involved in the clearance of amyloid beta from the brain and that the epsilon 4 variant is not as effective the epsilon 2 or epsilon 3 variants in clearing amyloid beta (Peterson, 2002).

Researchers have recently begun to examine the role of diet in the etiology of this disease (Heude et al 2003, Kalmijn et al 1997, Engelhart et al 2002, Luchsinger et al 2002, Morris et al 2003 and Laitinen et-al 2006). Diet is a particularly interesting field for research because dietary interventions are relatively cheap and easy to initiate. This research hopes to provide the public with a cost effective method for preventing Alzheimer's disease, or at least delaying its onset. Dietary fats in particular have been the focus of much research. The central nervous system has a



very high concentration of lipids, second only to adipose tissue. Additionally, lack of essential fatty acids has been shown to be a limiting factor in brain development (Winocur and Greenwood, 1993). It follows that intake of fatty acids would have a significant impact on the health of the brain later in life. The link between apolipoprotein E and Alzheimer's disease also suggests a role for lipid metabolism in the etiology of Alzheimer's disease.

Dietary fatty acids are ingested as triglycerides, groups of three fatty acids attached by an ester bond to a glycerol backbone. In the small intestine, these triglycerides are broken down to monoglycerides and fatty acids by the enzyme lipase. After being absorbed by the epithelial cells of the small intestine, they are recombined to form triglycerides. Because triglycerides are not water soluble, they cannot be transported through the blood stream without a carrier. Instead, they are combined with proteins to form lipoproteins. Lipoproteins are spherical particles with an outer shell of proteins, phospholipids and cholesterol surrounding an inner core of triglycerides. There are a number of different types of lipoproteins, each with a different composition and a different function. The lipoprotein formed in the epithelial cells of the intestine is called a chylomicron. The chylomicron passes from the intestinal epithelial cells to the lacteal, and is delivered to the blood stream via the lymph system (Tortora and Derrickson 2006).

Studies have shown that rats raised on a diet high in saturated fat were impaired on behavioral tests (Winocur and Greenwood 1993). Changes in the morphology of the hippocampus consistent with a loss of dendritic integrity and immune response were also observed (Granhölm et al 2008). Additionally, animals that were fed a diet high in saturated fat were found to have higher concentrations of the protein amyloid beta (Galloway et al 2007), the main component of amyloid plaques, and compromised blood brain barrier integrity (Farrall and Wardlaw 2009, Taketchi et al 2010, Taketchi et al 2013a, Taketchi et al 2013b, and Freeman and Granhölm 2012). In humans, however, the link between saturated fats and Alzheimer's is not as clear. A number of studies have been conducted, and they have yielded conflicting results. This paper will review and elucidate the relevant research with the goal of answering the following two questions: Is there a definitive link between high intake of saturated fat and incidence of Alzheimer's disease? If yes, what is the mechanism of this relationship, and does this suggest any possible interventions other than diet modification?

### Methods

A literature search was conducted using pubmed.gov. The following search terms were used: Alzheimer's disease AND saturated fat, Alzheimer's disease AND dietary fat, Alzheimer's disease AND amyloid beta, Alzheimer's disease AND blood brain barrier, and

Alzheimer's disease AND statins. To exclude irrelevant papers the search terms were restricted to the title and abstract. Of the papers that were found using these search terms, relevant papers were selected based on their abstract. Additional sources of information were found in the reference sections of these papers.

### Discussion

#### *Saturated Fat and Amyloid Beta*

In 1992 Hardy and Higgins proposed that the deposition of amyloid plaques is the initiating event in Alzheimer's disease. This hypothesis, called the amyloid cascade hypothesis, suggests that an influx of calcium caused by the toxic effects of amyloid plaques leads to hyperphosphorylation of the tau proteins which then form neurofibrillary tangles and kill the neurons. A number of findings support this theory. Firstly, it has been observed that mutations of the gene encoding the tau protein lead to Parkinson's-like symptoms, but did not lead to the deposition of amyloid plaques. Secondly, it has been observed that mice expressing mutant variations of both amyloid beta and tau protein experience increased formation of tau protein tangles when compared to mice expressing only the mutant version of tau protein, while the number of amyloid plaques is the same. Both of these observations indicate that the deposition of amyloid plaques leads to the formation of tau protein tangles, but not the other way around. The discovery that familial Alzheimer's disease is caused by a genetic mutation in the amyloid precursor protein also supports the amyloid cascade hypothesis. It seems likely that both familial and sporadic Alzheimer's disease share an underlying cause (Hardy and Selkoe, 2002).

It has been shown that the degree of dementia is correlated with the amount of amyloid beta present in the brain (Naslund et al. 2000). However, there is no evidence of increased production of the amyloid beta protein in the brains of Alzheimer's disease patients, indicating that the observed increase is caused by decreased clearance of amyloid beta from the brain or increased uptake of this protein from circulation (Pallebage-Gamarallage et al. 2009). Using iodine-125 labeled amyloid beta, researchers have shown that amyloid beta from circulation does indeed cross the blood brain barrier (Mackic et al. 2002). This suggests that factors which increase the levels of circulating amyloid beta may increase the level of amyloid beta in the brain, and lead to the formation of plaques.

The protein amyloid beta is involved in the metabolism of lipids. In vivo, it is associated with chylomicrons and lipoprotein B, and it increases the uptake of triglyceride rich lipoproteins (TRLs) by fat rich organs (James et al. 2003). Consistent with this, it has been found that a diet high in saturated fat increases the levels of amyloid beta in enterocytes (Galloway et al. 2007). The link between lipid metabolism and the deposition of amyloid plaques

in the brain is further strengthened by the finding that high serum cholesterol induced by high cholesterol feeding is correlated with increased levels of amyloid beta in the brain, as well as increases in the number and size of amyloid plaques (Refolo, 2000). These studies investigated the effect of cholesterol only, but not the effect of saturated fat.

The link between saturated fat and amyloid plaques suggests that reducing intake of saturated fat would reduce the incidence of Alzheimer's disease. However, it must be noted, that a ketogenic diet, a diet very low in carbohydrate content and very high in fat content, has been shown to lower the amount of amyloid beta in a rat's brain (Van der Auwera et. al. 2005). This indicates that the increase in amyloid beta observed in rats fed a high saturated fat diet is not simply because of the high fat content. Rather, it is the result of a synergistic effect between saturated fats and carbohydrates.

### **Saturated Fat and Blood Brain Barrier Dysfunction**

Saturated fat may also have a detrimental effect on the health of the brain by disrupting the blood brain barrier. The blood brain barrier was discovered more than 100 years ago by Paul Ehrlich when he observed that blue dye injected into an animal's blood stream will not reach the spinal cord or the brain. The blood brain barrier is formed by the endothelial cells of the blood vessels in the central nervous system. In most capillaries there are gaps between the endothelial cells that allow substances to diffuse in and out of the blood. In contrast, the endothelial cells in the central nervous system are tightly packed, and held together by tight junctions. This prevents many substances that normally diffuse out of the blood stream from diffusing into the central nervous system (Carlson, 2011).

A disruption of the blood brain barrier can lead to Alzheimer's disease through two distinct pathways. As previously mentioned, there is evidence that the increase in the amount of amyloid beta in the brains of Alzheimer's patients may be due to an influx of amyloid beta from circulation. We previously explored the possibility that this is because intake of certain lipids increases the amount of amyloid beta present in the blood. Another possibility is that the influx of amyloid beta is due to the breakdown of the blood brain barrier. In addition to affecting the level of amyloid beta in the brain, a breakdown of the blood brain barrier would allow toxins to enter the brain, possibly leading to Alzheimer's disease through a completely different pathway.

There are a number of methods by which blood brain barrier integrity can be assessed. The levels of plasma derived proteins in the brain can be measured, with a greater amount indicating influx through the blood brain barrier. Similarly, the levels of

cerebrospinal fluid derived proteins in the plasma can also be measured to determine efflux through the blood brain barrier. Immunofluorescent antibodies can be used to detect the presence of tight junction proteins and other proteins integral to the maintenance of the blood brain barrier. Imaging techniques such as contrast CT, contrast MRI, and positron emission topography (PET) have also been used.

Whether blood brain barrier dysfunction is linked to Alzheimer's disease has been debated for many years. A number of experiments have been performed using both biochemical and imaging techniques. A meta-analysis of sixteen studies found that there was a statistically significant link. There was, however, great heterogeneity among the results of individual studies (Farrall and Wardlaw, 2009).

To examine the effects of saturated fat on the blood brain barrier, researchers examined the brains of mice fed a diet high in saturated fat, high in unsaturated fat and a control group fed standard laboratory chow. Immunoglobulin G (IgG), and apolipoprotein B (a hepatically derived lipoprotein) were used to measure influx. The cerebrospinal fluid protein, S100B was used to detect efflux. After three months, the mice were euthanized, and the relative abundance of occludin, a tight junction protein, was also assessed. It was observed that levels of immunoglobulin G and apolipoprotein B were greatly elevated in the brains of mice in the saturated fat group, indicating increased influx of proteins and lipoproteins. The increased presence of apolipoprotein B, a lipoprotein that is associated with amyloid beta in enterocytes, supports the theory that blood brain barrier dysfunction can cause Alzheimer's disease by increasing the influx of amyloid beta. Plasma levels of S100B were also greatly elevated indicating efflux from the cerebrospinal fluid into the brain. Finally, levels of occludin were found to be lower in the saturated fat group, indicating a breakdown of the tight junctions that form the blood brain barrier. The effects of the high saturated fat diet were exacerbated when the mice were examined after six months of feeding (Taketchi et. al. 2010 and Taketchi et. al. 2013a).

Another experiment examined the effect of a high saturated fat/high cholesterol diet on three blood brain barrier proteins. The proteins investigated were endothelial barrier protein and the tight junction proteins occludin, and ZO-1. Using immunofluorescent antibodies the high saturated fat/high cholesterol fed rats were found to have markedly less endothelial barrier protein in the cortex and the CA-1 region of the hippocampus. When analyzed by western blot, a hippocampal homogenate of the experimental rats was found to have more occludin and the same amount of ZO-1 as that of the control rats. However, immunofluorescent imaging showed that the localization of occludin was markedly different in the two groups. In the control group

occludin was found to be present mostly in the walls of blood vessels, as would be expected for a blood brain barrier protein. In contrast, the blood vessels of the experimental group stained very weakly for occludin, while there was a strong signal in axon fibers and granule cells of the dentate gyrus. It may be that the increased level of occludin detected in the brain of the experimental group was because expression of occludin had been up-regulated in response to blood brain barrier damage (Freeman and Granholm, 2012).

### Saturated Fat and Cognitive Decline in Animals

The evidence linking saturated fat to increased production of amyloid beta and blood brain barrier dysfunction suggests a link between saturated fat and Alzheimer's disease. Indeed, a link between saturated fat and cognition has been observed in rats. Researchers found that rats fed a diet high in saturated fat performed worse on memory tests than rats fed a diet that had unsaturated fat as its main fat component (Winocur and Gordon 1993). Again, it must be noted that rats fed a ketogenic diet did not experience any cognitive decline, despite the extremely high saturated fat content in their diets. This is consistent with the finding that a ketogenic diet reduces the levels of amyloid beta in the brain (Van der Auwera et al 2005).

In addition to memory impairment, researchers found that a diet high in cholesterol and saturated fat significantly altered the morphology of the hippocampus, one of the areas of the brain responsible for memory and learning. After being fed a diet high in saturated fat and cholesterol, sections of the hippocampus were tested for Map2, an integral membrane protein that is considered a marker for dendrites, and Iba1 a marker for microglia. Compared to the control group, rats fed a diet high in saturated fat had significantly less Map2 and considerably more Iba1, indicating a loss of dendritic integrity and activation of microglia, both hallmarks of Alzheimer's disease (Granholm et al 2008).

### Saturated Fat and Alzheimer's Disease

A link between saturated fat and Alzheimer's disease cannot be definitively asserted unless there is evidence of such a link in humans. A number of epidemiologic studies have been conducted to determine the link between dietary fats and Alzheimer's disease (Heude et. al. 2003, Kalmijn et. al. 1997, Engelhart et. al. 2002, Luchsinger et. al. 2002, Morris et. al. 2003, and Laitnen et. al. 2006). Although the information gleaned from these studies is enlightening, the results must be approached with caution. All of these studies rely on self reporting. Because participants do not know how much fat they have ingested, researchers use food frequency questionnaires that ask participants about their dietary habits. This information is used to estimate their intake of various micronutrients. This method is an estimate at best. As with all studies relying on self reporting there is an inherent inaccuracy.

Additionally, responses on a questionnaire reflect only the respondent's current diet, but offer no information about their dietary habits in the past. Discrepancies among these studies can arise from differences in the questionnaires used, length of follow up time, the age of the population being studied, and the ethnicity of the population.

In the Rotterdam study, 7,983 residents of a suburb of Rotterdam over the age of 55 were administered questionnaires that were followed up by clinical examinations. After a two year follow up, researchers found a statistically significant link between increased intake of saturated fat and incidence of Alzheimer's disease (Kalmijn et. al. 1997). Interestingly, the link was significant even after eliminating patients with cerebrovascular disease, indicating that the mechanism mediating this link is not vascular in nature. However, a six year follow up of the same group showed no significant link between saturated fat intake and Alzheimer's disease (Engelhart et. al. 2002). An examination of the methods used in the two studies does not reveal an obvious reason for this discrepancy. Both studies used the same method of assessing dietary intake of fats, and both adjusted their results for similar confounding variables. The authors of the second study do not address the discrepancy directly, but they do suggest that the longer follow up may introduce selective survival. For instance, it may be that subjects who live longer are less susceptible to the detrimental effects of a high fat diet leading to a longer life span, and a lower incidence of dementia as well (Engelhart et. al. 2002). It may also be that saturated fat lowers the age of onset of Alzheimer's disease, but does not increase overall incidence.

Using data from the Washington Heights-Inwood Columbia Aging Project (WHICAP), researchers found a link between total intake of fat and increased incidence of Alzheimer's disease. Saturated fat alone was linked to increased incidence of Alzheimer's disease; however, the hazard ratio was slightly lower than that of total fat intake and was less than that of monounsaturated fats, indicating that saturated fat is not more harmful than other fats. Although increased intake of saturated fat overall was linked to increased incidence of Alzheimer's disease, no difference was found between those reporting moderately high intake and those reporting very high intake. When the data was analyzed using only people who had tested positive for the apolipoprotein E epsilon 4 allele (both homozygous and heterozygous) the link between total fat intake and Alzheimer's disease was much stronger and successively greater intake of saturated fat indicated successively greater risk of Alzheimer's disease (Luchsinger et al 2002).

Another study using data from the Chicago Aging Project and a median follow up of 3.9 years found a strong link between saturated fat and Alzheimer's disease. Participants in the top fifth of saturated fat intake were 70% more likely to develop Alzheimer's

disease. This is in contrast to other fats, some of which seemed to lower the risk of Alzheimer's disease. Adjusting for confounding variables led to 120% increase in risk. In contrast to the findings of Luchsinger et al (2002), this study found that the link between saturated fat and Alzheimer's disease was not any stronger in participants possessing the apolipoprotein E epsilon 4 allele (Morris et al 2003).

The previous studies focused on elderly people and had a follow up between two and seven years. A short follow up can potentially underestimate the link because of the possibility of subclinical dementia (i.e. the patients who were discovered to have dementia might have had subclinical dementia at baseline, and the poor diet is a symptom rather than a cause of the disease). In contrast, Laitinen et al (2006) studied the link between fat intake at midlife and the incidence of Alzheimer's disease later in life. In this study participants of the CAIDE study who had been examined in either 1972, 77, 82, or 87 were called for reexamination in 1998. The mean follow up was 21 years. This study found that participants with moderate fat intake (second quartile) had a lower risk of Alzheimer's disease than either low fat or high fat intake. When broken down to specific fats, high polyunsaturated fats and monounsaturated fats were correlated with a decreased risk of Alzheimer's disease, while high intake of saturated fats was correlated with a higher incidence of Alzheimer's disease. Only the data for polyunsaturated fats and saturated fats yielded results that were statically significant. When adjusted for the Apolipoprotein E epsilon 4 allele, the results were similar; suggesting that effect of saturated fat is not related to the Apolipoprotein E epsilon 4 allele. It is noteworthy that this study calculated relative intake of fatty acids based only on intake of different types of dairy, excluding two major sources of dietary fatty acids, meat and fish.

As previously mentioned, studies that rely on self reporting food frequency questionnaires are inherently inaccurate. To address this problem, researchers studied the link between erythrocyte membrane content and cognitive decline. This study found a significant link between saturated fatty acid content in erythrocytes and cognitive decline. Interestingly, the results for polyunsaturated fatty acids were mixed; n-6 polyunsaturated fatty acids were linked to cognitive decline while n-3 polyunsaturated fatty acids seemed to protect against decline. This study investigated only the link between fatty acids and general cognitive decline. A link between membrane composition and Alzheimer's disease specifically was not investigated (Heude et al 2003).

### Lipid-Lowering Pharmaceuticals

The link between saturated fatty acids and Alzheimer's disease suggests that drugs that lower plasma lipids may prevent Alzheimer's disease, or slow its progression. Probucol is a lipid-lowering agent

with anti-inflammatory and antioxidant properties that has been used to treat cardiovascular disease (Santos, 2012). Experiments with mice have shown that Probucol ameliorates the detrimental effects of a diet high in saturated fat. It was previously mentioned that mice fed a diet high in saturated fat and cholesterol were found to have a greater abundance of the protein amyloid beta in enterocytes. When Probucol was added to their diet, the levels of enterocytic amyloid beta in the saturated fat group were comparable to those in the control group (Pallebage-Gamaramillage et al 2012).

It was also found that Probucol prevented the damage to the blood brain barrier normally associated with a high saturated fat diet. Mice were randomly assigned either to a control diet, a diet high in saturated fat, a diet high in cholesterol, or a high saturated fat/high cholesterol diet with Probucol for three months. Consistent with previous experiments the mice in the high saturated fat and high cholesterol diets were found to have damage to the blood brain barrier as measured by the concentrations of immunoglobulin G and apolipoprotein B in the brain, and the concentration of S100B in the blood plasma. High saturated fat and high cholesterol mice were also found to have a greater abundance of activated microglia, a sign of inflammation. However in mice treated with Probucol the levels of immunoglobulin G, apolipoprotein B and S100B were comparable to that of the control group. There was also no evidence of inflammation in mice treated with Probucol (Taketchi et al 2013a).

When the experiment was repeated and mice were kept on the high saturated fat diets for 12 months the effect of the high saturated fat diet on blood brain barrier permeability and enterocytic abundance of amyloid beta was even more pronounced. Yet, despite the long term exposure to saturated fat, the group treated with Probucol was not effected (Taketchi et al 2014). In addition to preventing damage to the blood brain barrier and lowering enterocytic accumulation of amyloid beta, Probucol was also found to increase synaptic density in mice (Poirier, 2008).

A pilot study examined the effect of Probucol on 12 patients with mild-to-moderate Alzheimer's disease. A clinical assessment after six months of treatment found that Probucol led to a stabilization of symptoms (Poirier, 2008). This was a small scale study that did not include a control group. A long term controlled study is needed to definitively determine whether Probucol is an effective treatment for Alzheimer's disease and whether its effect is long lasting.

It is possible that the positive effects of Probucol are not related to lipid metabolism. Probucol was found to attenuate the effects of an injection of amyloid beta in mice. Mice injected with amyloid beta exhibited cognitive decline, and a histological examination of

their brains shows decreased synaptic density (as measured by the presence of the presynaptic protein synaptophysin) and increased acetylcholinesterase activity. Injection with amyloid beta does not increase plasma cholesterol. Treatment with Probucol for 15 days attenuated the effects of the amyloid beta injection. This is despite the fact that the pathological conditions observed in these mice were not due to increased lipid intake or dysfunctional lipid metabolism. This suggests that Probucol may directly affect the pathway responsible for Alzheimer's disease pathology in addition to its positive effect on lipid metabolism (Poirier, 2008).

Statins (HMGCoA reductase inhibitors) are another group of lipid lowering agents prescribed to treat cardiovascular disease. Similar to Probucol, statins also have anti inflammatory properties. In an experiment similar to that performed with Probucol it was shown that treatment with statins will reverse the blood brain barrier damage caused by a high saturated fat diet. Mice were fed either a low fat diet or a diet high in saturated fat. Consistent with previous experiments the mice fed a diet high in saturated fat were found to have significantly higher levels of immunoglobulin G and apolipoprotein B in the brain parenchyma, an indication of blood brain barrier damage. Treatment for two weeks with Atorvastatin, a lipid soluble statin, led to a decrease in the amount of immunoglobulin G present in the brain. Treatment with pravastatin, a water soluble statin did not lead to any reduction after two weeks. After eight weeks of treatment it was found that both atorvastatin and pravastatin completely negated the effects of the high saturated fat diet (Pallebage-Gamarallage et al 2012). Unlike other studies, this study tested the effect of the experimental compound after the mice had been on the saturated fat diet for an extended period of time. The effectiveness of the compounds after the effects of the saturated fat diet had presumably set in suggests that statins may be effective at reversing damage to the blood brain barrier as opposed to simply preventing it.

An analysis of data obtained from the General Practice Research Database in the UK revealed that individuals who had been prescribed statins had a significantly reduced incidence of Alzheimer's disease. This was true even for individuals who had taken statins in the past, but were no longer taking these drugs. Interestingly, use of other lipid lowering agents was not correlated with a lower risk of Alzheimer's disease (Jick et al 2000). Another study used data from the databases of three different U.S. hospitals. This study also found a statistically significant relationship between use of lovastatin (Mevacor) or pravastatin sodium (Pravachol), and a lower incidence of Alzheimer's disease. Simvastatin (Zocor), a different statin, was not correlated with decreased incidence of Alzheimer's disease in this study (Wolozin et al 2000).

Ibuprofen, a non-steroid anti inflammatory drug (NSAID) that does not have any lipid lowering properties was also tested alongside simvastatin and pravastatin in the previously described experiment. After two weeks of treatment Ibuprofen was found to be as effective as simvastatin, and eight weeks of treatment completely reversed the effects of the high saturated fat diet (Pallebage-Gamarallage et al 2012). Antioxidants are another group of compounds that have been found to ameliorate the effect of saturated fat. Inflammation is caused by reactive oxygen species (ROS). Anti oxidants combat the effects of reactive oxygen species and prevent inflammation. Four compounds were tested: aged garlic extract, alpha-lipoic acid, niacin, and nicotinamide. Mice were fed either a low fat diet a high saturated fat diet or a high saturated fat diet containing one of the four compounds. All four compounds were found to prevent the blood brain barrier dysfunction and inflammation caused by a high saturated fat diet (Takechi et al 2013b). These studies suggest that the protective property of statins and Probucol that was observed in humans and mice may be related to their anti inflammatory properties and not their lipid lowering properties.

### Conclusion

Saturated fat is clearly linked to an increase in amyloid beta and to blood brain barrier dysfunction in animals. Although not definitive, epidemiologic evidence seems to indicate that increased intake of saturated fat is a risk factor for Alzheimer's disease. Based on the evidence presented, it is reasonable to suggest limiting intake of saturated fat as part of an effort decrease the risk of Alzheimer's disease. In particular those with the apolipoprotein E epsilon 4 allele should limit their intake of saturated fat. However, it must be noted that saturated fat is only one among many micronutrients that make up our diet. Saturated fat alone is not responsible for inflammation, blood brain barrier dysfunction, or deposition of amyloid plaques. In fact, we have cited evidence that a ketogenic diet, which is high in saturated fat, reduces the amount of amyloid beta in the brain. When designing a diet low in saturated fat one needs to ensure that saturated fat is not replaced with an equally unhealthy substitute. Furthermore, there is no evidence that limiting saturated fat intake after the onset of Alzheimer's disease will reverse or even slow the disease's progression. At best, limiting intake of saturated fat throughout the course of one's life will lower the incidence of Alzheimer's disease and delay its onset, but it will not cure it.

The use of Probucol, statins, and other anti-inflammatory medications in the treatment of Alzheimer's disease is strongly supported by the evidence cited. Anti inflammatory medication has been shown to reverse damage to the blood brain barrier caused by saturated fat. Probucol has been shown to lower enterocytic accumulation of amyloid beta and to slow the progression of Alzheimer's disease. Use of statins has also been linked



to a decreased risk for Alzheimer's disease. Further research is needed to determine which of these drugs is most effective, and whether these drugs can be effective in the long term.

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# Regulation of Ghrelin a Possible Treatment Option for Obesity and Diabetes

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## Abstract

Obesity is a mounting problem in America today. One major concern about obesity is that it is a risk factor for type 2 diabetes, a disease that impairs insulin sensitivity and secretion. This interferes with blood glucose levels and can cause hyperglycemia, which is when there is too much circulating glucose in the blood. Ghrelin, an amino acid peptide responsible for appetite stimulation and energy balance, plays a direct role in insulin secretion and glucose metabolism. In many experiments, elevated ghrelin levels are associated with decreased insulin secretion from pancreatic islet cells. Although ghrelin concentration is decreased in obese individuals and diabetics, researchers attempt to use the ghrelin system as a treatment option for these people. It seeks to accomplish this by regulating the ghrelin produced by the body, diminishing its activity, affecting its receptor, the growth hormone secretagogue receptor (GHS-R1a), and by targeting ghrelin directly. Studies observed these methods' effects on insulin secretion and sensitivity as well as blood glucose concentration. The octanoylation of ghrelin, which activates it, is catalyzed by ghrelin Oacyltransferase (GOAT). Therefore GOAT inhibitors are one way of minimizing ghrelin's effects on metabolism. Additionally, cAMP concentrations decrease in islet cells of the pancreas in the presence of ghrelin. Using cAMP analogs can counter ghrelin's consequences as well. On the other hand, targeting the GHS-R1a receptor with an antagonist can also help enhance insulin secretion. Another research option entails using immunoneutralization to build antibodies against endogenous ghrelin. Lastly, studies have examined ghrelin knockout models in mice, by deleting the ghrelin gene, to examine the results that the lack of ghrelin has on insulin-glucose metabolism. Each of these methods has been proven to affect insulin and glucose metabolism. Further advances in the clinical application of these methods may lead to viable treatment options for obesity and diabetes.

## Introduction

A big problem facing the world today is the rise of obesity. According to the CDC, approximately one third of the American adult population is obese. Obesity is characterized by a Body Mass Index (BMI), of 30 or higher, while healthy weight BMI is in the range of 18.5-24.9 (CDC, 2012). One possible consequence of obesity is the development of type 2 diabetes. Diabetes is a disease characterized by elevated glucose levels as a result of insufficient insulin production or a malfunctioning insulin system. Of the American population 29.1 million or 9.3%, had diabetes in 2012. This number was an increase from 2010 when the statistics were 25.8 million and 8.3% (American Diabetes Association, 2014). In type 1 diabetes mellitus, the  $\beta$  cells of the pancreas that are responsible for producing insulin are destroyed. In the absence of insulin, these individuals are at risk for hyperglycemia, or dangerously elevated blood glucose levels. Type 2 diabetes usually has a later onset. People with type 2 diabetes experience insulin resistance so that the insulin does not help glucose enter the body cells. Eventually the body stops producing insulin altogether (CDC, 2014). Most cases of diabetes, 90-95% are type 2 diabetes. Statistics show that 90% of cases of type 2 diabetes in western countries can be attributed to excess weight gain or obesity. Improving glucose tolerance while losing weight is the aim of new treatments for diabetes. Examining the effects the hormone ghrelin has on glucose metabolism can be a valuable therapeutic option (Rudolph, et al. 2007). Ghrelin is a 28 amino acid peptide hormone that plays a role in many different body functions. It is the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) and has been proven to increase growth hormone secretion. Additionally, ghrelin is known as an orexigenic, or appetite-stimulating hormone, and is also responsible for energy balance. It is produced predominantly in the stomach but is also

produced in other places including the pancreas. One important function ghrelin serves is the ability to control insulin release from  $\beta$  cells of the pancreas. In the presence of ghrelin, insulin secretion is reduced, and blood glucose concentration increases. Research shows the effect of ghrelin on pancreatic islet  $\beta$ -cells. Ghrelin suppresses insulin secretion from  $\beta$ -cells by interfering with the K and  $\text{Ca}^{2+}$  channels that are stimulated by glucose metabolism. Obese individuals and diabetics have lower levels of plasma ghrelin than lean individuals. It is reasonable to suggest that this may be a regulated response in order to minimize the ghrelin activity that reinforces obesity. Subsequent research implicated possible regulation of ghrelin to treat obesity and diabetes. Since insulin levels are decreased in the presence of ghrelin, investigation of techniques to reduce or diminish the effects of ghrelin is in effect. Various treatment options that have undergone research and experimentation show positive outcomes in regard to treating diabetes and obesity. Many techniques that influence ghrelin's role in decreased insulin secretion are still in the experimental phase but may eventually be effective to treat diabetes and obesity. Ghrelin's effects on insulin sensitivity are also being explored. Can regulation of ghrelin serve as a possible treatment option for diabetes and obesity?

## Methods

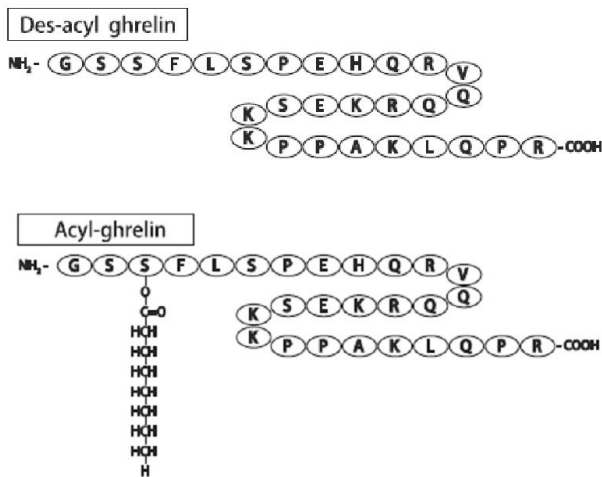
The research obtained about ghrelin's effects on the metabolism of diabetics and obese individuals was collected from a variety of sources. The majority of articles were collected using Touro's library to access databases such as Pubmed, ProQuest and more. Additionally, other articles were found by searching Google scholar for relevant journals on the topic. After reviewing numerous articles on ghrelin's effects on obesity and diabetes, evidence was compiled to answer the research question.

## Discussion

### Ghrelin and its forms:

Ghrelin is a 28-peptide amino acid that is produced mainly in the stomach. This hormone is responsible for signaling hunger and maintaining an energy balance. Acylated ghrelin, or acyl ghrelin, the active form of ghrelin consists of noctanoylation on the third serine. This form of ghrelin is able to bind to the GHS-R1a, enabling it to have a biological effect in areas such as growth hormone secretion and glucose metabolism. However, most of our plasma circulating ghrelin is unacylated ghrelin, or des acyl ghrelin, which has been discovered to have biological effects as well (Kiewiet, et al. 2009).

Figure 1



The effects of each form of ghrelin on insulin concentration and sensitivity has been studied. Acylated ghrelin (AG), the active form of ghrelin has been found to be responsible for decreased insulin levels, while unacylated ghrelin's (UAG) affects are being studied. It has been noted that ghrelin levels have a preprandial increase and a postprandial decrease indicating its role as an appetite stimulant (Kiewiet, et al. 2009). The hormone insulin acts in the opposite way, so investigators seek to explain the relationship between the two. Some studies examined the effects of AG and UAG on the insulin metabolism. Findings were that AG stimulated glucose release by hepatocytes, while UAG inhibited these effects. In addition, in an experiment done with adult-onset growth hormone-deficient patients, AG was found to decrease insulin sensitivity, and coinjection of UAG countered the effect. Thus, the circulating plasma ghrelin's two forms act in contrasting ways (Ukkola, 2009). An experiment was conducted in order to isolate the actions of AG and UAG. Subjects were administered a combination of AG and UAG and then each form of ghrelin on its own. The subject group consisted of 8 morbidly obese female Caucasians. The mean age was  $45.4 \pm 10.3$  in the range of 28-62 years old. The average BMI of the subjects was  $42.4 \pm 4.8$  kg/m<sup>2</sup>.

This experiment was a repeated measures design in which each of the 8 subjects received all the experimental conditions with a two-week break in between. Ghrelin was administered after overnight fasting conditions after a saline infusion. The three experiments were: administration of 200 µg of UAG on its own, administration of 100 µg of AG together with 100 µg UAG, and a placebo. Subjects were then given a 595 kcal breakfast, a similar lunch three hours later, and then they were free to eat what they pleased until midnight. Blood samples were collected to measure the total ghrelin, glucose, and insulin concentrations. After the first hour of administration, while subjects were fasting, administration of UAG on its own did not change insulin levels or glucose concentrations, nor did it differ from the placebo. Receiving the combination of UAG and AG resulted in significantly decreased insulin levels as compared to UAG administration alone and the placebo ( $58.2 \pm 6.3\%$  insulin concentration when AG and UAG were administered combined, compared to  $91.8 \pm 3.0\%$ , the insulin concentration when UAG was administered alone). This experiment tested for insulin sensitivity using the glucose to insulin ratio. The results indicated an almost 50% decrease in insulin levels and almost no change in glucose concentration. This demonstrated the effect of UAG to improve insulin sensitivity when administered along with the AG that lowered the insulin levels. Also, subjects with a smaller AG:UAG ratio possessed increased insulin sensitivity indicating the combination effect of coinjection of AG and UAG. This may lead to a possible treatment for diabetes for many diabetics have problems with insulin sensitivity. However, further research is needed to exactly determine insulin sensitivity, for the glucose-insulin ratio does not consider secretion, distribution and degradation of insulin (Kiewiet, et al. 2009).

However, a recent study tested the effect of AG in combination with UAG on insulin and glucose metabolism. Healthy volunteers were given pharmacological doses of AG and UAG as a bolus and at 210-minute intervals under fasting conditions and intravenous glucose administration. However, The UAG did nothing to plasma insulin levels after AG decreased them. This study did not examine but encourages further study on insulin sensitivity following AG and UAG administration (Barazzoni, 2014). Previous research tested the effects of ghrelin on insulin sensitivity by using the insulin-glucose ratio. Although the insulin levels stayed the same, the glucose levels decreased indicating greater insulin sensitivity (Kiewiet, et al. 2009). Although this may not give the most reliable results because it does not consider other factors that can contribute to insulin sensitivity, it is a start in observing the effect that UAG has on insulin sensitivity. Nevertheless, further research is needed to confirm this relationship.

### Insulin Sensitivity

Although it may be hypothesized that obese individuals have

elevated ghrelin levels contributing to their obesity, on the contrary, obese individuals have lower ghrelin concentrations than normal. According to a study obese patients' plasma active ghrelin levels compared to lean subjects were  $180 \pm 18$  vs.  $411 \pm 57$  pg/ml. Serum total ghrelin levels differed from  $3650 \pm 408$  in obese patients and  $5263 \pm 643$  pg/ml in lean subjects. Ghrelin levels were correlated with insulin resistance that was calculated using the HOMA approach which is:  $\text{insulin (micro-units/milliliter)} \times \text{blood glucose (millimoles/liter)} / 22.5$  (Marzullo, et al. 2003).

Similarly, in a different study, lower ghrelin levels were found in obese subjects. This study, shows the correlation between body fat and circulating ghrelin levels after obese and lean subjects were fed a weight maintaining diet and abstained from exercise two days. The results indicated that the obese subjects' ghrelin levels were 32% lower than the lean subjects'. Additionally, a negative correlation was found between ghrelin concentration and body fat ( $r = -.45$ ). Insulin was also negatively correlated to ghrelin concentration ( $r = -.45$ ) (Tscöp, et al. 2001). Since ghrelin is responsible for energy balance and homeostasis, this study is consistent with present knowledge. Obese individuals have stable energy levels so the body lowers the ghrelin response. However, although the circulating ghrelin levels are minimized, its effects on insulin and body fat are still present.

Studying children with type I diabetes mellitus (T1DM) indicated lower levels of total ghrelin in comparison to healthy subjects. Also, higher levels of AG contributed to lower insulin levels and greater insulin resistance. Therefore, a possible way the body protects diabetics from hyperglycemia can be the decreased concentration of circulating ghrelin. Insulin therapy after four months elevated ghrelin levels in twenty children with T1DM (Ukkola, 2009). This can indicate the body considering lower ghrelin levels unnecessary due to the insulin therapy.

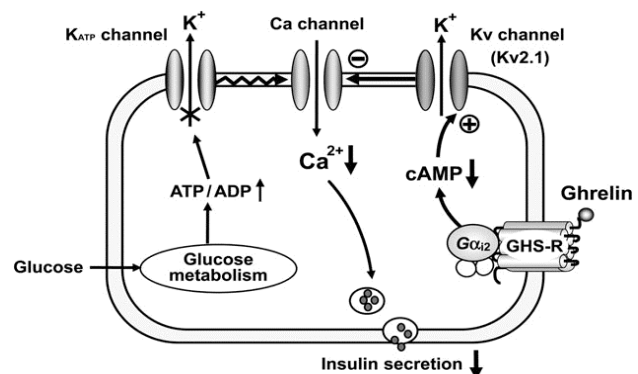
Although we can understand how insulin resistance is a problem in diabetic patients, obesity can contribute to insulin resistance as well. One way in which obesity contributes to insulin resistance is through the immune system's activation by obesity. Adipose tissue macrophages produce pro-inflammatory cytokines that can lead to insulin resistance by blocking insulin action in adipose tissue, skeletal muscle, and liver. Therefore, ghrelin as a possible therapeutic option can improve both diabetics and obese individuals' health (Ota, 2014).

### Ghrelin's Metabolic Effects

An experiment examined ghrelin levels in lean, obese, diabetic and nondiabetic subjects. Blood samples were collected after overnight fasting and again after monitored eating. Compared to normal weight subjects, mean plasma ghrelin concentrations were 225% for those with anorexia nervosa and 68% for obese

subjects. A negative correlation exists between BMI and plasma ghrelin concentration. The body maintains homeostasis by decreasing ghrelin levels in the presence of positive energy balance. When the body has excess energy from nutrition, the ghrelin levels are decreased to postpone hunger. This justifies the postprandial, or after eating a meal, decrease of ghrelin levels (Shiyya, et al. 2002). Ghrelin's effects on feeding and energy balance refer to the ghrelin produced in the stomach; however, the pancreas also produces ghrelin contributing to glucose metabolism. Ghrelin is an insulinostatic hormone, meaning it controls the release of insulin. Ghrelin levels were compared from the pancreatic vein that leaves the pancreas and the pancreatic artery that brings new blood to the pancreas. Results showed 8 times the amount of ghrelin levels in the pancreatic vein, indicating the pancreas produces that ghrelin (Yada, et al. 2014). The GHS-R1a, the ghrelin receptor, is located on pancreatic islet cells. Ghrelin binds to the receptor and decreases insulin release from the cell and elevates blood glucose concentrations. When glucose concentrations rise from 2.8 mM to 8.3 mM, insulin release from  $\beta$  cells is stimulated. The intensification of  $\text{Ca}^{2+}$  concentrations, stimulated by glucose metabolism is a crucial step in insulin secretion. However, insulin release is weakened when ghrelin is exogenously administered at a high concentration of 10 nM. Ghrelin suppresses the peaks of  $\text{Ca}^{2+}$  by increasing amplitudes of  $\text{K}^+$  currents that block the influx of  $\text{Ca}^{2+}$  ions into the cell thereby slowing down the process of glucose-induced insulin release (Yada, et al. 2008).

**Figure 2**



The binding of ghrelin to GHS-R reduces cAMP expression and increases the  $\text{K}^+$  current. This slows down the influx of  $\text{Ca}^{2+}$  into the cell, leading to a decreased insulin secretion response to glucose metabolism. (Yada, et al. 2014)

Research has proven that ghrelin's effect on the  $\text{Kv}$  channels result from decreased cAMP levels. cAMP, cyclic AMP (adenosine monophosphate) signaling plays one of the most vital roles in insulin secretion from beta cells (Kashima, et al. 2001). Ghrelin uses G-protein  $\text{G}\alpha_{i2}$  to obstruct the cAMP response to glucose metabolism. cAMP activates PKA, protein-kinase-A, and suppresses  $\text{Kv}$  currents in islet cells. Since the insulinostatic effect of ghrelin

occurs through the cAMP pathway, one possible way to minimize it is by presenting cAMP analogues along with ghrelin. One molecule used in testing the effects of cAMP analogs on insulin secretion is 6-Phe-cAMP. In the presence of cAMP analogs, ghrelin has no effect on insulin secretion. Hence, by reinstating the cAMP activation pathway in the presence of ghrelin, diabetics can experience enhanced insulin secretion (Dezaki, et al. 2011). This study demonstrates a new remedial approach to reducing ghrelin's effects. cAMP analogs may aid diabetes by enhancing insulin secretion in pancreas islet.

## Ghrelin Antagonism

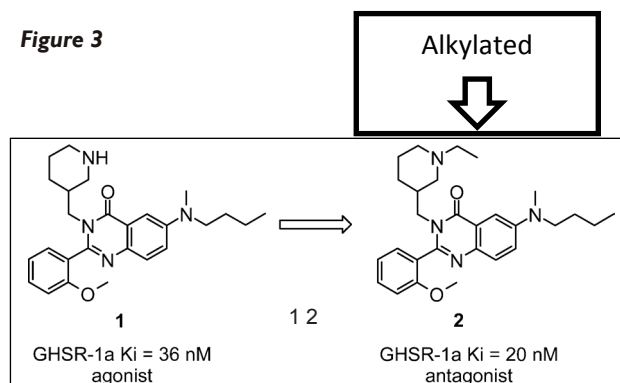
Since type 2 diabetics do not have enough insulin, anti-ghrelin treatments can enhance insulin secretion. In obesity, insulin resistance is a problem, so eliminating ghrelin can produce a positive effect on hyperglycemia. Ghrelin's effects on insulin and glucose metabolism, food intake, and body weight all need to be assessed in order to determine if antagonism of ghrelin can relieve symptoms of diabetics and obese individuals. One popular method used to remove ghrelin's effects is by antagonism of the GHS-R. If the receptor does not accept ghrelin, it has no effect on the pancreatic islets. In an experiment using fasted mice, ghrelin receptor antagonists [D-Lys3]-GHRP-6 enhanced insulin secretion and lowered blood glucose levels. This research indicated that a ghrelin blockade increases insulin release to help maintain normal glucose concentrations (Yada, et al. 2008). Experiments done with gastrectomized mice, that lack stomach-derived ghrelin, show the role of pancreatic ghrelin in the insulin response. After GHS-R antagonist was administered, both groups of mice showed the same increased insulin response. This proves that the pancreatic ghrelin is responsible for these effects. Therefore, targeting the GHS-R can be a therapeutic approach in enhancing insulin secretion (Dezaki, et al. 2006). Administering intra-peritoneal ghrelin antagonist to mice also decreased food consumption (Asakawa, et al. 2003). These findings can be beneficial in aiding obese individuals control their food intake.

Additionally, an experiment done on mice implied ghrelin's effects on body weight, insulin levels, and blood glucose concentrations. Injection of ghrelin twice a day for five days increased body weight in mice compared to the control. Depending on the diet and whether the mouse received ghrelin or saline, determined the increase in body weight. For example, one group of mice were fed a high fat diet and given saline. These mice exhibited a .60 g/day increase in body weight, while those fed a high fat diet with ghrelin had a .92 g/day increase. Serum cholesterol and insulin levels increased and glucose concentration increased as well. The same experiment tested how ghrelin antagonist, [D-Lys3]-GHRP-6, affected the same factors. After 7 days of administration to obese mice, weight gain, fat pad mass, and blood glucose concentrations decreased (Asakawa, et al. 2003). This study does

not seem to be consistent with other research that has linked elevated ghrelin to decreased plasma insulin secretion; but a moderate increase in glucose concentration was observed. This can indicate a case of insulin resistance instigated by an increase in free fatty acids (FFA) that are known to contribute to insulin resistance. GHS-R antagonists increased insulin sensitivity and reduced glucose levels. In addition, it simultaneously decreased FFA concentration aiding in fostering insulin sensitivity (Asakawa, et al. 2003).

One class of drugs that is being studied as possible ghrelin receptor antagonists are quinazolinone derivatives. In the lab, quinazolinone derivative 1 was discovered to have affinity to GHS-R1a. Yet, it was found that the molecule had agonistic binding to the receptor rather than the desired antagonistic affect. Alkylation of the N group on the molecule altered its functionality by transforming it into a GHS-R1a antagonist. Piperidine-substituted quinazolinone derivatives that are phenyl or phenoxy substituted were found to act as GHS-R antagonists (Del-porte, 2011).

**Figure 3**



In a structure-activity relationship study, many derivatives of the compound were tested for their effects in vivo. Two quinazolinone compounds that were used for study were compounds 26 and 43. These antagonists were administered at doses 3, 10, and 30 mg/kg. While compound 26 lowered body weight at the 10 mg/kg dose, compound 43 only was effective at 30 mg/kg. Compound 26 at 30 mg/kg is known as the first orally administered GHS-R antagonist possible to induce weight loss in animals. This renders it a feasible way to treat obesity, a leading cause of diabetes. Compound 26 administered at the highest dose had similar effects to an anti-obesity drug that is still under evaluation by the FDA, CBI antagonist rimonabant, 5-(4-choophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide. This study also tested glucose tolerance. Both compounds decreased glucose excursion by around 20%. However, the cause behind these decreased blood glucose levels, was not tested for. The study would like to go further in confirming that this result from the GHS-R antagonist stemmed from an enhanced insulin secretion (Rudolph, et al. 2007). This research



brings practical application of a drug that may be available to help treat obesity and diabetes. As a receptor antagonist, it minimizes ghrelin's insulino-static effects and enhances glucose metabolism.

### Immunoneutralization

Immunoneutralization of ghrelin also is a potential method of enhancing insulin secretion. Creating a vaccine to stimulate antibody production against acylated ghrelin may be a viable treatment for obese individuals. However, when a trial was conducted using a CYT-009 Ghr Qb vaccine, antibodies were developed, but weight stayed the same (Delporte, 2011). In another instance, mice were immunized against ghrelin to produce an antibody response. After being fed identical diets, the rats with more ghrelin-specific antibodies gained less weight per calorie ingested. Since their food intake did not vary from other control mice, this experiment shows that ghrelin produces a metabolic change rather than feeding changes. However, ghrelin has been known to increase feeding in a high-fat diet, these mice were given low-fat, less appetizing meals. This could be a factor that might have influenced the ghrelin-vaccinated mice's feeding habits, so the reliability of their conclusion is questionable. Additionally, there can be possible draw-backs to this course of treatment. Long-term or other side effects of eliminating ghrelin are unclear. It may have possible ramifications on energy balance that is regulated by ghrelin. The absoluteness of inactivating ghrelin by use of antibodies is also irreversible, unlike ghrelin receptor antagonism that can be stopped when undesired effects occur. Another obstacle that we may be facing with immunoneutralization is the negative feedback loop of the body producing more ghrelin to compensate for lack of ghrelin signals. Although the immediate results of an anti-ghrelin vaccine may look promising, further research is needed to confirm the safety and reliability of this method to reduce body weight. Controlling the immune response can keep ghrelin antibodies from getting out of hand (Carlson, Cummings, 2006). However, until research can safely develop and monitor such a vaccine, it would be best to put this method on hold.

### Ghrelin Knockout

An alternative to a ghrelin vaccine is the ghrelin knockout model. The ghrelin gene is deleted from rats and they are then monitored for feeding habits, metabolism, weight gain, and glucose concentration. One point to remember is the unknown effects of ghrelin's complete absence from the body. In one experiment, ghrelin's effects on insulin secretion were tested using ghrelin knockout mice. In particular, glucose-induced insulin release was higher in knockout mice. On the contrary, basal insulin levels remained the same in knockout and wild-type mice. Therefore, ghrelin knockout mice had improved insulin responses to glucose. Afterwards, an insulin tolerance test (ITT) was given to both groups of mice. No significant differences were found between them. The experiment advanced to testing the effects of a high-fat

diet, known to increase glucose concentrations. When fed a high fat diet, blood glucose levels increased in wild-type mice, and no increase was identified in the knockout mice. This can be attributed to enhanced insulin secretion in response to elevated glucose levels. After the wild-type mice did a glucose tolerance test (GTT), mice with a high-fat diet showed higher glucose concentration levels compared to a control diet, implicating glucose intolerance. On the other hand, the ghrelin knockout mice exhibited no difference in glucose tolerance between the high-fat diet and the control group. However, there was an increased insulin response in the mice fed a high-fat diet (Dezaki, et al. 2006). This research shows ghrelin's role in glucose-insulin metabolism. In the absence of ghrelin, increased insulin levels and improved glucose tolerance were found. Although ghrelin knockout cannot be done to humans, it serves as an indication of ghrelin's metabolic effects. Regulating ghrelin levels in obese people can possibly improve their body weight and glucose tolerance.

### GOAT

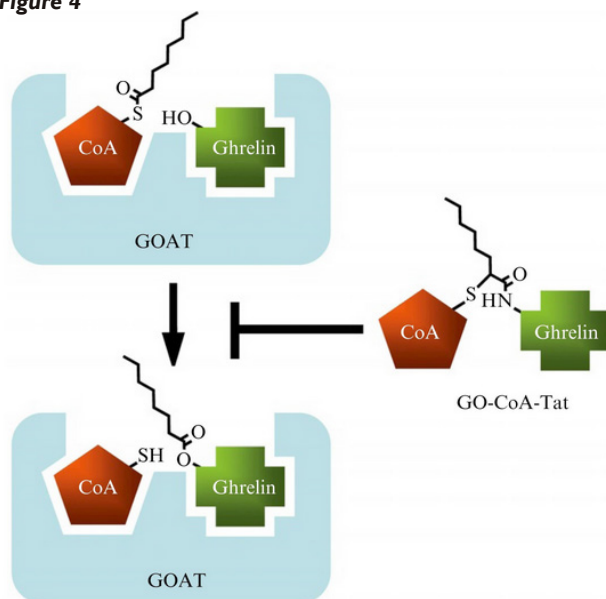
Ghrelin O-acyltransferase is a membrane-bound O-acyltransferase. It catalyzes the octanoylation of ghrelin allowing it to be acylated and able to connect with the GHS-R1a receptor. Since it catalyzes ghrelin to be in its active form, one idea is to target GOAT to slow the process of the body producing acylated ghrelin. In GOAT knockout models, there is no acylated ghrelin found circulating (Pulkinen, et al. 2010). As discussed earlier, studies show that unacylated ghrelin counteracts acylated ghrelin's effect on insulin, and did nothing to insulin metabolism when administered on its own (Kiewiet, et al. 2009). So, inhibiting GOAT action can minimize the effects that acylated ghrelin has on insulin and glucose metabolism.

One method studied to inhibit GOAT is the administration of GO-CoA-Tat in mice. The GHS-R binding property of ghrelin is determined by the octanoylation on serine 3, making it acyl ghrelin. This inhibitor was found to be most active in binding to GOAT. After administering GO-CoA-Tat to mice at a 40 mg/kg dose, acyl ghrelin levels decreased while des acyl ghrelin levels remained unchanged. Additionally, after 1 month, weight gain due to decreased fat mass was assessed in the treated mice. Interestingly, no difference in weight was found compared to ghrelin knockout mice. This indicates that GOAT inhibition may have similar effects to ghrelin knockout. The mice's response to glucose was measured to observe the effects of GO-CoA-Tat. The insulin response was increased and blood glucose levels subsequently decreased. This did not occur in ghrelin knockout mice thereby reinforcing the mechanism of GOAT inhibition to prevent ghrelin's acylation (Taylor, et al. 2012). Additionally, use of this GOAT inhibitor may be a better option than using a ghrelin antagonist. Some advantages that a study done by Barnett et al. in 2010 explains that GO-CoA-Tat does not need to cross the



blood brain barrier unlike the GHS-R antagonist which targets receptors in the brain as well. Additionally, there is no feedback of the body trying to compensate by producing extra acylated ghrelin to bind to the receptors. Another reason given is that targeting an enzyme like GOAT may be safer than targeting a widespread receptor (Barnett, et al. 2010). However, GOAT is still being studied and its use in treatment is not yet confirmed. Further experimentation would need to be done to examine the viability of such a method of treatment in humans, and the possible negative side effects of a drug using this mechanism.

**Figure 4**



*This simple diagram shows the catalytic activity of GOAT on ghrelin. Additionally, the inhibitor GO-CoA-Tat is shown to demonstrate interference with the GOAT mechanism.*

In conclusion, currently research is in the process of determining the possibilities of using the ghrelin system to treat or cure diabetes and obesity. No definite answer can be given about its viability in human subjects as most experimentation is done in mice. However, the facts have been proven. Ghrelin's influences on insulin secretion, blood glucose concentration, weight-gain, and fat mass have been assessed in various studies. The regulation of ghrelin by reducing its impact on pancreatic islets or by eliminating it altogether have been found to enhance insulin secretion and lead to less weight gain. Therefore, further research on the pharmacological effects of drugs used to regulate ghrelin's effects may lead to a more certain conclusion.

## Legend

AG: Acylated ghrelin UAG: Unacylated ghrelin

GHS-R: Growth hormone secretagogue receptor

GOAT: Ghrelin O-acyltransferase

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# Transposon Based Gene Therapy as a Treatment for Cancer

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## Abstract

Gene therapy is the use of genes to treat or prevent diseases. Diseases such as cancer, which are difficult to treat using conventional methods, can be treated using gene therapy. The transport of the therapeutic transgene can be accomplished using viral or non-viral methods. However, widespread use of viral vectors is limited due to its high cost of manufacture and safety concern. Non-viral vectors are limited in their effectiveness. The use of transposons such as the Sleeping Beauty transposon system can effectively deliver the transgene with less concern than viral vectors. This review discusses the various vectors and treatment strategies using gene therapy to treat cancer.

## Introduction

The molecular basis for cancer is now well understood to involve the genetic control of multiple genes that control cell cycle and tissue growth. The random mutations that activate dominant oncogenes or inactivate tumor suppression genes result in a cell cloning itself with an abnormal pattern of growth control forming a tumor. Traditional cancer therapies aim to destroy or remove the tumor by chemotherapy, radiation or surgery. The problem with both radiation and surgery is the ability of tumors to metastasize and spread cancerous cells to areas inaccessible to these treatments or may not be noticeable at the time of treatment allowing a secondary tumor to appear. The trouble with chemotherapy is its low therapeutic index for many cancers and the rapid development of drug resistance in cancerous cells.

Gene therapy is a fundamentally different approach to the treatment of diseases. Originally proposed as treatment for inherited autosomal recessive Mendelian disorders, such as hemophilia, gene therapy is now being used to treat multiple acquired conditions including infections, degenerative diseases and cancer. Gene therapy is the use of genetic material (genes) to express a specific protein in a cell or to reduce the amount of protein by interfering with its synthesis. Replacing a defective gene with a functional one is the essence of gene therapy.

Cancer is widely believed to arise from mutations to the cell's DNA by either carcinogens or random mutations during cell division. This genetic basis coupled with the limitations of the traditional treatments, makes cancer a great candidate for gene therapies. More than 60 percent of all gene targeted therapy clinical trials since 1989 aimed to treat cancer (Ginn et al, 2013).

## Strategies for Gene Therapy for Cancer

Cancer is a disease that involves multiple genetic changes to oncogenes, tumor-suppressor genes and modifier genes. There is also the intracellular interactions with multiple cells that regulate the body's immune response and the interaction with other cancer cells to maintain a solid tumor. Gene therapy can be used alone or together with conventional treatments. It can be used to sensitize cancerous cells to radiation or chemotherapy. It can increase the body's overall resistance to chemotherapy so larger

doses can be used. Gene therapy can declump or shrink tumors to allow for surgical removal. Various approaches are being examined both in preclinical studies and in clinical trials for gene therapy for cancer.

## Downregulation of Genetic Targets

To downregulate the expression of protein coded by oncogenes, antisense molecules are used. Antisense molecules are synthetic oligodeoxynucleotides (ODN) that hybridize with the coding (sense) mRNA of a specific gene. The antisense and sense molecules form double stranded RNA which cannot be translated destroying the mRNA. ODNs are designed to be highly resistant to nucleases which will destroy the mRNA (Stein et al., 1988). In a phase I-II clinical study using antisense ODNs to target BCL2 (an anti-apoptotic oncogene) mRNA combined with chemotherapy in patients with advanced malignant melanomas, the antisense ODN was found to successfully downregulate the target protein and has shown anti-tumor effects in 6 of the 14 patients (Jansen et al., 2000).

## Immunomodulation by Gene Therapy

Because cancer cells originate from "self" cells, they generally do not cause a strong immune response. However, the immune system can be augmented by gene therapy to increase their function. Therapeutic genes can be introduced into tumor cells or into effector cells such as T lymphocytes.

To elicit a greater immune response, tumor cells have been modified with the insertion of cytokine genes. Cytokines are small cell signaling polypeptides involved in immunity and inflammation. Systemic and local administration of cytokines from the interleukin (IL) family has shown significant reduction in tumor size but have systemic side effects and short half-lives, making injection of interleukins deficient in long term tumor control. However, tumor cells modified to express IL-12 or IL-2 have shown the same size reduction as well as long term remission and metastasis control (Gao et al., 2005, Tahara et al., 1995).

The receptors on T lymphocytes can be modified to target tumor-associated antigens. The most common method of creating an artificial T cell receptor (TCR) is by protein fusion of single-chain

variable fragments derived from monoclonal antibodies that acts as the TCR ectodomain with the CD3-zetatransmembrane and endodomain. The endodomain can be further modified with co-stimulatory receptors to increase immunologic activity. Leukemias from B cell lineages are suitable targets for this therapy. The CD19 antigen is expressed in differentiated cells with B cell lineages and is rarely lost in cancerous cells. Anti-CD19 T cells can safely target and destroy cells expressing CD19 including cancerous cells, and they will later be replenished with healthy cells (Scheuermann & Racila, 1995).

For effective activation of T cells, non-specific signals are needed as well as the antigen-specific signal received by the T cell receptor. Co-stimulatory molecules interact with receptors, such as CD28, on the T cell to provide the non-specific signals. Martinet et al. (2000) has shown that transfection of both IL-12 and 4-1BB ligand genes into tumor cells resulted in long-term remission of liver metastases in mice. The 4-1BB ligand, a co-stimulatory molecule that binds with the 4-1BB receptor on T cells, synergizes with the CD28 pathway to increase the immune response (Melero et al., 1998).

### 'Suicide' Gene Therapy

A commonly used idea for gene treatment of solid tumors is 'suicide' gene therapy, where the cells expressing the therapeutic gene are killed. Suicide genes code for enzymes that can activate a drug with an otherwise low toxicity. When suicide genes are expressed only in the targeted cancer cells, the healthy cells avoid the drug's toxic effect. One enzyme commonly used is herpes simplex thymidine kinase, which converts the nontoxic drug Ganciclovir, into a toxic form by phosphorylation (Song et al., 2009). The suicide gene can be placed under the control of tumor specific promoters such as c-erbB2 in breast cancer, ensuring specific drug activation in the tumor (Pandha et al., 1999). Because the enzyme can bleed into and kill neighboring non-transduced cells in what is termed the bystander effect, suicide genes can be optimized to only require ten percent transduction in a solid tumor greatly increasing its efficiency (Xiong et al., 2012).

**Apoptosis-inducing genes** One of the problems involved in treating solid tumors by conventional therapies is that cancerous cells are often resistant to apoptosis and will not die with chemotherapy or radiation therapy alone. The gene that codes for the anti-tumor protein p53 is either mutated or deleted in more than 50 percent of human tumors (Hollstein et al., 1991). Inserting a copy of wild type p53 into cancerous cells has been shown to induce apoptosis and make the cells more susceptible to chemotherapy and radiation therapy.

### Anti-angiogenesis

Tumors, just like any tissue, require a constant supply of nutrients,

oxygen, hormones and growth factors for their growth and progression. This is provided by the formation of new blood vessels or angiogenesis. Therefore, inhibition of angiogenesis would stunt tumor growth. Patients with glioblastoma multiforme, a highly vascularized form of brain cancer that does not respond well to conventional treatments, would benefit greatly from an anti-angiogenic treatment. Even poorly vascularized tumors can be reduced by anti-angiogenic treatment (Beecken et al., 2001). Clinical trials involving systemic administration of angiogenic inhibitors such as angiostatin and endostatin showed no dose-limiting toxicity. However, they did not show marked signs of tumor regression due to the continuous release of pro-angiogenic factors released by the tumor (Ohlfest et al., 2005, Shepherd & Sridhar, 2003). Therefore, the delivery of angiogenic inhibitors by gene transfer is favored over systemic administration.

### Gene Transfer Techniques

The success of gene therapy lies in the efficient delivery of the gene of interest to the target cell. The therapeutic gene is cloned into a vector together with appropriate regulatory regions (promoters/enhancers) as well as any supporting proteins needed. Selecting the right vector is a crucial part of gene therapy. The ideal vector will protect and easily deliver the genetic information across the cell membrane and into the nucleus. It should have the ability to regulate the expression of the gene of interest and be able to successfully target specific cells to minimize toxicity. It should be easy and inexpensive to produce in large quantities. Once the gene is cloned into the vector it can be introduced to the target cell. The gene can be delivered in vitro or in vivo. In in vitro transfer, cells from a specific tissue are removed from the patient and exposed to the gene-carrying vector. The transformed cells are selected by biomarkers and are reintroduced into the patient's body. In in vivo transfer, the vector is injected into the patient's body directly, usually into the target tissue or the tumor if a tumor is the target.

### Viral Vectors

Currently, the most effective method of gene transfer is through viral vectors. Viruses have evolved over the years to enter the cell and efficiently hijack the cell's machinery to produce its own viral proteins. An ideal viral vector uses the viral infection pathway but avoids the expression of the viral genes that facilitate replication and the subsequent host cell death. This is achieved by deleting most of the viral genome, leaving intact the sequences (usually the long terminal repeats) required for capsid packaging and integration of the vector DNA into the host's chromatin. The most commonly used viruses for gene therapy are retrovirus, lentivirus and adenovirus.

Retroviruses carry their genetic material as RNA and integrate their genome into host DNA using the viral enzymes reverse

transcriptase and integrase. The integrated DNA acts as a provirus which replicates to make multiple copies of the virus and is released outside the cell. Therapeutic retroviral vectors are made replication-deficient by replacing the structural genes with the therapeutic gene. Since retroviruses only gain access to the host's DNA when the nuclear membrane is broken down, they only infect actively dividing cells and integrate the gene of interest into the target cell.

Lentiviruses, a subclass of retroviruses, have recently been adapted to be used as gene transmission vectors. Lentivirus vectors can naturally enter an intact nuclear membrane and integrate their genome into non-dividing cells, which a retrovirus vector cannot do. HIV-1 is the most common lentivirus used in gene transfer. Because of its dangerous nature, lentivirus vectors do not carry the gene required for replication. As an added precaution, self-inactivating (SIN) lentivirus vectors are being developed which contain deletions in the downstream LTR.

Adenoviruses carry their genetic material as double stranded DNA, but as opposed to the other viruses mentioned, the DNA is not integrated into the host's genome. However, adenovirus can successfully infect broad range of cell type and is not limited to dividing cells. In 2003 the first gene therapy to be approved for commercial production was a recombinant adenovirus-p53 gene therapy for head and neck squamous cell carcinoma approved by China's State Food and Drug Administration and is sold under the name Gendicine (Pearson et al., 2004).

### Limitations of Viral Vectors

The obvious concern regarding the use of viral vectors is the possibility of the virus eliciting a strong immune response. In 1999, an 18-year-old male died due to an extreme immune response triggered by the administration of an adenoviral vector. He was participating in a phase I clinical trial to determine the safety of a gene therapy for ornithine transcarbamylase deficiency, an X-linked genetic disease of the liver (Raper et al., 2003). Since then most work with adenovirus vectors used genetically crippled constructs which would minimize the likelihood of an immune response.

The integration of the therapeutic gene when using retroviral or lentiviral vectors carries a possibility of oncogene activation. Since the point of insertion is mostly random, the transgene can insert into the upstream regulatory region of an existing gene and activate or upregulate the existing gene due to the proximity to the promoters in the downstream LTR of the transgene. The transgene can also insert itself into a transcriptional unit of an existing gene causing a loss of function in that gene. This has been shown to be a real threat when four patients in a clinical trial that successfully treated X-linked severe combined immunodeficiency

disease developed a form of leukemia. The cause in at least two of the patients appeared to be the integration of the therapeutic murine leukemia virus retroviral vector close to the LMO2 oncogene (Kohn et al., 2003).

Adenovirus vectors, since they do not integrate the gene into the host's genome, carry no risk of insertional mutagenesis. However, since adenovirus vectors do not integrate the gene into the cell's DNA they are only expressed transiently and require multiple administrations to be effective and repeated delivery may compromise efficacy and might induce a severe immune response (Hartman et al., 2008).

While self-inactivating (SIN) retroviral vectors may reduce the risk of insertional mutagenesis (Ellis, 2005), the clinical use of retroviral vectors is curtailed due to the limited packaging capacity of viral vectors. Most retroviral vectors can carry a transgene of up to 8 kb, as larger genes would compromise the efficiency of viral reverse transcription (Thomas et al., 2003). This excludes the transport of multiple or large transgenes. Finally, the high costs involved in the manufacture of clinical-grade retroviral vector and regulatory issues keep viral vectors from widespread translation into clinical practice.

### Non-viral Methods

The simplest method of gene delivery is injecting naked DNA directly into the target tissue. Since naked DNA lacks any mode of transport through the cell membrane, there have been various methods developed to increase the efficiency of cellular uptake.

In smaller animals, hydrodynamic injection can overcome the low efficiency of cellular uptake. This procedure involves the injection of a large volume, about 10% the weight of the mouse, of DNA/saline solution through the tail vein in less than 10 seconds, with most of the protein being expressed and accumulating in the liver (Liu et al., 1999). In larger animals, the volumes required for hydrodynamic injection become prohibitive. This limitation can be overcome by isolating an organ's blood flow using catheters or part of a limb using external tourniquets.

The efficiency of delivery can be enhanced by physical methods such as microinjection directly into the cell, electroporation, sonoporation and the use of microparticle in gene guns and magnetofection. Most of these methods are only feasible in *in vitro* gene delivery. Electroporation has been used successfully in gene delivery to mice skeletal muscle *in vivo* (Miyazaki et al., 2002), but the larger energy required to increase the permeability of the cell membrane across a human limb risks destroying too many cells. Sonoporation is limited to acoustically accessible organs. Although these methods are efficient, they require expensive specialized equipment reducing their benefit over viral vectors.



The efficiency of delivery can also be increased by chemical methods. The negatively charged phosphate on DNA can bind to a variety of cationic polymers to form a DNA-polymer complex called a polyplex. The polyplex interacts with the cell membrane and is absorbed into the cell by phagocytosis where the polyplex is released and the DNA can migrate into the nucleus. Polyethylenimine (PEI) is one of the most commonly used non-viral vectors based on polycations for DNA delivery both in vitro and in vivo. PEI is a polymer with repeating units composed of an amine group and two carbon spacers. PEI can be linear, branched or as a highly branched dendrimer, though linear PEI is more commonly used. The exact mechanism by which the DNA-PEI polyplex escapes the endosome is unknown but is thought to be a result of the increased influx of protons, chloride ions, and water during endosome acidification causing it to rupture from the high osmotic pressure. Using confocal laser scanning microscopy, Merdan et al. (2002) observed the dispersal of the genetic cargo soon after the rupture of the endosomes on living cells.

Cationic lipids are also used to condense the DNA into a liposome. These liposomes can protect the DNA from damage during transport, something that the other non-viral delivery methods fail to do. Suzuki et al. (2010) successfully transfected murine ovarian tumors in vivo with IL-12 using self-prepared bubble liposomes combined with ultrasound applied to the tumor. The bubble liposome protocol was more effective than using the more expensive commercially available transfection agent, Lipofectamine 2000. However, the Lipofectamine control in the study was not conducted together with ultrasound.

Non-viral methods present many advantages over viral vectors. Most non-viral methods are simpler and cheaper to produce and elicit a smaller host immune response. The transfer capacity of non-viral methods is functionally unlimited compared to viral capacity. Low levels of transfection used to be the limiting disadvantage, but advances have resulted in methods with transfection rates that are clinically viable and can compete with viral vectors. However, the efficient delivery of DNA to the nucleus is not enough for long-term transgene expression if it cannot integrate into the host genome. Transposable elements (transposons) could potentially offer such an alternative.

### Transposon Systems for Gene Therapy

Transposons or transposable elements (TE) are mobile regions of DNA that can change their position in the genome. DNA transposons rely on a transposase enzyme to cut the TE from the donor site and insert it at the receiving site in a 'cut-and-paste' like manner. The transposase gene is the trans-acting element of the transposon system. The cis-acting element is a pair of inverted repeats at either end of the gene which are the target sites for the transposase as well as associated enhancers and

promoters. Most regions coding for transposase also contain the cis-acting elements and are considered autonomous TEs. While transposons seem to have played a large role in evolution with close to fifty percent of the human genome made up of TEs, most species have accumulated mutations in the transposase genes.

One family of transposons, the mariner/tc1-like superfamily, has members in a wide range of species including nematodes, insects, fish, and humans with analogs reported in prokaryotes. This is a result of horizontal gene transfer between species. Unfortunately, not a single autonomous element has been isolated from vertebrates. Ivics et al. (1997) reconstructed the first man-made transposon system called the Sleeping Beauty (SB) transposon system based on the consensus sequence of members of the mariner/tc1 family isolated from fish. The reconstructed SB transposase was successfully able to transfer elements not only in fish but also in other species including human cells. The use of a SB transposon based system of gene transfer was proposed in 2000 when successful integration and long term expression using SB was shown in mice (Yant et al., 2000).

Because transposons have to survive along with the host cell, traits that posed less of a threat to the cell were selected evolutionarily. A transposase with a high activity increases the chance of insertional mutagenesis. Therefore, early iterations of the SB transposase had lower activity that could not compete with the fast acting retroviral enzymes. It was known early on that exchanging certain amino acids in SB transposase could increase its efficiency, but guessing the right combination of changes needed to construct a hyperactive transposase would be near impossible. Mates et al. (2009) conducted a large scale genetic screen of SB mutants and yielded a transposase (SB100X) that was ~100 times more efficient than first generation SB transposases. This hyperactive transposase allows the SB transposon system to compete with viral vectors and was awarded the title of "Molecule of the Year, 2009."

Since SB transposon based vectors integrate the transgene into the host's DNA, just as retroviral and lentiviral vectors do, they also pose the risk of insertional mutagenesis. Statistical analysis of the integration sites of both viral integrase and SB transposase suggests the SB vectors are safer. SB transposons integrate at TA dinucleotides and shows a preference ( $p < 0.01$ ) for a short TA palindromic consensus sequence. This preference can be explained by the greater bendability at TA sites that is possibly required to allow the transposase access, but with ~108 TA sites in the human genome this can still be considered random on a genomic level. A study mapping over 1,300 SB integration sites found 39 percent ( $p = 0.02$ ) integrated into known genes with a weak preference for regions slightly upstream or within 5kb of the start site (Yant et al., 2005). These results were compared to

10,000 computer-simulated insertions that showed truly random insertions would only result in 33 percent of genes. It is hard to avoid any preference for transcriptionally active genes since those are accessible due to chromatin modification such as CpG islands. Even though SB transposon shows a statistically significant preference for genes, the percentages are low compared to viral vector insertions. Schröder et al. (2002) reported 69 percent ( $p < 0.0001$ ) of in vivo integration sites using HIV-1 vectors were in known genes. Adding insulator sequences to the ends of transposable element can further reduce the risk of unwanted activation of neighboring genes (Walisko et al., 2007).

Long term expression of the transposase gene can result in the transposon being excised and reinserted in a different location in what is known as “re-hopping”, increasing the risk of insertional mutagenesis. To limit the amount of time in which transposase will be produced the transposase gene can be transported on a second plasmid that will be transcribed ectopically and destroyed. The use of mRNA as a source of transposase is also being explored (Wilber et al., 2006).

The greatest weakness of the SB transposon system is its inability to infect cells. Any of the non-viral delivery methods mentioned above can be used to transport the SB-containing plasmid. There is also the possibility of using hybrid vectors that transport the transposon packaged into virions. These hybrid vectors may prove more effective than either method alone. The integration sites of an HIV-1/SB hybrid was shown to be closer to truly random integration profile with only 30 percent inserting into known genes (Staunstrup et al., 2009), and a herpes simplex virus/SB hybrid has successfully increased the capacity of the SB transposon (de Silva et al., 2009). SB transposon have also been successfully delivered to target tissue using modified liposomes that used Asialoglycoprotein receptor (ASGPR)-mediated endocytosis to target hepatocytes in vivo (Wang et al., 2009) and hybrid vectors may employ similar targeting techniques being researched for viral vectors (Waehler et al., 2007).

Since transposons simply ‘cut-and-paste’, they do not have to rely on reverse transcriptase which has a tendency to incorporate mutations. The transposase can possibly be modified in the future to target a specific region of DNA using a synthetic zinc finger domain (Yant et al., 2007). SB transposons are easier and cheaper to manufacture than replication-incompetent viral vectors, allowing researchers without the resources required to produce viral vectors to continue exploring transposon based systems.

## Conclusion

Non-viral vectors with the ability to integrate the transgene are far more efficient than viral vectors or non-integrating DNA plasmids. The increase safety risks associated with viral vectors

and resultant bureaucracy has limited the widespread use of commercially licensed gene therapy treatments. There are only two approved gene therapies worldwide in over 25 years of clinical trials, mostly due to safety and toxicity concern and not effectiveness. There is a need for a safer delivery system and the SB transposon system may be it. Most of its early limitations have been resolved to the point that it can compete with viral vectors. SB-mediated gene transfer has been shown to be effective in a variety of treatment strategies including anti-angiogenesis (Ohlfest et al., 2005), suicide therapy (Song et al., 2009), and others.

The first human clinical trial using SB transposons is under way. The trial will attempt to transfect T cells ex vivo with engineered receptors against the CD19 antigen as described above (Williams, 2008). Regardless of what the results of this trial will be in terms of efficacy, the trial will prove the safety of this method and provide the data necessary for further improvement in future trials.

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# Understanding the Hygiene Hypothesis and its Mechanisms

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## Abstract

The hygiene hypothesis provides an explanation for the sharp increase in atopy over the past several decades by proposing that it is inversely related to the lack of infectious diseases in our society. Although atopy, as well as other hypersensitivity reactions, do have a genetic basis; studies clearly show that environmental and socioeconomic factors play a large role in determining which people will develop allergies. Proposed mechanisms leading to this disorder include an imbalance in the Th1/Th2 complex in the immune system, and a deficiency in Regulatory T cells which controls excessive T cell activity.

## Introduction

The immune system is a continuously adapting organ system with many defense mechanisms in place to prevent infection. Thousands of microbes are constantly trying to find a way to infiltrate and take residence in the body and the components of the immune system together find a way to prevent infection from penetrating, or killing it once it does pierce the initial barrier.

Throughout history, the persistence of different infections has constantly evolved to reflect our continually changing ecosystem. As we learn to adapt to a specific disease, a new one will fill the void that was left. *Yersinia pestis*, the bacteria responsible for killing hundreds of millions of people throughout the 14th century is now almost completely eradicated (Haensch, et. al., 2010).

Many methods have been discovered which successfully wiped out several illnesses plaguing the globe for many years. Vaccinations, healthier diets, and personal hygiene have all had a positive effect in reducing the number of bacterial and viral infections throughout the past century. Mumps, measles, polio, and diphtheria are all examples of sicknesses that have practically been eliminated in most developed countries resulting in a healthier environment.

Despite the decreased incidence of microbial infections, many other disorders have quickly filled the void left by these infections. Crohn's disease, type I diabetes, and allergies have seen exponential growth over the past seventy years, indicating an increase in hypersensitivity reactions. This paper explores the recent increase in hypersensitivity reactions, specifically atopy, to see if it is a direct result of the decreased number of microbial infections.

## Materials and Methods

Materials for this paper were mined using Touro College's library to access databases such as Proquest, Pubmed, and Ebsco. Additionally, some research was done using Google Scholar as well.

## Discussion

The number of cases of autoimmune disorders skyrocketed over the past half century. Asthma, diabetes, and Crohn's disease are all examples of hypersensitivity disorders which have increased

exponentially in recent years while bacterial infections have steadily decreased.

Bacterial infections and autoimmune disorders are believed to be inversely related. Evidence for this theory first appeared in 1989, in a paper written by David Strachan who conducted a study showing the relationship between the number of children born and the likelihood of receiving hay fever. Strachan found that children born into large families were less likely to contract the disease. He suggested the reason was that children born into large families were more susceptible to bacterial infections being transmitted by older siblings, preventing them from contracting allergies (Strachan, 1989). He did not provide an explanation for this correlation.

Strachan's hypothesis opened a door for many others who followed in his footsteps trying to explain the relationship between autoimmune disorders and bacterial infections. This theory later became known as the "hygiene hypothesis," and scientists today are still trying to find the mechanism which causes this correlation.

In order for scientists to properly study the mechanisms of infections leading to these disorders, it is important to understand what causes autoimmunity. Is a person's genetic makeup the primary factor causing the disease, or do socioeconomic factors determine if a person will develop the disorder?

## Genetic Factors

It seems that there definitely are genetic factors that contribute to an individual's susceptibility to contract the diseases. Many human autoimmune diseases have a tendency for familial clustering. Familial clustering, represented by  $\lambda_s$  in Table I (Vyse & Todd, 1996), gives the ratio of clustering in families relative to the rest of the world. A  $\lambda_s$  of 15 means that a sibling is at 15 times greater risk of developing the disease than a member of the general population (Vyse & Todd, 1996). All of the autoimmune diseases listed contain a high rate of clustering, indicating that genes do play a role in autoimmune disorders. For type I diabetes, the MHC linked locus IDDM1 was the primary allele affecting the disorder, although other alleles played a smaller role as well.



Crohn's disease, another autoimmune disease affecting different areas of the gastrointestinal tract, is the result of many single nucleotide polymorphism (SNPs) throughout a person's genome (Mao & Jeonghwa, 2008).

Such evidence would disprove Strachan's hypothesis. If autoimmunity is solely a genetic disorder, it is impossible that environmental factors would be able to change an individual's chances of getting infected.

However, there are numerous studies that counter the notion that autoimmunity is affected exclusively through genetics. Firstly, evidence from observing distinct groups of individuals as they migrated from one region to another shows that the rate of developing diabetes is directly related to the location. A study regarding Jews emigrating from Yemen to Israel showed that the number of cases of type I diabetes sharply increased with the duration of their stay in Israel. In fact, the numbers were higher than any other ethnic group in Israel (Airaghi & Tedeschi, 2006).

Additionally, many studies have indicated that autoimmune disorders are not evenly distributed amongst the continents. Geographic data concerning Crohn's disease, multiple sclerosis, and type I diabetes demonstrates that a north-south gradient exists (Bach, 2002). In the northern hemisphere, the incidence of autoimmune disease decreases towards the equator. One example of this is seen with the incidence of Crohn's disease in the US which shows a clear geographic pattern of occurrence. Data collected from hospital discharges suggests that Crohn's disease clearly occurs more often in northern parts of the country (Sonnenberg, et. al., 1991). Data exists showing similar results regarding type I diabetes, multiple sclerosis, and atopy as well (Bach, 2002). Genetic factors alone would not be enough to explain all of these phenomena.

### Socioeconomic Status

Many studies have indicated that environmental factors, as well as socioeconomic status play an even greater role in determining one's chances in developing autoimmune disorders (du Prel, et. al., 2007). We already stated that autoimmunity is not evenly distributed among the continents. Developed countries usually have a higher rate of autoimmunity than developing ones (Corvalan, et. al., 2005). Even in developed countries, there are several factors that will determine an individual's chances of developing such a disorder. Socioeconomic factors can include a number of different variables. A study regarding type I diabetes in Germany went into greater detail to determine which factors will have a greater influence. The results indicated that some aspects have a greater effect than others. Income level, education, and other similar trends had a small effect while living space per person was a much larger influence in determining a child's risk of developing

type I diabetes (du Prel, et. al., 2007). This study would support Strachan's theory that infections play a direct role on autoimmunity. Less living space would explain a child's susceptibility to get an infection which would inhibit his chances of developing type I diabetes. A similar study was done in Chile pertaining to asthma. The results supported the belief that overcrowding was associated with less asthma symptoms (Corvalan, et. al., 2005).

The general consensus is that although there are genetic variables in an individual that will determine if he is prone to develop an autoimmune disorder, these can be triggered based on socioeconomic conditions. A child raised in an environment that was exposed to infections was more prone to develop infectious diseases which in turn provided a sense of protection from developing autoimmune disorders.

### Hypersensitivity

Although asthma and allergy are both subsets of hypersensitivity reactions, they are caused by very different mechanisms. Allergy is an immediate hypersensitivity reaction (Type I Hypersensitivity) caused by activation of Th2 cells (a subset of T cells) which release cytokines to produce IgE antibody. When atopic individuals encounter certain foods or pollen, they produce a dominant Th2 response which stimulates unchecked IgE production leading to an allergic response.

Autoimmunity is a delayed type (or Type 2) hypersensitivity reaction resulting from the immune system acting on self tissue. Although the direct cause for this disorder is still unknown, it is believed Th1 activity, normally responsible for activating this pathway, may be involved in causing this disorder (Lohoff, et. al., 1998).

Most of this paper focuses on allergy, because this is where most of the research on the hygiene hypothesis occurred, though some of the information may shed light on autoimmunity as well.

A simple explanation regarding why less infections are correlated with a higher rate of autoimmune disorders is that the more infections an individual undergoes in his lifetime causes a certain amount of pressure on the immune system, which has to fight off the antigen. Fewer infections means a delayed pressure on the immune system, which is free to mount inappropriate responses against self antigens as occurs in type I diabetes (Airaghi & Tedeschi, 2006).

This explanation would not fit into any of the mechanisms previously explained in triggering autoimmunity. Just because the immune system is not pressured to fight off antigens, this doesn't explain why it would fight self antigens. The hypothesis would be consistent if the immune system was weakened against foreign microbes but not for an entirely new disorder. Nothing in this

theory explains the inverse relationship between bacterial infections and autoimmunity.

### Th1/Th2 balance

The immune system consists of T cells and B cells. T cells can be either CD8+, cytotoxic, which are responsible for killing viruses, or CD4+ helper T cells which assist in other immune functions. The CD4+ T cells can be either a subset of Th1 or Th2. Each subset helps the adaptive immune system in its own way.

Th1 cells are primarily instrumental in killing intracellular infections. Neutrophils are the immediate response to the body's defense against an attack. However, under some circumstances, these neutrophils are not successful in phagocytosis of the pathogen. Either the pathogen has evolved to evade the neutrophil, or the neutrophil has not been activated properly. These situations require help from Th1 activated T cells. These T cells recruit macrophages to kill the microbe through ROS (Reactive Oxygen Species). Although this is an effective way of destroying a pathogen, there are certain drawbacks. One issue is that the agent that kills the microbes may harm healthy tissue as well. Macrophages do not discriminate which species they attack so tissue damage is usually accompanied with hypersensitivity to an infection. One way of inhibiting this response is through Th2 activation.

The Th2 subset of T cells has an entirely different role than Th1. While Th1 is effective in activating macrophages, Th2 cells are primarily responsible for producing cytokines that signal antibodies such as IgE and activate eosinophils, which are responsible for killing Helminthic parasites (Abbas & Lichtman, 2009).

Th2 cells also secrete cytokines that activate the alternative macrophage pathway, which inhibits the classical macrophage pathway. When Th1 cells activate macrophages to kill a pathogen, they kill host tissue as well. Th2 cells are responsible for inhibiting this pathway, as well as producing cytokines to express mannose receptors to aid in tissue repair to the host.

A proper immune response to an infection requires Th1 cells to activate macrophages to fend off the infection and Th2 cells to slow down this response. Different organisms have different amounts of Th1 and Th2 which will directly affect how they respond to infections (Cohn, et. al., 2004).

An overabundance in Th2 response is what causes an allergic reaction. When a person is exposed to certain pathogens, namely Helminth parasites, Th2 cells secrete cytokines to bring IgE and mast cells to the area to kill the infection. Atopic individuals mount an especially strong immune response to these antigens causing an allergic response, known as type I hypersensitivity.

Th1 and Th2 develop from a common precursor termed Thp. Development into mature Th cell depends on the conditions present during maturation. Several cytokines such as IL2 and TGF- $\beta$  have a tendency to cause Th2 proliferation. However in the presence of a normal amount of antigen in the host, antigen presenting cells produced IL-12 which caused Th1 proliferation (Lohoff, et. al., 1998).

Thus, a healthy balance of Th1 and Th2 is necessary for a proper immune response. Th1 is necessary to fend off pathogenic microbes at the site of infection while a proper Th2 response is necessary to prevent too much tissue from getting affected by the macrophages. Different amount of Th1 and Th2 in the body determines the outcome of an infection. An experiment with different strains of mice with different amounts of Th1 or Th2 helps determine the outcome of an infection. *Leishmania major*, an infection normally killed by activated macrophages, was placed in a strain of mice that had effective Th1 cells. The infection was successfully eradicated. However, when placed in a strain of mice that has a dominant Th2 presence, the mice succumbed to the infection (Elso, et. al., 2004). *Mycobacterium leprae* as well, if dominated by a Th2 response will lead to a much more severe form of leprosy.

This imbalance of Th1/Th2 might explain why less infection may cause more allergic hypersensitivity. Developed countries are certainly more hygienic and individuals are less prone to develop infections. The body has less of a need for Th1 cells and may suppress Th1 production. Once an imbalance is formed, Th2 secretes cytokines which further inhibits Th1 and cause an overabundance of Th2 production (Holt, 2000).

### Regulatory T cells

In healthy individuals, regulatory T cells (Tregs) are responsible in suppressing dominant T cell activity. In response to infection, T cells often cause heavy responses that can cause serious harm to the individual if left unchecked. Activation of these Tregs, such as CD4+CD25+, were shown to suppress the Th2 response leading to a decrease of atopic responses in individuals exposed to pollen. Studies show that individuals who experience atopic symptoms do not express sufficient amounts of CD4+CD25+ (Ling, et. al., 2004). Additionally, lack of Tregs are also responsible for several disorders such as type I diabetes, multiple sclerosis, and inflammatory bowel disease.

Tregs utilize different mechanisms of control in response to different infections. In response to airway hypersensitivity reaction (AHR), Tregs secrete IL-10 along with TGF- $\beta$  which controls the Th2 response. In NOD mice, evidence showed that Tregs were instrumental in preventing type I diabetes through activation of CTLA-4 (a cell surface molecule) together with TGF- $\beta$  (Workman, et. al., 2009).

Alternatively, there are a number of factors that lead to healthy Treg development. FoxP3 is the transcription factor that develops immature T cells into regulatory cells. A mutation in this may lead to several autoimmune diseases such as diabetes, anemia, and eczema. Another cell necessary for healthy Treg development is IL-2. IL-2 is not secreted by Tregs but it is necessary for Treg survival. IL-2 is secreted by activated T cells and a healthy balance of it is needed to maintain homeostasis. Effector T cells compete with Tregs for IL-2 so Tregs can upregulate CD25 production to suppress effector T cells, providing a healthy feedback mechanism between responder T cells and Tregs (Goleva, et. al., 2005).

The mechanism leading to this deficiency in Tregs is unclear. One theory suggests that the effector T cells in individuals with asthma may be toxic towards Tregs in these individuals (Thorburn & Hansbro, 2010).

Another theory is that Tregs may actually be induced by pathogenic infections as indicated in Table 2 (Thorburn & Hansbro, 2010). Antigen presentation by the lungs was shown to promote TGF- $\beta$  dependent induction of Foxp3 expression. Lack of infection may lead to an uncontrolled immune response. Several infections are known to have a direct link to regulatory cells so by inhibiting these cells; it would lead to an increase in allergic response.

One particular study tried to find if there is a direct correlation between asthmatic infection and *H. pylori*- a common bacteria found in the gut. Experimentally induced asthma in mice had fewer symptoms when the mice were previously infected with *H. pylori*. The study also showed that these mice had significantly higher numbers of Tregs leading to the belief that Tregs is one of the underlying mechanisms controlling an asthmatic response (Arnold, et. al., 2011).

## Microbiota Theory

Although this study definitely supports the theory that Tregs are one of the most influential factors in developing asthma, it also supports a different belief entirely in what causes the hygiene hypothesis.

In an essay concerning the diminishing numbers of microbiota in the body, Blaser and Falkow postulated that the main cause in the increased numbers of autoimmune disorders is related to the number of microbiota in body, rather than the number of pathogenic infections a person encounters throughout life. For the past several centuries, humans have adapted to allow a diverse subset of microbial organisms to live inside the mucosal surfaces of the intestines and lungs, forming a symbiotic relationship between host and microbe. Humans provide a home for these microbes while they are helpful in digestion and metabolism. A

change in human behavior may have a direct effect on how people develop certain microbiota as shown in Table 3. The changes in the conditions listed have had a direct effect on many of the known microbes that reside in the human body. Although reduced colonization of *H. pylori* from the gastrointestinal tract is beneficial in the sense that reduces susceptibility to gastric cancer, it is also shown to contribute to many metabolic disorder as well as childhood asthma. Blaser and Falkow (2009) maintain that the recent epidemic of autoimmune disorders is not due to the lack of pathogenic infection as much as it is due to the human microbiome as a whole (Blaser & Falkow, 2009). Other research supports this premise by saying the hygiene hypothesis may not a product of less exposure to pathogenic infections itself, as much as it is caused by lack of exposure to all microorganisms (Bloomfield, et. al., 2006)

## Conclusion

Despite all the evidence supporting the hygiene hypothesis, there still is a fierce debate whether less hygiene will in fact cause a downward trend in autoimmune diseases. The direct mechanism remains elusive, and without definitive evidence demonstrating what causes the rise in allergic diseases, the microbial exposure hypothesis may be just as plausible in explaining the rise in atopy. And considering the many benefits our modern ecosystem provides for us on a daily basis, the pros of less microbial exposure far outweigh the cons. Clean drinking water and Cesarean sections are just a few examples that have saved many lives over the past few decades. So although decreasing the number of allergy cases is a priority, the current evidence should not support exposing oneself to additional microorganism than those already prevalent in society.

**Table 3**

Change	Consequence
Clean water	Reduced faecal transmission
Increase in Caesarean section	Reduced vaginal transmission
Increase use of pre-term antibiotics	Reduced vaginal transmission
Reduced breastfeeding	Reduced cutaneous transmission and a changed immunological environment
Smaller family size	Reduced early life transmission
Widespread antibiotic use	Selection for a changing composition
Increased bathing, showering and use of antibacterial soaps	Selection for a changing composition
Increased use of mercury-amalgam dental filling	Selection for a changing composition

*Changes in human ecology that might affect microbiota composition*

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# Creutzfeldt - Jakob Disease and Alzheimer's Disease: Does Overlap of Mechanism Mean Overlap of Treatment Methods?

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## Abstract

Alzheimer's disease is a neurodegenerative disorder that is the 6th leading cause of death in the United States. More than 5.5 million People over the age of 65 are currently diagnosed with Alzheimer's disease with predictions of 13.8 million to be diagnosed by the year 2050 (Sultana, et al., 2013) (Hebert, Weuve, Scherr, & Evans, 2013). With few treatments available, scientists are desperately looking for a solution to this growing epidemic. Creutzfeldt-Jakob disease is also a neurodegenerative disorder, but with a far less prevalence of only 4.6 persons per million per year. It was discovered that Alzheimer's and Creutzfeldt-Jakob disease share many pathophysiological mechanisms with each other. Being that both of these illnesses are currently incurable, a thorough critical analysis of mechanisms and potential treatments were performed to ascertain if knowledge in one disorder can help find a cure for the other. With the strong relationship between these two disorders, it was found that many treatments intended for one illness had positive results for the other (some with slight modifications). The discovery of this correlation improved scientist's knowledge of the pathological mechanism of these ailments along with finding new and creative ways for treatment. Experiments geared towards the relationship between Alzheimer's and Creutzfeldt-Jakob disease has brought researchers closer to finding a cure for several neurodegenerative disorders.

## Introduction

Alzheimer's Disease (AD) and Creutzfeldt-Jacob Disease (CJD) are two disorders characterized by their neurodegenerative symptoms. These ailments are both considered forms of dementia consisting of phenotypes portraying a patient's loss of memory, mood irregularities, paranoia, and several other neurological symptoms. Physiologically, AD is characterized by its Amyloid- $\beta$  and Tau protein plaques while CJD is characterized by its prevalence of misfolded prion proteins throughout the brain. Albeit these two diseases are believed to have different modes of physiological symptoms, they share many similarities with each other and other neurodegenerative disorders causing many scientists to theorize a possible correlation between their mechanisms. It is partly due to these similarities that physicians often misdiagnose various dementia relating disorders as either CJD or AD. In a study of 304 autopsies that showed no prion disease prevalence, 71 (23%) of the patients had Sporadic CJD (a variant form of CJD characterized by unknown cause) as a possible diagnosis in their medical records (Chitravas, et al., 2011). Another study showed that "between 12% and 23% of patients diagnosed with AD do not have sufficient AD pathology at autopsy to account for the presence of dementia ("misdiagnosed") (Gaugler, et al., 2013)." Due to the vast likeness between many dementias, there has not been a definitive noninvasive procedure to precisely diagnose disorders such as CJD and AD (Mayo Clinic Staff, 2014).

Although AD and CJD share similarities, their prevalence in American society is far dissimilar. AD is the 6th leading cause of death with an estimated 5.5 million people over the age of 65 who are afflicted with this neurodegenerative disorder in the United States (Sultana, et al., 2013). CJD on the other hand, displays a prevalence of a mere 4.6 cases per million people over the age of 60 (Centers for Disease Control and Prevention, 2014). This is far fewer than the thousands of people who are diagnosed each year with AD.

Despite the rarity of CJD, numerous research endeavors have been conducted in understanding this prion disease. One explanation for this attention is due to the unique properties of a prion; a protein which acts as an infectious pathogen misfolding other proteins into additional prions (Wadsworth, et al., 2008). What is more surprising is that prions have been found in patients diagnosed with AD (Armstrong, et al., 2005). This would further suggest a possible correlation between pathogenic mechanisms of AD and CJD. Today various treatments for AD and CJD are currently in clinical trials to try to treat these incurable diseases. If there is a correlation between pathological mechanisms, further research into one of these neurodegenerative disorders can help scientists better understand and treat the other.

The purpose of this research article will be to define and explain the physical and physiological symptoms and mechanisms of AD and CJD, suggest possible correlations between the two, discuss current treatments, and lastly, the different forms of possible treatments will be compared to postulate a possible venue to determine if they would be effective to treat the other illness.

## Methods

Several online databases were used such as: Google Scholar, PubMed, and Jstor which were accessed through Touro College's online library. Additional references were gleaned from papers found in these databases

## Discussion

### CJD Pathophysiology

CJD is quite unique due to its pathophysiological mechanism. While most infectious diseases are viral or bacterial based, CJD uses a prion to spread its illness. In actuality, every individual contains Prion Proteins (PrPc) which are believed to be necessary for normal synaptic function (Collinge, et al., 1994). It is when this human-coded PrPc is post-translated and converted into



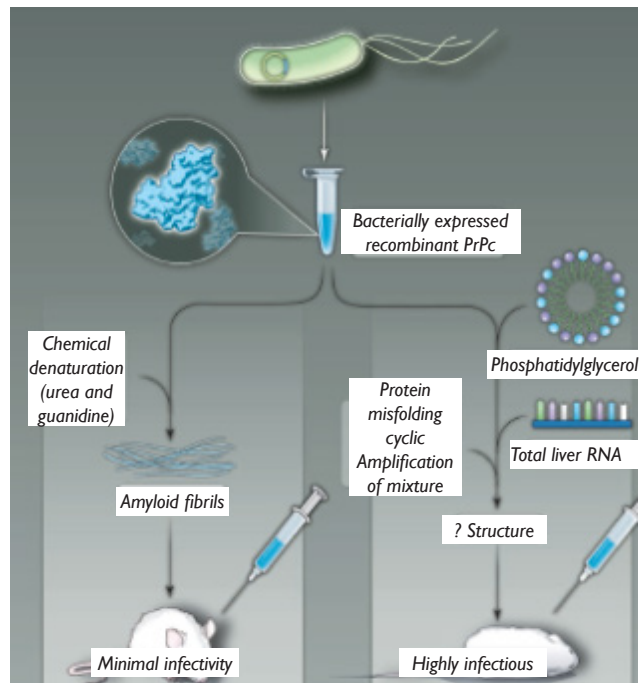
an abnormal isoform (PrP<sup>sc</sup>) that it starts to exhibit pathological tendencies. Data suggests that this abnormal protein is the main, if not the solitary, component of the transmissible disease (Wadsworth, et al., 2008). Once the PrP<sup>sc</sup> comes into contact with a normal PrP<sup>c</sup>, it causes a transmutation of residue 129 turning it into a pathological agent. This replaces the amino acid by residue 129 on the protein into either valine or methionine.

Whereas many scientists agree that the prion protein is the main constituent of CJD, there is debate over the validity of this claim. Manuelidis, et al (2007), argue that the abnormal PrP<sup>sc</sup> form of the prion protein is a result, not the cause, of CJD. They claim that a 25 nm virus is the cause of the abnormal prion isoform. They discredit the long accepted hypothesis, that prions are the sole pathological constituents of CJD, for several reasons. Studies show, "high PrP-expressing transgenic brains, as well as abnormal recombinant PrPs, have failed to show significant or reproducible infectivity". Additionally, the authors claim that after several decades of research, scientists found no "infectious conformation" of PrP<sup>sc</sup>. Furthermore, sheep with scrapie showed signs of these 25 nm viruses while uninfected controls showed no prevalence of these particles. These facts caused many researchers to share doubt on the prion-only hypothesis.

Another theory with tangible evidence suggests a different mode of pathological mechanism. Several studies suggest that non-proteinaceous cofactors are needed to facilitate the infectious properties of PrP<sup>sc</sup>, hypothesizing that prions work via multiple components in order to function (Deleault, et al., 2012). While there are studies that show that purified PrP<sup>c</sup>s which were folded chemically into amyloid fibrils showed little infectivity, a combination of PrP<sup>c</sup>, liver RNA, and synthetic 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) lipid molecules facilitated the production of authentic infectious prions. The contrasts between the infectivity of PrP<sup>c</sup> obtained from amyloid fibrils and from PrP<sup>c</sup> combined with lipids and RNA support this theory (see figure 1). However, further study should be conducted to determine whether the cofactors are required for pathology or are merely a catalyst for a slow progressive disorder (Supattapone, 2010). These experiments cast doubt on the viral hypothesis of prion disease. If viruses were the cause of prion pathology, why were scientists able to reproduce CJD symptoms with only PrP<sup>c</sup> and various cofactors?

There are several ways for an individual to contract CJD. Factors include ingestion of prion-infected tissue, contamination during a procedure (most commonly a contaminated tissue transplant or equipment), inheritance of a mutation in the PrP<sup>c</sup> gene (PRNP), or sporadically (no apparent cause). When tissue infected with abnormal prions is ingested, such as meat from a cow infected with Bovine Spongiform Encephalopathy (BSE, also known as Mad

**Figure 1**



*Extra ingredients: Two different biochemical protocols yield recombinant PrP<sup>c</sup> with different infectivity. (Left) Minimally infectious amyloid fibrils are formed by incubating recombinant PrP<sup>c</sup> with chemical denaturants. (Right) Mixing recombinant PrP<sup>c</sup> with phospholipid and RNA produces highly infectious prions. (Supattapone, 2010)*

Cow Disease), a variant form of CJD is contracted named variant CJD (vCJD). Contamination via a procedure causes a form of CJD termed iatrogenic CJD while a genetic mutation that is inherited is called Genetic CJD. CJD that is acquired sporadically is referred to as Sporadic CJD (sCJD) (Wadsworth, et al., 2008).

According to Wadsworth et al (2008), approximately 85% of cases with CJD occur as the sporadic variant (sCJD) while 15% are associated with a pathogenic mutation in the PRNP gene (Genetic CJD). Wadsworth further explains that the rare iatrogenic form of CJD occurs most frequently due to the transmission of prions via contaminated growth hormones from cadavers or by implementation of contaminated dura mater grafts. Additionally, it has been speculated that iatrogenic CJD can be transmitted via blood transfusions although it is extremely rare (Ricketts, et al., 1997). Prion disease prevalence has been tied to vCJD in the United Kingdom by the consumption of cattle with BSE. Another unique variant of CJD, named Kuru, is found in the Eastern Highlands of Papua New Guinea where members of the community would feast on the deceased relatives as a sign of respect and grief. From the community in Papua, scientist discovered that the incubation period of individuals infected with PrP<sup>sc</sup> could exceed 50 years (Wadsworth, et al., 2008).

## Creutzfeldt - Jakob and Alzheimer's Diseases: Overlap of Treatment Methods?

Individuals with CJD often exhibit phenotypical signs of dementia and various other neurodegenerative symptoms. The primary indication of CJD is the observation of sponge-like holes in a person's brain giving the appearance of a sponge; for this reason, many scientists refer to prion diseases as transmissible spongiform encephalopathies (TSEs). Additionally, patients have been known to portray feelings of anxiety, depression as well difficulties of cognition such as impaired thinking, memory loss, personality changes, insomnia and difficulty speaking. Blurred vision, difficulty swallowing and sudden jerky movements are also typical signs of CJD. As the progression of the illness takes its course, cognitive phenotypical symptoms worsen. A majority of individuals lapse into a coma and eventually die due to cardiac arrest, respiratory failure, or an infectious pathogen. The duration of duress is typically 7 months for classical CJD and 12-14 months for variant CJD; however, patients have been known to live up to 2 years after diagnosis (Mayo Clinic Staff, 2012).

Currently, the only way to verify the diagnosis of CJD is via an autopsy to confirm the presence of PrPsc plaques and to observe the formation of spongy-like holes and structures within the patient's brain; however, doctors can accurately identify this disease via several diagnostics. First, a physician might assess the patient to determine if he/she portrays any of the symptoms of CJD. Additionally, doctors may administer an Electroencephalogram (EEG) to measure the individual's brain's activity. People with CJD typically portray an abnormal pattern. Magnetic resonance imaging (MRI) may be used to produce a high resolution image of a person's brain in order to determine a signs of abnormality. Another known method of testing is a spinal tap where a physician withdraws a small amount of cerebral spinal fluid that surrounds an individual's brain and spinal cord. The presence of several proteins in the CSF may be an indication of illness (Mayo Clinic Staff, 2012). A simple blood test to detect trace amounts of PrPsc may be an additional venue to test for CJD, but further testing is needed to determine if this is a viable method of diagnosis (Castilla, Saa, & Soto, 2005). Furthermore, in a study conducted by Moda et al (2014), 13 out of 14 patients with vCJD showed signs of PrPsc in their urine samples suggesting a future possible diagnostic for vCJD.

### AD Pathophysiology

AD is a form of dementia characterized by the formation of Amyloid- $\beta$  protein ( $A\beta$ ) plaques or the hyperphosphorylation of Tau proteins along with neuron degradation in the brain. This is accompanied with numerous cognitive relating symptoms. While the symptoms of AD are well documented, there is much debate over the exact mechanism in which the disease carries out its pathology.

One suggested mechanism is through the formation of Amyloid- $\beta$

proteins which in turn form amyloid- $\beta$  plaques. The  $A\beta$  is formed from a far larger protein called the  $\beta$ -Amyloid Protein Precursor (APP). The cleavage to the  $A\beta$  form is catalyzed by the enzyme  $\beta$ -CITEAPP cleaving enzyme-1 (BACE1) (Small & Cappai, 2006). It is believed that when there is an overproduction of  $A\beta$ , either via a faulty  $A\beta$  production mechanism or a failure of the body's clearance of the protein, Amyloid plaques are formed (Verkhratsky, et al., 2014). These aggregations of  $A\beta$  are believed to be toxic and are the main cause of AD. The conclusion that  $A\beta$  is involved with the pathogenesis of AD is strongly supported by an experiment involving APP transgenic mice. These mice contained human APP and when induced with an amyloid disease, showed very similar pathophysiological symptoms as AD. However, within recent years, studies have shown that  $A\beta$  plaques are not the most toxic form of  $A\beta$ . Experiments have shown that a low molecular weight diffusible form (non-plaque forming) of  $A\beta$  is more toxic. This would explain why in APP transgenic mice, abnormal cognitive symptoms are seen before  $A\beta$  plaque formation (Small & Cappai, 2006).

Another well-known hypothesis to the pathological mechanism of AD is the formation of toxic hyperphosphorylated tau proteins. Normal or dephosphorylated microtubule-associated protein (MAP) tau aid in the assembly and stability of microtubule networks in neurons. If an abnormality in the MAP tau arises, there are two other MAPs (MAP1A/B and MAP2) that can compensate; however, when the dephosphorylated tau turns into the hyperphosphorylated form, it turns toxic. This pathological form of tau sequesters normal tau (MAP1A/B and MAP2) while inhibiting and disrupting microtubule structure. The neuron that has been afflicted with this pathogenic MAP tries to dispose of the toxic substance by synthesizing additional normal tau and by turning the hyperphosphorylated form into an inert polymer. Eventually, this afflicted neuron begins to degenerate at a progressively slow pace. Aggregation of this abnormally hyperphosphorylated tau has been linked to dementia and neurofibrillary degradation. This abnormal MAP tau appears to be the main contributor to tau pathologies, or tauopathies, for dephosphorylation returns the protein back to its normal functioning state. One possible reason for this abnormality may be due to a conformational change in the MAP tau. This causes it to be a better substrate to be phosphorylated or a worse substrate for dephosphorylation. In either case, these conformational changes appear to cause the pathology of AD (Iqbal, et al., 2005).

AD is typically defined via 3 diverse categories: early-onset AD (EOAD), late-onset AD (LOAD), or early-onset familial AD (EOFAD). With increasing age as a known risk factor, patients who contract the disorder past the age of 65 are said to have LOAD which resembles the majority of all AD cases. People who are diagnosed with AD before the age of 65 is said to have EOAD

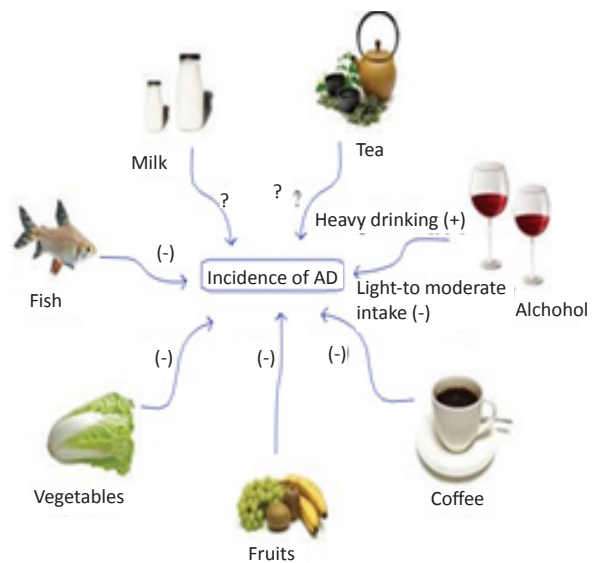
which encompasses up to 5 percent of all cases (WebMd, 2014). EOFAD is a form of the disorder where the onset of duress is under 65 years of age and where the individual has one or more of the genes associated with AD. These genes are believed to cause the person to attain a genetic disposition towards AD (Bird, 1999).

Genetic linkage studies have identified three different genes associated with EOFAD. The first gene identified was the APP gene (the gene that is responsible for APP production). It is believed that a missense mutation within this gene is a risk factor for EOFAD. Another gene found to be correlated with AD is PSEN1. Family studies have shown that mutations in this gene are pathogenic. The third gene found in junction with AD is PSEN2. Although it is far rarer than the PSEN1 mutation, it is believed that mutations in this strand of the gene are a precursor for pathogenesis of EOFAD. It is believed that the PSEN1 and PSEN2 genes are involved with  $\gamma$ -secretase, an enzyme involved with the cleavage of APP, and a mutation in these genes cause pathology via this pathway. The majority of EOFAD cases (81%) can be accounted for by a mutation in the PSEN1 gene, the second most (14%) by a mutation in the APP gene, and the least (6%) associated with the PSEN2 gene (Ertekin-Taner, 2007).

Similar to EOFAD, a gene has been found to be associated with LOAD. The Apolipoprotein (ApoE) gene codes for a protein that transports cholesterol in the bloodstream. It is found as different forms named ApoE  $\epsilon$ 2,  $\epsilon$ 3, and  $\epsilon$ 4. The ApoE  $\epsilon$ 3 form is the most common form of the gene while ApoE  $\epsilon$ 2 and  $\epsilon$ 4 are less prevalent. It is believed that ApoE  $\epsilon$ 2 reduces the risks of AD and ApoE  $\epsilon$ 4 has the contrasting effect of increasing AD pathogenesis. If an individual attains two sets of the ApoE  $\epsilon$ 4, it greatly increases his/her chances of AD. Unlike other known genetic mutations, inheriting both sets of the ApoE  $\epsilon$ 4 gene does not guarantee AD pathology (Alzheimer's Association, 2014).

While there is no definitive method of ascertaining if an individual will contract AD, there are several disease-modifying factors that decrease or risk factors which increase one's probabilities of pathology. Nam Hu et al (2013) characterizes a list of nutrients and their risks towards AD. Antioxidants obtained in an individual's diet were shown to reduce the risks of AD. Studies show that maintaining a healthy level of vitamin C in a person's diet can have a protective effect towards AD. In regards to metals, Iron is believed to be a risk factor for dementia while Zinc is believed to be a cause in the delay in AD pathology. In regards to fats and carbohydrates, while there is an assumption that these can pose as risk factors for AD, not enough reliable evidence is available to make a firm conclusion. Hu et al made a graph (figure 2) illustrating the effects of various everyday foods if whether or not they increase or decrease a person's risk towards AD.

Figure 2



*Foods and beverages that influence the incidence of AD. Fish, vegetables, fruits, coffee, and light-to-moderate alcohol intake are reported to reduce AD incidence. Milk and tea are reported to influence cognition, but their influence on AD is not clear. (Hu, et al., 2013)*

Various other theories about the pathophysiology of AD contribute to our understanding of the disease. One proposed hypothesis is the degradation of the blood brain barrier (BBB). The BBB protects the brain from various pathogens and unwanted particles within the bloodstream. The deterioration of the BBB can cause various proteins in the blood to enter the cerebral spinal fluid and form plaques (Sardi, et al., 2011). Another theory involves the metal aluminum. While the correlation is not entirely known, it is believed that a high level of aluminum in the brain is a cause for AD (Shcherbatykh, 2007). Further research suggests, as mentioned by Moulton and Yang (2012), that air pollution too plays a role in AD. While the pathogenesis of AD is multifactorial, air pollution can accelerate "age-related oxidative changes observed in the brain", increasing one's risk of contracting the disorder.

Analogous to CJD, patients who suffer from AD share many similar phenotypical symptoms. Patients have been known to exhibit emotional symptoms such as depression, anxiety, and mood swings and cognitive symptoms such as loss of memory, difficulty speaking, and difficulty thinking. Additionally, those who contract AD are known to have delusions, such as paranoia, change in sleeping patterns, and loss of inhibitions. Towards the late progression of the disorder, important skills such as reading, hobbies, and reminiscing are lost (Mayo Clinic Staff, 2014).

Currently, there is no specific diagnostic to confirm the presence of AD. A physician determines whether you meet the criteria of

## Creutzfeldt - Jakob and Alzheimer's Diseases: Overlap of Treatment Methods?

dementia and often they can determine if it is due to AD. Doctors have an arsenal of diagnostic tests that they can perform to assist in narrowing the diagnosis. One possible test is a neuropsychological test where they can identify cognitive changes. Different types of dementia can have different cognitive patterns. A blood test can be administered to rule out other causes of memory loss such as vitamin deficiencies. Frequently, brain imaging is used to identify any abnormalities within the brain. An MRI can be used to produce a high resolution image of the brain for further study. A computerized tomography (CT) scan uses x-rays and a computer to generate cross-sectional images of the brain. This can help rule out head injuries and tumors. A positron emission tomography (PET) scan is used in conjunction with an isotope tracer which is injected into an individual. With this machine, a doctor can track the movement of this tracer and determine which areas of the body are dysfunctional. All of these diagnostics aid in the identification of AD; however, similar to CJD, the only way to confirm illness is to perform an autopsy and to observe plaque buildup within the brain (Mayo Clinic Staff, 2014).

### Pathophysiological Comparison of AD and CJD

AD and CJD are diagnosed via their dissimilar pathological mechanism and symptoms. AD is associated with A $\beta$  plaques and Tau proteins and CJD with abnormal PrP<sup>sc</sup>. AD is a slow progressive disorder with an extremely high prevalence, afflicting a third of octogenarians, while CJD is rapidly progressing with an extremely low prevalence, afflicting one per million per year. However, no matter the dissimilar characteristics between the two disorders, there is evidence that there is an analogous mechanism between the two.

It was shown in a study that PrP<sup>sc</sup> prevalence affects the function of BACE1. BACE1 is a cleaving enzyme which catalyzes the cleavage of APP to either A $\beta$ -40 or A $\beta$ -42, two versions of the A $\beta$  differing in residue length. A high expression of PrP<sup>sc</sup> reduces the severing of the APP into peptide fragments by more than 95. This in turn reduces the secretion of A $\beta$ -40 by 92% and A $\beta$ -42 to undetectable levels. The support for this conclusion arises from the study that showed that reduced PrP<sup>sc</sup> prevalence showed an increase in A $\beta$ -40 and A $\beta$ -42 production. This provides strong evidence between the correlation between AD and CJD mechanisms (Hooper & Turner, 2008).

These findings increase the probability that a slight reduction in PrP<sup>sc</sup> at a young age could cause an inconsequential accumulation of A $\beta$ -40 and A $\beta$ -42 over many years, furthering the accumulation of A $\beta$  plaques and the onset of AD. Patients with AD showed lower levels of PrP<sup>sc</sup> in their occipital lobes with an increased activity of BACE1. Additionally, studies show that a polymorphism in codon 129 of the PrP<sup>sc</sup> gene is a risk factor for early-onset AD. This polymorphism changes the peptide coded in that location

to a valine or methionine causing the transconformational alteration to the PrP<sup>sc</sup> pathogenic form. This is consistent with our assumed PrP<sup>sc</sup>-BACE1 relationship. Hooper and Turner (2008) suggest that if PrP<sup>sc</sup> affects A $\beta$  production, we can assume that the absence of PrP<sup>sc</sup> would lead to early-onset AD.

If prion proteins regulate A $\beta$  accumulation thus preventing AD, does A $\beta$  regulate and/or prevent prion pathology? In an experiment involving mice infected with scrapie, a form of prion disease in animals, it was found that in the terminal stages of the disease there was a considerable increase in the concentration of A $\beta$ . Hooper and Turner comments on this that, "[i]nterestingly, the amounts of A $\beta$  peptides were high in scrapie-infected mice, with a shorter disease incubation time raising the possibility that the higher levels of A $\beta$  might exacerbate the disease process." If this theory is correct that high A $\beta$  levels contribute to the rate of prion pathology, then this can be used as a way to slow down the progression of these ailments. However, they point out that we cannot rule out that the increased levels of A $\beta$  are perhaps a result of the scrapie pathology, as stated before, and that further study is necessary to confirm this hypothesis (Hooper & Turner, 2008). Furthermore, Debatin et al (2008) point out that the increased levels of A $\beta$  could be the result of the saturation of clearance mechanisms; since the body is preoccupied with PrP<sup>sc</sup> maintenance, the same mechanism that also deals with A $\beta$  accumulation becomes saturated leading to a buildup of the AD associated protein. This theory is supported by evidence that suggests a common pathway for protein degradation of PrP<sup>sc</sup> and A $\beta$ .

Alternatively, there are those that argue that overexpression of PrP<sup>sc</sup> increase A $\beta$  plaque production. Lara Ordonez-Gutierrez et al (2013) disregard the evidence that suggest that PrP<sup>sc</sup> directly inhibits A $\beta$  production by altering the activity of BACE1. They state that most of these tests were performed in cell cultures while studies involving older animals showed no change in the expression of BACE1 in regards to PrP<sup>sc</sup> concentrations. Furthermore, in vivo experimentation conducted by Ordonez-Gutierrez et al (2013) discovered that older animals that expressed a high level of PrP<sup>sc</sup> expression also illustrated a high level of A $\beta$  deposits with no significant consequence on BACE1 levels. They speculate that since BACE1 levels stay the same while A $\beta$  deposit levels increase, perhaps this surge in plaque formation is a result of increased protein degradation and/or production via an alternate pathway involving PrP<sup>sc</sup>.

It's been suggested that a divergent reasoning for the accumulation of A $\beta$  deposits in patients with a high PrP<sup>sc</sup> expression. They explain, given the fact an unaltered expression of PrP<sup>sc</sup> is observed during the pathology of prion disease, it is unlikely prions are the cause for A $\beta$  accumulation in specific sCJD patients. Being that the aggregation of misprocessed proteins is caused by



the differences between de novo generation and its elimination, it is a possibility that the increased levels of A $\beta$  deposits in sCJD are a result of a saturation of common clearance mechanisms. This is validated by studies that show a similar clearance mechanism is in place for both PrPsc and A $\beta$ . Furthermore, in a group study of sCJD patients, individuals who exhibited high levels of A $\beta$ -42 showed little deposits of PrPsc while individuals with high levels of PrPsc had low amounts of A $\beta$ -42. The correlation between A $\beta$ -42 and PrPsc concentrations clearly indicates a shared mechanism between the two (Debatin, et al., 2008).

There are also reports discussing a few intriguing similarities between AD and CJD. Both AD and CJD are characterized by their peptide aggregations resulting in plaques; A $\beta$  in AD and PrPsc in CJD. Additionally, the authors explain how AD and CJD can coexist in the same individual and that the A $\beta$  and PrPsc plaques in sporadic AD and sCJD share a similar spatial distribution throughout the brain, "suggesting a consistent pattern of

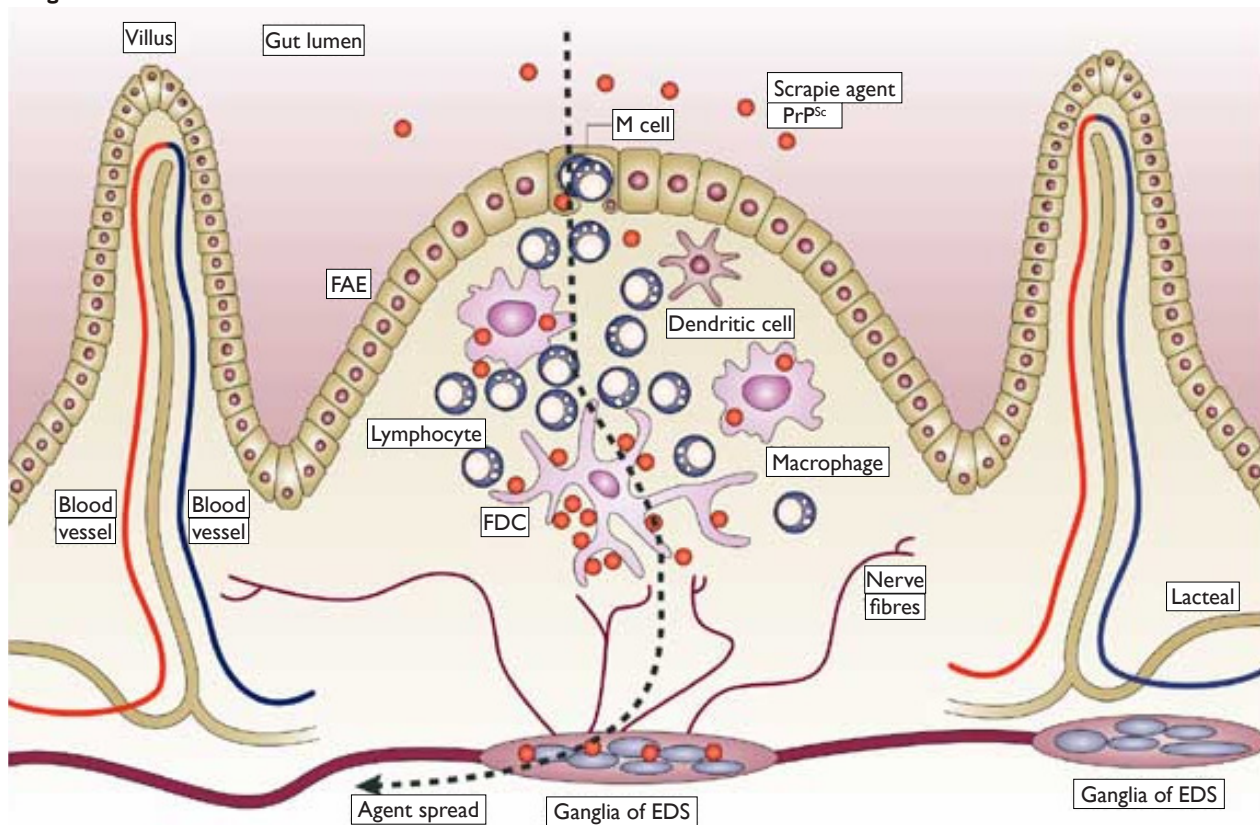
cortical degradation of the two disorders (Armstrong, Lantos, & Cairns, 2005)." With all of these similarities, including the ones mentioned earlier, it is hard to deny the correlation between these two neurodegenerative diseases.

### Possible Treatments for CJD

In persons with vCJD, it was observed that when ingested orally, infectious prions aggregated towards gut-associated lymphatic tissue and the spleen (Cashman & Caughey, 2004). From this location, the prions are transported via splanchnic

innervation to the spinal cord and brain (Figure 3). Cashman and Caughey point out that replication of these prions occur in locations accessible to immunotherapy and antibody neutralization. Due to the pathogenic pathway observed in vCJD, it is a possibility to artificially induce an autoimmune response against prions affecting its replication. This can effectively slow down or block pathogenesis of the disease. It has been illustrated in studies that

**Figure 3**



Possible spread of scrapie infectivity from the gut lumen to the nervous system following oral infection (route indicated by dotted line). Soon after ingestion, the abnormal prion isoform (PrPsc) is detected readily within Peyer's patches on follicular dendritic cells (FDCs), within macrophages, within cells with morphology consistent with that of M cells and within ganglia of the enteric nervous system (ENS). These observations indicate that, following uptake of scrapie infectivity from the gut lumen, infectivity accumulates on FDCs in Peyer's patches and subsequently spreads via the ENS to the central nervous system. FAE, follicle-associated epithelium. © Elsevier Ltd (2000). (Cashman & Caughey, 2004)



antibodies targeted against PrPc, cleared cells that were scrapie-infected with PrPc in vitro. However, in vivo, antibodies targeted against normal PrPc can have adverse effects since these cells in their non-pathogenic configuration are detrimental to normal neurological function. The autoimmune response to PrPc can prompt complement-dependent lysis in many cells disrupting normal function leading to apoptosis in the brain. It is entirely possible for an induced antibody response to reduce the immunological tolerance of PrPc with the subsequent induction of an autoimmune disease (Cashman & Caughey, 2004).

Often when a protein misfolds causing pathology, a sidechain of peptides that originally was sequestered within the molecule becomes exposed providing a means of distinguishing between pathogenic and non-pathogenic conformational isotopes. This specificity can aid in devising a means of targeting PrPc alone without affecting the normal PrPc. The therapeutic potential of this approach is immense with the benefit of leaving non-pathogenic proteins unharmed (Paramithiotis, et al., 2003).

There are several chemical inhibitors of CJD progression in vitro. Cyclic tetrapyrroles have been shown to slow the progression of scrapie in mice; however, this was only if treatment began immediately after infection due to its inability to cross the BBB. Other tetrapyrroles that do cross the BBB could prove to be efficacious further in the stages of infection. It is believed that cyclic tetrapyrroles inhibit PrPc by direct interaction with the protein. Polyene antibiotics and dimethylsulphoxide are also thought to inhibit PrPc pathology. Polyene antibiotics target prions while dimethylsulphoxide helps reduce accumulations and excretion of PrPc. Unfortunately, no chemotherapeutic drug has been shown to significantly alleviate the pathology of CJD once clinical symptoms are present (Cashman & Caughey, 2004).

Several other therapies have been suggested for CJD treatment. One is through gene therapy via a lentiviral vector-mediated RNA interference (RNAi). A lentivirus is a genus of viruses that can be modified to implement specific genes within a host's genome or silence others. This is achieved through short hairpin RNAs (shRNAs) that can silence select genes. A study conducted with scrapie-infected neuronal cells that involved shRNAs that suppressed the PrPc gene, PRNP, showed an efficient suppression of PrPc aggregation. Mice infected with lentiviruses with shRNAs showed a reduced expression of PrPc and an extended lifespan of those infected with scrapie. Although further testing needs to be done, a lentiviral vector-mediated RNAi appears to be a valid approach for the treatment of CJD (Pfeifer, et al., 2006). However, reducing PrPc expression may hinder CJD pathogenesis but it may also contribute to various adverse effects. additional research will need to be conducted to attain a viable solution to these concerns.

Another suggested therapy is by the use of antihistamines, specifically astemizole. Experiments involving astemizole indicated that it prolonged the lifespan of scrapie-infected mice. Additionally, astemizole has the capabilities of crossing the BBB making it a favorable drug for the treatment of CJD. Except for a rare case of heart arrhythmia, astemizole is an over-the-counter drug that is relatively safe in recommended doses. It is theorized that astemizole's antiprion properties arises from its activity on autophagy, which is the degradation and recycling of cells and proteins. It is suggested that individuals containing mutations in the PRNP gene and who are genetically susceptible to CJD should take astemizole regularly as a preventative measure against disease. The effects of astemizole on autophagy could provide new methods for treating other neurodegenerative diseases (Karapetyan, et al., 2013).

### CJD treatments vs AD pathology

Can several potential treatments for CJD be administered or modified to treat AD? AD is associated with the accumulation of abnormal proteins (A $\beta$  and/or Tau proteins). It is possible to target these molecules via an induced autoimmune response such as that observed in CJD treatments (Cashman & Caughey, 2004). However, this can initiate a negative cascade in which the body targets normal functioning A $\beta$  or Tau proteins resulting in cell lysis and the reduced immunological tolerance towards normal cells causing autoimmune disease.

Abnormal proteins are often accompanied by the exposure of a previously sequestered side chain. This side chain can be used to target pathogenic proteins alone without interfering with normal function. These findings can be applied to AD in treating A $\beta$  and Tau fibril accumulations. Although further experimentation is needed to produce a safe autoimmune induced treatment, this can be a viable method to combat AD pathology (Paramithiotis, et al., 2003).

Lentivector-mediated RNAi can be used to treat AD as well. ShRNAs that repress specific gene expression could be used to suppress a faulty gene that causes pathology. For instance, if an individual has two genes coding for a molecule involved in the pathogenic AD pathway with one of the genes corrupted and the other in its normal function, shRNAs can be utilized to silence the faulty gene's expression without affecting the normal one. However, Lentivector-mediated RNAis can suppress the function of both normal and faulty genes causing unwanted adverse effects (Pfeifer, et al., 2006). This prototype treatment for CJD can ultimately be utilized to treat other disorders such as AD.

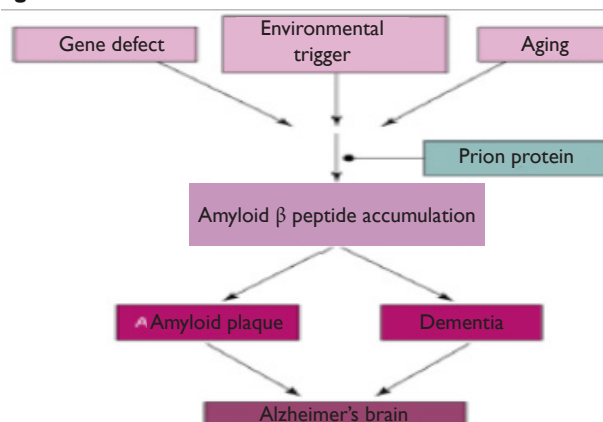
Direct modification of PrPc/PrPc levels is another option for AD treatment. With a theorized mechanism correlation between PrPc and A $\beta$  proteins, it is possible to indirectly treat AD by

inducing prion production. For instance, Hooper and Turner believe that PrPc regulates A $\beta$  levels by inhibiting enzyme catalyzed cleavage of APP to A $\beta$ -40 and A $\beta$ -42. The greater the PrPc levels, the lower the levels of A $\beta$  in the body. If a patient has AD due to high levels of A $\beta$ , according to Hooper and Turner (2008), it should be possible to reduce these levels by increasing expression of PrPc production (see figure 4). Ordonez-Gutierrez et al (2013) argue that increased levels of PrPc increase A $\beta$  plaque formation. This suggests that reducing PrPc concentrations is the viable method in treating A $\beta$  aggregations in AD. More data has to be obtained to procure the exact mechanism between PrPc and A $\beta$ . It seems that both Hooper and Turner and Ordonez-Gutierrez et al agree that a related mechanism exists between the two proteins, it is the effect one has on the other that is in question. Either way, understanding how these two molecules interact can be important in treating either of these two neurodegenerative disorders. Prion diseases can offer scientists a prototype on the processes of other neurological disorders such as AD (Cashman & Caughey, 2004).

### Possible treatments for AD

Currently, AD patients are treated with two different types of drugs, either acetylcholinesterase inhibitors or memantine. Acetylcholinesterase inhibitors act by increasing cell-to-cell communication chemical levels that were diminished during AD onset. This is accomplished by inhibiting enzymes that facilitate the breakdown of acetylcholine, a neurotransmitter found within the brain. Progression of symptoms is usually maintained for

**Figure 4**



*The role of the prion protein in Alzheimer's disease prevention: In the amyloid cascade hypothesis of AD, a genetic mutation (for example, in the genes encoding APP or the presenilins), ageing or some other environmental trigger (e.g. oxidative stress) causes an accumulation of the neurotoxic Ab peptide, which aggregates to form the characteristic amyloid (senile) plaques found post-mortem in the brains of AD patients. Through mechanisms that are poorly understood, the increased levels of Ab peptides, particularly oligomeric and fibrillar forms, cause neuronal cell death and dementia. The normal cellular form of the prion protein, through inhibiting the production of the Ab peptide, might help to prevent the development of AD (Hooper & Turner, 2008).*

a time before AD pathology continues to worsen. Memantine works via a different pathway within the brain, slowing the development of symptoms relating to AD. The mode of action for memantine is to target and inhibit NMDA receptors which has shown to improve cognitive function compared to a placebo. Unfortunately, medication only forestalls pathology since there is no cure for AD (Gotz, Ittner, & Ittner, 2012) (Eubanks, et al., 2006) (Mayo Clinic Staff, 2014).

Research into the immunization against A $\beta$  showed promising results. Mice immunized with A $\beta$ -42 showed reduced A $\beta$  burden and preserved cognitive function. In an interrupted clinical trial, patients who were injected with one, two, or three immunizations of A $\beta$ -42 showed decreased levels of tau proteins with postmortem reports of diminished A $\beta$  in the neocortex. The trial was interrupted because 6% of the patients developed meningo-encephalitis (Gilman, et al., 2005). Using natural anti-A $\beta$  antibodies derived from healthy humans may provide a better method in reducing adverse effects such as encephalitis in patients (Sardi, et al., 2011). Although the clinical trial was suspended, immunotherapy seems to be a viable method in treating AD (Gilman, et al., 2005).

In a recent pharmaceutical study, a monoclonal antibody aimed at A $\beta$  named Bapineuzumab was intravenously administered for 78 weeks. It was found that there was a diminished phosphorylated tau along with a reduced accumulation of A $\beta$  in the brain of AD individuals carrying the pro-pathological gene, ApoE  $\epsilon$ 4. These findings show promise in the treatment of AD through reductions of plaque buildup. Interestingly, these reductions of the disease-inducing molecules were not able to forestall the rate of pathological progression in the disorder. One suggested hypothesis is that Bapineuzumab was administered too far along in the AD pathology. To remedy this concern, the drug would have to be administered before the appearance of clinical symptoms. This poses an issue since it is extremely difficult to diagnose a patient before symptoms appear; however, it may be able aid those who are high risk for AD. Another hypothesis is the actual pharmaceutical mechanism of the drug. As of yet, Bapineuzumab does not target phosphorylated tau that aggregated into neurofibrillary tangles, another hallmark of AD (Cedernaes, et al., 2014). While further study is needed to improve the efficaciousness of antibodies against AD, immunotherapies are strong contenders in discovering a feasible treatment for neurodegenerative diseases.

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be a preventative measure for AD. There are reports that suggest that BBB degradation plays a central role in AD. As the BBB degrades, many previously unwanted molecules cross into the fluid surrounding the brain possibly eliciting a response of inflammation. Inflammation can further increase the

## Creutzfeldt - Jakob and Alzheimer's Diseases: Overlap of Treatment Methods?

permeability of the BBB allowing circulating A $\beta$  to enter the brain where it can start a degenerative and inflammatory process. NSAIDs are drugs that reduce pro-inflammatory responses in the body. Studies have shown that patients who were medicated with NSAIDs over a long period of time exhibited a reduced incidence of AD. This indicates the presence of pro-inflammatory molecules in the pathology of AD (Sardi, et al., 2011).

Many therapies for AD focus on ameliorating A $\beta$  levels in the brain without much focus on tau proteins concentrations. Treatments aimed at pathogenic tau proteins produced reasonable results. Abnormal tau proteins are phosphorylated versions of the normal tau form. One method of treatment is to inhibit this phosphorylation. Administered lithium chloride inhibited glycogen synthase kinase-3, an enzyme that catalyzes the transfer of a phosphate group onto other molecules, and reduces the levels of hyperphosphorylated tau, insoluble tau, and behavioral impairment in various mice models. Inhibition of other kinases also showed promising results. Furthermore, immunization with a tau phospho-peptide prevented pathology in tau transgenic models with no apparent adverse effects (Gotz, et al., 2012). With the promise of tau directed therapies, it is worthwhile to formulate treatments involving both A $\beta$  in conjunction with tau proteins.

The active component of marijuana,  $\Delta^9$ -tetrahydrocannabinol (THC), appears to also be an efficacious and viable method in the treatment of AD. THC competitively inhibits the enzymatic protein acetylcholinesterase which catalyses the breakdown of acetylcholine. It is believed that acetylcholinesterase is a key component in the pathological progression of AD by inducing A $\beta$  plaque formation. THC effectively inhibits this enzyme, reducing the degradation of acetylcholine transmitters in the brain while simultaneously diminishing A $\beta$  plaque formation. This treats both the progression and the symptoms of the disease. THC is a superior inhibitor of acetylcholinesterase as opposed to other acetylcholinesterase inhibitors that are currently used to treat AD. Additionally, THC appeared more effective with only half the dosage compared to these other medications. (Eubanks, et al., 2006).

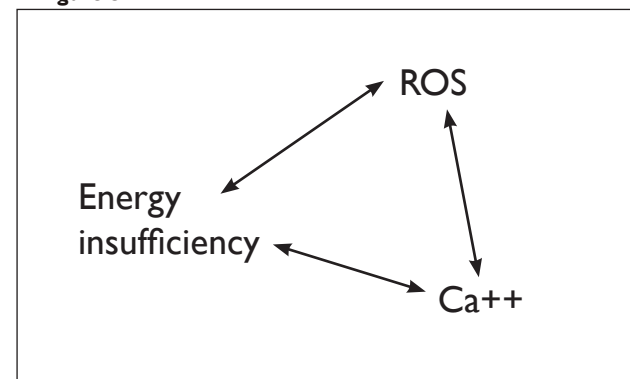
The treatment of metabolic deficiency is another suggested alternative in treating AD. An abnormality in the energy metabolism, calcium metabolism, or free radical pathways showed to contribute to deficiency within the other two metabolic routes creating a "mitochondrial spiral". This spiral forms a negative cascade effect where the defects of one metabolic pathway continue to degrade other pathways which in turn further damage the original faulty pathway (see figure 5). It is thought that mitochondrial spiral causes abnormalities in the metabolism of the APP and the segments of A $\beta$  it produces. It may also be a proximate reason for clinical debilities found in AD. Ameliorating the spiral

may be an alternative route in AD treatment. Studies involving an induced increase in metabolic activity by administering a concoction of glucose and several Krebs cycle intermediates into patients showed an increase in cognitive function with less neurodegeneration. Improving a deficient mitochondrial spiral may prove to be effective in treating AD (Blass, 2001).

### AD treatments vs CJD pathology

With AD and CJD sharing many pathological pathways, it seems it may be possible to treat CJD via techniques previously reserved for AD. Acetylcholinesterase inhibitors that reduce the enzymatic degradation of acetylcholine perhaps can be used to alleviate symptoms of dementia found in patients with CJD (Cummings, 2000). With the increased levels of the neurotransmitter acetylcholine in vivo, there can be an increased level of cell-to-cell communications within the brain possibly easing several cognitive symptoms. However, inhibition of this enzyme is most likely not a viable method of treatment for there is little research conducted on the effects of acetylcholinesterase inhibitors on CJD. There is some evidence that memantine, another drug used to treat AD,

**Figure 5**



*The mitochondrial spiral. Impairments of energy metabolism, alterations in cellular calcium homeostasis, and excess free radicals (ROS) interact with each other in mitochondria; inducing any one of them leads to abnormalities in the other two. The interaction can set up a deleterious, downward cycle (Blass, 2001).*

has some inhibitory effects towards PrPsc (Muller, et al., 1993).

Immunizations for CJD have been thought of as a method of treatment as well. Similar to AD, antibodies targeted against proteins associated with CJD have showed some promising results. However, being that adverse effects were found with immunizations, research has to be done before it is safe for public use as a treatment for CJD (Cashman & Caughey, 2004). NSAIDs have been suggested as a treatment for CJD. Inflammation has been associated with both AD and CJD. In contrast to AD, there is little data available on the effectiveness of NSAIDs on CJD. In vivo studies showed that NSAIDs may have some effect on prion pathology, nevertheless, experiments using patients showed

disappointing results. It is possible that the NSAIDs were given too late in the disease pathogenesis. Further study is needed to determine if antiinflammatory drugs are a suitable treatment for CJD (Eikelenboom, et al., 2002).

Treatment of the “mitochondrial spiral” may play a role with individuals with CJD. In regards to AD, it was found that a deficiency in one of three different metabolic pathways can cause deficits in the other two creating a mitochondrial spiral. Attempts to alleviate the stress on this spiral showed to improve cognitive symptoms in patients (Blass, 2001). In CJD, some studies show oxidative abnormalities plays a role in pathogenesis (Petersen, et al.). This can cause defects in one of the three metabolic pathways mentioned earlier. Alleviating these deficiencies may be a viable venue of treatment. It should be mentioned that other studies found no role of oxidative stress in CJD (Bleich, et al., 2000). Further study is needed on the topic to fully understand the effects of the mitochondrial spiral on disease pathogenesis.

It was suggested that it is possible that A $\beta$  contributes to CJD pathology. It has been hypothesized that A $\beta$  exacerbated the disease process by possibly accelerating the pathology (Hooper & Turner, 2008). If this hypothesis proved to be correct, any treatment formulated towards AD can in turn be prescribed for the mitigation and delaying of symptoms in CJD. Additionally, it was found that the other protein linked with AD, tau proteins, were associated with prion pathology (Han, et al., 2006). As additional experimentation is required to determine the exact impact of one protein on the other, treatments made to diminish tau pathology may ameliorate symptoms of CJD. These hypothesized venues of remedy will not cure prion pathology but can most likely hinder CJD neurodegenerative impacts on the brain. The correlation between these two diseases is too strong to suggest otherwise. Furthering research into understanding CJD is a viable method of ascertaining a feasible treatment for the growing epidemic that is AD.

## Conclusion

AD and CJD are both neurodegenerative disorders that share a common pathophysiological mechanism. PrPc and A $\beta$ , proteins associated with pathology in CJD and AD respectively, are believed to interact with each other. There is a slight debate whether they interact directly by contact or indirectly by affecting enzymatic proteins, such as BACE1. Additionally, there is an argument how they influence each other in regards to inhibiting/promoting pathology; for example, does PrPc induce or inhibit A $\beta$  aggregations that cause pathology? Either way, there is a strong correlation of pathologies that most scientists agree with. It is this connection that helped researchers discover new and creative treatments for AD and CJD. Treatments that were previously reserved for either disease are now being tested against the other. This is

enhancing the understanding of the mechanisms of each ailment, bringing researchers one step closer to discovering a cure for both neurodegenerative diseases. Perhaps, discovering the means of pathologies for AD and CJD will lead to cures for many other forms of dementia.

## Abbreviations

AD – Alzheimer’s Disease  
ApoE – Apolipoprotein  
APP –  $\beta$ -Amyloid precursor protein  
A $\beta$  – Amyloid- $\beta$  protein found in excess amounts in patients with AD  
BACE1 –  $\beta$ -CITE APP Cleaving Enzyme-1  
BBB – Blood Brain Barrier  
CJD – Creutzfeldt – Jakob Disease  
EOFAD – Early-Onset Familial Alzheimer’s Disease  
LOAD – Late-Onset Alzheimer’s Disease  
MAP – Microtubule-Associated Protein  
NSAIDs – Non-steroidal anti-inflammatory drugs  
POPG – 1-Palmitoyl-2-oleoyl-phosphatidylglycerol  
PrPc – The normal form of a prion protein  
PrPsc – the abnormal variant of a prion protein associated with CJD  
RNAi – RNA interference  
sCJD – Sporadic Creutzfeldt-Jakob Disease  
shRNAs – Short Hairpin RNAs  
THC –  $\Delta^9$ -Tetrahydrocannabinol, the active component in marijuana  
TSE – Transmissible spongiform encephalopathies  
vCJD – Variant Creutzfeldt-Jakob Disease

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# Pitch-count and its Effects on Shoulder Injuries.

## How to keep a Pitcher Healthy

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### Abstract

Baseball is currently the fourth most popular sport in high schools across the country. However, pitchers are at a high risk to develop career ending injuries. Overuse can result in SLAP tears as well as torn rotator cuff muscles that can end a dream of pitching professionally. Major League Baseball has teamed up with leading sports medicine researchers to determine safe pitch-count guidelines for pitchers of all ages. Articles were found using Touro's e-Journal database as well as Pubmed.gov to find pertinent research on this topic. Studies on ball velocity and scapular kinematics were done on three different levels of pitching. Data showed that over time fatigue sets in and pitching mechanisms change. It is important for pitchers to realize this and to act in a safe manner.

### Introduction

Baseball, America's Pastime, is a sport enjoyed by men and women of all ages. The look of joy on a young child's face when receiving his first baseball glove is testament to the love of the game. Who doesn't remember going to their first baseball game? The glamour of the game as well as the part time relaxation of lazing around in the field allows for baseball and softball to be among the most popular sports for adolescents in the United States. A survey from the National Federation of High School Athletes (NFHS handbook) found in the 2013-2014 academic year, that a total of 15,789 schools listed baseball as a sport of choice for men (behind basketball and track), with 541,054 students participating (behind football as well). Fast pitch softball for females was listed as the fourth most popular sport as 15,225 schools list it as a sport (behind basketball, track, and volleyball), with 364,297 students participating (behind soccer as well). However, contrary to popular belief, as well as studies classifying baseball as a safe sport, baseball and softball are not without their fair share of injuries. Although not as prevalent or life threatening as football injuries can be, the results can be season ending, or at times, even career ending. A study done by Conte et al. (2001) on Major League pitchers has shown that 57% of pitchers suffer from a shoulder injury during a season. The study also showed a growth in injury occurrences over an 11 year period from 1989 to 1999. Estimates of injury rates for youth (ages 9-14) are a 5% risk of serious arm injury (shoulder or elbow) within 10 years (Littleleague.org).

### Pitch-count Recommendations

As a result of such injury rates there has been an increasing emphasis on the pitch count during a game, as well as cumulative pitch count throughout the season. Pitch count is more strongly enforced in younger populations due to concern over how the repetitive stress that occurs with pitching alters both structural as well as functional mechanics of the young shoulder complex. Arm pain is common in youth pitchers with studies showing that risk of pain increases after 75-80 pitches per game (Lyman et al 2002). This has led to recommendations by little-league baseball for the following daily pitch maximums: ages 7-8, 50; 9-10, 75; 11-12, 85; 13-16, 95; and 17-18, 105

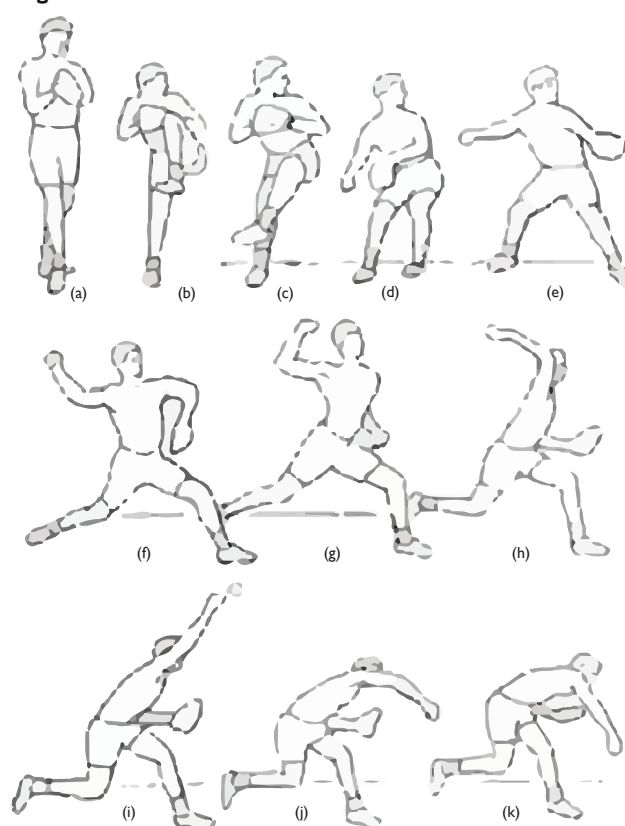
### Methods

Journal articles were found by searching the terms "pitcher fatigue causes", "pitch-count", and "fatigue related injuries". Articles were accessed using the Touro Library databases of EBSCO, ProQuest, and Sage Premier Collection. As the topic is a sports medicine related issue, the index of The American Journal of Sports Medicine was utilized as well. Search terms were later refined to specify shoulder injuries and pitching biomechanics. A final method of research was an interview with a biomechanical shoulder researcher at the Hospital for Special Surgery (HSS).

### Pitching Biomechanics

Biomechanical studies have determined that the arm is not the

Figure 1



Sequence of Motion in Pitching (Dillman et al 1993)

only body part involved in pitching. A pitch can be split into 6 separate sections: wind-up, stride, arm-cocking, arm acceleration, arm deceleration, and the follow through (Dillman et al 1993), with the upper and lower limbs working in tandem for a proper pitch.

For purposes of this paper the focus will be on the shoulder, but details from other locations are needed in moderation. During the windup stage the pitcher takes a step backward with the front foot, or stride foot. As the pitcher begins, he rotates 90° and slowly lifts, elevates, and flexes the stride leg. The stride stage follows with the other leg slightly flexing, and the stride leg moving toward the catcher. This phase is most important to a safe pitch, as a key element is to control proper trunk location to contribute to a pitch. If the trunk is not as back as possible it would affect the pitch velocity. Additionally, the landing spot of the stride leg contributes to hip rotation which will interfere with the contribution of the trunk to the pitch. As the hips start to rotate, the arm is flexed at the elbow, and the shoulder undergoes maximum external rotation (arm-cocking stage). The humerus then internally rotates along the glenoid as the arm accelerates toward the plate. During the release of a pitch the scapula rotation goes from a maximum of 178° to 105° in approximately 0.029 seconds. It is important to note that a decrease in lower extremity force will often result in either a decrease in momentum or an increase in upper extremity force leading to injury.

To date there have been three studies that compared ball velocity and shoulder kinematics over the duration of a game. Murray et al (2001) studied major league pitchers, Escamilla et al (2007) collegiate, and a recent yet to be published study from HSS, studied high school pitchers. Each study has advantages over the other, with specific revelations into pitch-count related injuries.

## Major League Pitchers

Murray et al (2001) was the first attempt to compare pitch velocity and pitcher kinematics over a period of time. They studied 7 pitchers (mean age 26) during spring training. 5 pitchers pitched for 5 innings with 2 pitching for 7. Cameras were placed in three different locations of the stadium to capture different angles of the pitcher. A computer program was used to manually digitize 20 different landmarks to compare over time. The program compared a fastball from the first inning as well as one from the last inning pitched.

They observed significant changes in the maximum external rotation angle of the shoulder as well as a decrease in ball velocity. A decrease of an average of 9° was noted with a 5 mph drop in velocity. Although fatigue is the probable cause for these discrepancies, the possibility of protective measures taken by the pitchers cannot be ruled out.

## Collegiate Level Pitchers

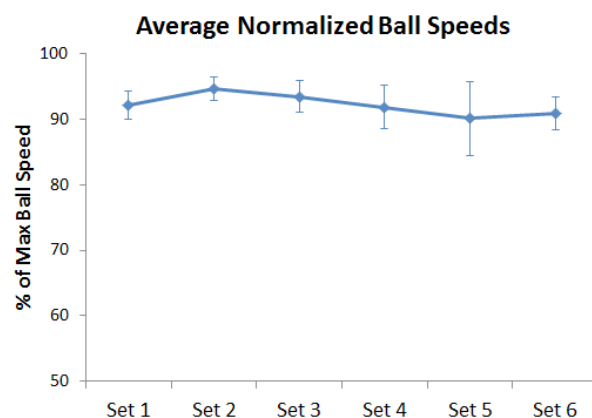
Escamilla et al (2007) chose a slightly different approach to study fatigue. They studied 10 Division I collegiate baseball pitchers with a mean age of 20. Each pitcher threw 15 pitches per “inning” with a rest period in between to simulate an actual baseball game. Five pitchers pitched the entire 9 innings (135 pitches), 2 pitchers pitched 8 innings (120 pitches), and 3 pitchers pitched 7 innings (105 pitches). Along the lines of a simulated game, the catcher called for a variety of pitches-not just fastballs. At the end of each inning pitchers were asked to scale their level of fatigue. Kinematic data were recorded using reflective markers on the pitcher to be studied using motion analysis software (more accurate than video markers used by Murray et al). (2001)).

Although pitch velocity decreased from the first inning to the last, the only significant kinematic difference was trunk position, as the trunk was more vertical at the end than the beginning. This led Escamilla et al. to conclude that there cannot be a specific amount of pitches that determine fatigue and therefore injury. They hypothesized that in real game scenarios there are more than 15 pitches during some innings, as well as an increased level of motivation that may lead to harder throws.

## High School Pitchers

A recent study from HSS (unpublished data) focused on young adults (average age was 15) during a simulated game. Participants were initially tested for range of motion in three different positions: 45° internal rotation, 0°, and 45° external rotation. After the average peak torque was determined the pitching sequences began. Once warmed up, the pitchers threw 90 fastballs in 6 sets (1 set = 15 pitches) with five-minute breaks between sets to simulate a game length situation. After the completion of the innings, participants were re-tested for range of motion. Kinematic data was recorded using the same method as Escamilla et al but with one major addition. They used a specialized scapula marker for more exact scapula tracking.

**Figure 2**



Average normalized pitching speeds across sets (unpublished data).

Although each pitcher threw with different velocities, normalized data showed that the second set of pitches were thrown the fastest, with the fifth and sixth being the slowest (Figure 2).

Isometric strength tests demonstrated decreased internal (19%-26%) and external strength (13% to 16%) for neutral and 45° internal rotation testing positions. No significant differences in strength were detected when participants were tested in 45° of external rotation.

Kinematic measurements at five pitching events (foot contact, maximum external rotation, release, and maximum internal rotation) revealed significant changes in scapulothoracic measures at maximum internal rotation. There was a 3.8° decrease in scapula backward tilt in set 6 compared to both sets 1 and 2 and scapula elevation increased by 3.6° in set 6 when compared to set 1. Abnormalities in scapula kinematics with increased scapula elevation are very common for symptomatic shoulders with possible rotator cuff tears.

This study shows that even before a pitcher feels fatigued, there is a drop in strength and velocity. Although no one complained about feeling fatigued when questioned, there were clear indications of muscular fatigue amongst the athletes, which was reflected in the reduction of the arm rotational strength and the decrease in ball speed between sets 2 and 6.

### Discussion

There are only a few studies that have examined baseball during an extended number of pitches to investigate how fatigue affects upper extremity kinematics. Escamilla et al. (2007) defined fatigue as the point at which the athletes could no longer continue pitching due to a subjective perception of muscular fatigue. This resulted in a variable number of pitches for each athlete that ranged from 105 to 135 pitches (7 to 9 innings). Although the subjects in that study didn't report fatigue, the rotational isometric strength post pitching in the HSS study clearly showed that the subjects experienced muscular fatigue during the simulated game of 90 pitches. The difference may be attributed to the higher age and level of competition of collegiate participants as studies have demonstrated that there are great differences in pitching kinematics between different levels of competition (high school, college, major league) (Fleisig 2009). An advantage in Escamilla's study over previous research was the introduction of an artificial game, allowing researchers to more accurately measure shoulder kinematics. However, this also opens a possibility of lack of effort on behalf of the pitchers leading to inaccurate data.

The advantage for Escamilla is a disadvantage for Murray et al (2001). Since Murray was analyzing major league pitchers in real game scenarios, (albeit spring training) the actual motion capture

can be inaccurate. However, a major clinical advantage was the conclusion of minimal kinematic variability as changes in external rotation can be attributed to protective measures. This shows that there is hope for young pitchers. Proper strength and conditioning, as well as being coached in proper mechanics can minimize injury risk to pitchers.

The study from HSS was advantageous in not letting pitchers wait until they felt fatigued, or let them complete a simulated game. Their research shows how fatigue affects strength and velocity before the pitcher realizes it. Add the fact that the study was done in a meaningless simulated game, and one can see the probable effect during a game. However, a major disadvantage was the type of pitch allowed. They only allowed fastballs which does not accurately portray real-life scenarios.

Muscular fatigue was also reflected in the ball speed results, as all three studies revealed a decrease in ball velocity from start to finish. This is to be expected as when one overworks any muscle there tends to be fatigue. However, the main implications are not just the ball velocity. Lyman et al (2001) surveyed a little league over the course of a season. Careful pitch counts were collected and players were interviewed at the end of each game, as well as the end of the season regarding pitch type and injuries. The results of the study led Lyman et al to recommend a maximum of 75 pitches per game as mentioned above. Interestingly enough, they also found that the more a person pitched the lower the risk of injury. This is due to lack of experience when it comes to pitchers, or possibly lack of conditioning. However, more than 600 pitches a season led to elbow pain as well, so that should be avoided.

Fleisig et al. (2011) added to this study by linking pitch type and amount to injury frequency. The American Sports Medicine Institute concluded that the players who pitched more than 100 innings in at least 1 calendar year had about 3.5 times as much chance of serious injury as those who pitched less. They also recommended not starting to throw curve-balls before the age of 13, to take a break of 2-3 months annually, and not to pitch on consecutive days (American Institute of Sports Medicine, 2013). However, subsequent reports have found that a large percentage of youth pitchers do not follow these guidelines (Yang et al. 2014).

### Specific Injuries

During the release of the ball the shoulder goes from 178° to 105° in approximately 0.029 seconds. This quick motion can cause the greater tuberosity to come near the acromion. Unfortunately, the biceps tendon or supraspinatus (rotator cuff) can get caught in between. Impingement pain is easily treated with rest and physical therapy (Hawkins 1980).

The sudden interior rotation of the humerus also leads to biceps tendinitis, or a frayed long head of the biceps tendon. Although this injury alone can be treated surgically, it is also treated with rest and therapy. However, a frayed biceps is the start of a cascade of injuries. If the biceps gets frayed or torn the shoulder loses some stability. When the shoulder loses some stability it applies stress on the labrum. This leads to tears in the superior part of the labrum an injury called a superior labrum anterior and posterior, or SLAP, tear. Instability can also lead to torn rotator cuff muscles, specifically the supraspinatus. Both SLAP tears and torn rotator cuff muscles require surgery to fix.

### Conclusion

Literature has shown a correlation between high pitch-counts and shoulder injuries. Although pitchers may think they are not fatigued, this has been shown to be false. Younger pitchers, who are not yet developed, both physically and in pitching mechanics, are at a higher risk for injury. This has led the American Sports Medicine Institute together with Major League Baseball to institute guidelines for young pitchers.

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# Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy (SMA): A Genetic Linkage?

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## Abstract

Neurodegenerative motor neuron disorders (MNDs) have devastating effects. Spinal Muscular Atrophy (SMA), for example, is a debilitating and sometimes lethal disease in children. SMA is monogenic, autosomal recessively inherited disorder caused by a loss-of-function mutation of surviving motor neuron 1 (SMN1). SMN2 is an identical copy of this gene and produces abbreviated transcripts without exon 7 though some full transcripts are produced that ameliorate the disease. Previous clinical trials for this disease have not produced consistent results. However, in a recent cross-sectional study, biomarkers for SMA (BforSMA), protein candidates and metabolite markers were identified (Finkel et al., 2012). These markers can be used for clinical assessment, identification of molecular pathways, and may guide response to treatment. Clinical trials of amyotrophic lateral sclerosis (ALS), another motor neuron disorder, have been uniformly disappointing without the benefit of a full understanding of ALS's mechanisms. Numerous theories attempt to explain ALS's selectivity for motor neuron degeneration, but none are conclusive. One hypothesis, gem depletion, emerges from the studies of superoxide dismutase 1 (SOD1) transgenic mice that have been discovered to contain low levels of SMN, thereby potentially linking SMA and ALS. Furthermore, SMN1 and SMN2 are seen as risk factors for ALS (Andersen & Al-Chalabi, 2011). Biomarker identification may also help in identifying ALS's pathogenesis and pathophysiology as it has begun to do for SMA. ALS and SMA may be more similar than previously thought. Both MNDs may interact in a variety of genetic and mechanistic pathways unknown at present. If so, this may serve to link seemingly disparate crippling diseases, and thereby promote efforts by government agencies and pharmaceutical companies to pursue research and development for these "orphan" diseases.

## Introduction

Progressive muscular atrophy (PMA) was first described in 1848 by F.A. Aran, who reported 11 cases of weakness and paresis of the upper limbs (Bonduelle, 1989). However, it was not until 1869 that French neurologist Jean-Martin Charcot classified amyotrophic lateral sclerosis (ALS) as a separate neurodegenerative motor neuron disorder (MND) from PMA. It was not until 1874 that ALS was given the name of amyotrophic lateral sclerosis in Dr. Charcot's "Oeuvres Completes." In his earliest studies, he noted that, "lesions within the lateral column in the spinal cord resulted in chronic progressive paralysis and contractures (no atrophy of muscles), while lesions of the anterior horn of the spinal cord resulted in paralysis without contractures (with atrophy of muscles)" (Goetz, 2000). ALS shares many clinical symptoms to other MNDs such as: spinal muscular atrophy, primary lateral sclerosis and bulbar palsy. The key to ALS's understanding was due to Dr. Charcot's unique method known as "anatomy-clinical method." Using this technique he was able to determine the correlation between clinical signs detected during life and anatomical lesions seen at death. Furthermore, post-mortem studies revealed both the anterior horn cell lesion typical of acute amyotrophy, and also the distinctive bilateral and symmetric sclerosis of the lateral spinal cord columns. Hence, he named the syndrome ALS, since it incorporated the two aspects of gray matter involvement (amyotrophy) and white matter damage (lateral sclerosis). Dr. William Gowers, though, argued with Charcot's terminology since it suggested that lateral sclerosis was primary and amyotrophy was secondary, and instead, postulated they are one event (Goetz, 2000). This is still debated over a century later.

ALS is an incurable disease that has an incidence of approximately

2 in every 100,000 people. The mean age of onset for ALS is 55-60 and affects men more than women. No diagnostic test exists for ALS. Physicians only diagnose ALS when both upper and lower motor neurons are affected and after ruling out all other causes. At first, one may notice weakness in an arm or leg, described as "limb onset" of ALS, or difficulty with speech production, known as "bulbar onset" ALS, which quickly spread to other parts of the body. Eventually, all limbs and movement cease, leading to complete paralysis. Since ALS shares several common symptoms with spinal muscular atrophy and other neurological conditions, several tests are required to diagnose. Presently, the only tests, aside from limited genetic testing, vary from electromyography (EMG) and nerve conduction study (NCS) to magnetic resonance imaging (MRI). After all these tests are performed, physicians still may not know until later stages of the disease.

Presently, the cause of ALS is unknown. Some research points out that ALS results from inaccurate protein formation. Other research demonstrates there is an excess of glutamate in the synapses causing neurite toxicity. Still other research shows changes to RNA processing. A fourth theory suggests environmental factors since U.S. military personnel in the Gulf War had higher incidence of developing ALS (Haley 2003).

Genetic mutations in ALS are numerous, for instance, superoxide dismutase 1 (SOD1), chromosome 9 open reading frame 72 (C9orf72), TAR DNA-binding protein 43 (TDP-43), senataxin (SETX) and fused in sarcoma (FUS) are a few of the growing list of genetic mutations associated with ALS. In contrast, spinal muscular atrophy (SMA) has only one genetic mutation in survival

motor neuron 1/2 (SMN1/2). SMA shares some common features with ALS, such as lower motor neuron degeneration, gem depletion and possibly biomarkers.

ALS and SMA share a biochemical pathway in gem depletion related to each disease's respective genetic mutation (TDP-43 and SMN) (Yamazaki, 2012; Groen et al., 2013; Turner, 2014; Rafałowska et al., 2014). Also, both selectively target motor neurons although SMN1, SMN2, TDP-43, SETX and FUS are ubiquitously expressed (Cauchi, 2014; Achsel et al., 2013; Tsuiji et al., 2013). A project called: Biomarker identification for SMA (BforSMA) has yielded significant results for future clinical studies (Finkel et al., 2012). ALS biomarker identification would also accelerate future clinical trials. SMA has had much more success in diagnosis and prognosis than ALS due to its monogenic nature, while many of ALS's genetic mutations are still being discovered (Keller et al., 2014). Nonetheless, there appears to be a strong relationship between these two disparate MNDs. By researching the overlap of these two MNDs a common therapeutic approach may be possible. As of yet, it remains unknown why ALS and SMA, which have genes that are ubiquitously expressed, selectively destroy motor neurons.

### Methods

Peer-reviewed articles from PubMed and UptoDate (a division of Wolters Kluwer Health) using keywords "Amyotrophic Lateral Sclerosis" "ALS" "Genetic basis of ALS" "Biomarkers for ALS" "Spinal Muscular Atrophy" "SMA" "SMA and ALS" were utilized as background for this paper. Dr. Alex Pearlman and Dr. Harry Ostrer also provided guidance in developing this thesis.

### Theories on Targeted Motor Neuron Degeneration in ALS

ALS is a heterogeneous disease with multiple pathogenic mechanisms and variable sites of disease onset and progression. Researchers currently are searching for the starting point of the disease and the reason why motor neurons are specifically targeted. Present research focuses on aberrant protein formation in axons, excess glutamate activity in neuromuscular synapses and gem depletion in cells as being responsible for ALS pathology.

The first hypothesis on ALS pathogenesis is faulty protein formation in axons. According to Dr. Zhang, at the University of Madison-Wisconsin, misfolded protein in neurons causes a cascade of events (Chen et al., 2014). Eventually, the protein is shuttled to the distal part of the axon, but becomes tangled in transport and axonal degeneration occurs. This may also explain Alzheimer's and Parkinson's disease and their pathophysiology. Through use of induced pluripotent stem cells (iPSCs), Dr. Zhang formed new nerve cells in vitro and tested his hypothesis (Chen et al., 2014). Indeed, when patient iPSCs were used, neurofilament

(NF) aggregation together with neurite swelling in spinal motor neurons (MN) resulted. "Such MN-selective NF changes were mimicked by expression of a single copy of mutant SOD1 (D90A) in human embryonic stem cells (hESCs) and prevented by genetic correction of the SOD1 mutation in patient iPSCs" (Chen et al., 2014). "A4V is the most common SOD1 mutation in the US and D90A is most common in Europe" (Giannini et al., 2010; Saeed et al., 2009). In ALS MNs bead like structures form along neurites. These bead like structures have heavy immunostaining for plasma phosphorylated neurofilament-H. Plasma phosphorylated neurofilament-H levels closely reflect disease progression in SOD1 (G93A) mice and are regarded as an ALS biomarker (Calvo et al., 2012). Therefore, the bead like structures that were heavily phosphorylated with plasma phosphorylated neurofilament-H, indicated pathogenicity. As opposed to control MNs and non-MNs that have an even staining pattern. This finding highlights the possibility of targeting NF regulation for therapeutic intervention.

A second hypothesis on the pathogenesis of ALS is excess glutamate accumulation, which causes neuro-degeneration. "Glutamate is generally acknowledged to be the most important transmitter for normal brain function" (Purves et al., 2001). Glutamate is an excitatory neurotransmitter. However, high extracellular glutamate can have toxic effects on neurons. It is synthesized in neurons from precursors. In a Human Molecular Genetics paper by Dr. Guo he explains that glial glutamate transporter, excitatory amino acid transporter (EAAT2 also known as GLT1), is responsible for removing glutamate from the synaptic cleft (Guo et al., 2003). In ALS patients, the glutamate transporter EAAT2 has been found inactive. Defective glutamate transport and loss of EAAT2 protein have also been observed in affected brain regions of patients with Alzheimer's disease (Masliah et al., 1996). Overactivation of glutamatergic neurons can result in a neurodegenerative process known as excitotoxicity i.e. cell death. (Guo et al., 2003). "However, it is still unknown whether it is a primary cause in the cascade leading to neuron degeneration or a secondary event to cell death" (Guo et al., 2003). After experimentation with transgenic mice, containing increased expression of EAAT2 and SOD1 variation (G93A) degeneration of neurons was slowed, but did not cease. The results "suggest that the loss of EAAT2 may contribute to, but does not cause, motor neuron degeneration in ALS" (Guo et al., 2003). Transgenic mice with the common mutations of SOD1 exhibit neurodegeneration comparable to ALS. The SOD1 mutation results in a toxic gain of function rather than a loss of enzymatic function (Wong et al., 1995). Furthermore, in a Nature paper by Dr. Rothstein he demonstrates  $\beta$ -lactam antibiotics, for example, ceftriaxone, a semi-synthetic, third generation cephalosporin antibiotic, has been used to treat bacterial infections (Rothstein et al., 2005). These antibiotics also show no substantial toxic CNS effects. Additionally, they have found it to increase gene expression

of GLT1. Leading to neuroprotection by increasing glutamate transporter expression. The mechanism of this overexpression appears to be activation of the genetic promoter for GLT1, although the pathway for promoter activation is, as yet, unknown. (Rothstein et al., 2005). This too provides another possible intervention of ALS, which is currently in phase 3 clinical trials (Berry et al., 2013). Note that the only drug currently available for ALS is Riluzole. It provides no relief of symptoms, but slows the progress of the syndrome by decreasing glutamate accumulation.

### ALS and SMA Overlap

A third hypothesis for ALS disease onset is gem depletion. Gems or gemini of Cajal bodies are protein products of SMN, TDP-43, FUS and SETX located in nuclear foci and in the cell cytoplasm responsible for the assembly of small nuclear ribonucleoproteins (snRNPs) (Tsuiji et al., 2013; Achsel et al., 2013; Cauchi, 2014). In spinal motor neurons with TDP-43 mutations there are depleted gems, snRNPs and small nuclear ribonucleotides (snRNAs) (Tsuiji et al., 2013). TDP-43 and FUS localize within nuclear gems together with the SMN complex and are involved in the maintenance of the spliceosome by controlling levels of snRNA (Tsuiji et al., 2013). It was further shown that accumulation of spliceosomes cause aberrant splicing of mRNAs resulting in motor neuron death in ALS and SMA. To clarify what gems are, how they relate to SMN, TDP-43 and FUS and the link between spliceosomes and motor function, Dr. Ruben Cauchi explains succinctly the RNA processing pathway. The pathway starts with the SMN-Gemins complex consisting of a nine-membered union of diverse proteins. More specifically, these are SMN, seven Gemin proteins (Gemin2-Gemin8) (Carissimi et al., 2006) and Unrip (Carissimi et al., 2005). The SMN-Gemins complex establishes the snRNP assemblyosome. "snRNPs are composed of one or two short noncoding RNA molecules (snRNAs) bound to a set of seven Smith (SM or Sm-like (Lsm) proteins, and a unique set of snRNP-specific proteins" (Matera, Terns, & Terns, 2007).

Working together with numerous non-snRNP splicing factors, U1, U2, U4/U6 and U5 snRNPs form the more abundant spliceosome that is responsible for splicing introns from pre-mRNA; as opposed to, U11, U12 U4atac/U6atac and U5 snRNPs that constitute the less available spliceosome (Patel & Steitz, 2003). The Lsm-class U6 and U6atac snRNPs, though, are synthesized in the nucleus while the core structure of the remaining Sm-class snRNPs is assembled in the cytoplasm. The published work on these interactions is fairly recent, and it is still not fully understood why in vivo requires the action of the SMN-Gemins complex (Otter et al., 2007) and involves the uploading of a heptameric Sm D1/D2/E/F/G/D3/B ring-shaped core domain onto the "Sm site," a conserved uridine-rich sequence motif intrinsic to snRNAs (Cauchi, 2014).

The snRNAs expelled from the nucleus following transcription are tagged by SMN-Gemins complex-independent Gemin5 protein. Once Gemin5 is charged with snRNAs it binds with SMN-Gemin complex, proximate to Gemin2, to form the Sm core assembly. Gemin2 is essential for the majority of Sm proteins recognition to form the ring-shaped domain. It also blocks foreign RNA binding supposedly until bona fide RNA substrates, snRNAs, are identified. (Zhang et al., 2011) (Figure 1). SMN's function in snRNP assembly, according to many, is the most decisive of all the SMN-Gemins complex members (Cauchi, 2014) and is probably linked to axonal mRNP trafficking (Fallini, Bassell, & Rossoll, 2012; Briesse, Esmaeili, & Sattelle, 2005). Proper Sm core assembly is necessary not only for stability and function of snRNPs, but also for snRNP biogenesis, including cap hypermethylation, 3' terminal trimming and eventual import into the nucleus. Once in the nucleus, snRNPs mature in Cajal Bodies (CBs) prior to pre-mRNA splicing. After multiple splicing procedures, snRNPs return to the CBs where they are regenerated or recycled (Staněk et al., 2008).

Mutations in the copy numbers of SMN1 and the identical gene of SMN2 are the principal etiologic basis of SMA cases. The number of copy numbers in SMN2 is inversely correlated with disease severity. One of the main theories explaining SMA is improper mRNA processing. According to the above, the fewer normal SMN proteins cause a lack of SMN-Gemins complex essential for pre-mRNA splicing (Borg & Cauchi, 2013; Briesse et al., 2009). Phenotypic SMA effects have been modeled in fly, zebrafish and mouse models with insufficient levels of SMN protein (Burghes & Beattie, 2009; Briesse et al., 2009). Gemins mutations, however, have not been associated with SMA. Investigators hypothesize SMN is unique in human genetics and a SMN2-like pseudogene in any of the Gemins would be incompatible with life. Dr. Cauchi conducted an experiment with organisms containing reduced levels of Gemins selectively in motor neurons. Organisms with this defect develop similar motor deficits found in the attenuation of SMN. Therefore, inadequate levels of any member in the SMN-Gemins complex can cause motor deficits. Decreased capacity of the SMN-Gemins complex can account for neuromuscular selectivity, based on sequencing of RNA from microdissected motor neurons of presymptomatic SMA mice; there are specific transcriptome abnormalities that link SMN deficiency to motor neuron pathology in SMA (Zhang et al., 2013).

The function of Gems is thought to colocalize with CBs in later developmental stages. Gems are viewed as storage depots for supplementary SMN-Gemins complexes since upregulation of SMN or Gemins induces gem formation or increases gem numbers (Cauchi 2011, Turner et al., 2014). Additionally, when there is loss of SMN or Gemins there is substantial decrease in gems and CBs.

ALS and SMA phenotypes affect lower motor neurons in the anterior horn of the spinal tract. There is a common tract and biochemical pathway that both MNDs share. ALS can also affect the upper motor neurons. Two of the more common genetic mutations found in ALS are TDP-43 and FUS. (Millecamps et al., 2010; Tsai et al., 2011). Surprisingly, these two genes engage in RNA processing. Under pathogenic conditions, the proteins of each are found concentrated in the cytoplasm and not in the nucleus in both neuronal and glial cells. This suggests there is a loss of proper nuclear function or toxic gain of function pertinent to ALS pathogenesis (Andersen & Al-Chalabi, 2011; Ferraiuolo et al., 2011; Lagier-Tourenne, Polymenidou, & Cleveland, 2010).

SMN has been found in decreased levels in transgenic SOD1 murine models (Turner, 2009) and SMN protein levels were reduced in ALS patients (Turner, 2014). This agrees with previous evidence relating decreased copy numbers of SMN2 to increased severity of ALS. (Veldink, 2005). Furthermore, loss of the SMN2 protein caused gem depletion in motor neurons, and knockout mice of TDP-43 showed altered numbers of gems (Shan et al., 2010). In ALS patient derived cells with TDP-43 or FUS mutations, the gem numbers are significantly reduced too (Yamazaki et al., 2012). Also, biochemical experiments have shown that TDP-43 and FUS interact with the SMN-Gemins complex (Yamazaki et al., 2012; Tsuiji et al., 2013).

### Biomarker Identification

Since both ALS and SMA are swift and crippling diseases, early diagnosis is essential for prognosis and future treatment options. Although, genetic testing is a current diagnostic tool for SMA, it does not test for all mutations in SMA, and is a premium that most cannot afford. Additionally, it does not provide a test for treatment response. Previous clinical trials for both ALS and SMA have produced no complete treatments for humans. There is a need to develop biomarkers that can help deliver quicker results from research into practice. At the same time, biomarkers facilitate the discovery of novel targets and pathways in pathogenesis.

One of the most successful approaches for diagnosis is biomarker identification, incorporating: proteomics, metabolomics and transcriptomics. Biomarker identification is not new to translational medicine. Biomarkers have been used tremendously in the cancer field and they are beginning to show promise in SMA too. After a recent cross-sectional study in Biomarkers for SMA (BforSMA), the top 5 biomarker candidates: CILP2, TNXB, COMP, ADAMTSL4 and CLEC3B may be used for diagnosis and testing response to treatment after further testing (Finkel (2012). A longitudinal study will be necessary to qualify the results of BforSMA. The BforSMA isolated the candidate plasma proteins, metabolites based on type of SMA presented since type I will produce possible differences than type II or III. Transcripts, though, did not

provide significant candidates in the BforSMA. The potential from the BforSMA is tremendous for future clinical trials and reduction in patients and costs associated with studies. ALS would also benefit from biomarker identification.

All of the BforSMA are only from peripheral blood mononuclear cells. As a result, there was an absence of change in gene expression or splicing suggesting that the degree of reduction of the SMN protein in this tissue is not enough to cause dramatic changes. This would be consistent with the fact that blood and other tissues do not exhibit the change of cellular or organ function except in Type I SMA. Since SMN protein is known to have reduction concurrent with genetic mutations in ALS, some of the biomarkers not tested for in BforSMA may serve as an interesting study for a common biomarker between these two seemingly disparate diseases.

Following the BforSMA study, a publication by Dr. Robert Bowser outlines biomarkers that have been discovered recently in transgenic models that may best represent ALS patients, along with prognostic determinants for ALS. Some of the biomarkers highlighted are SOD1 in the cerebrospinal fluid (CSF), increased levels of NF in blood/CSF, increased expression of CD4+ T cells (Bakkar, Boehringer, & Bowser, 2014). Some of the positive prognostic markers for ALS are low pNF-H, high sCD14 and low SI00B in blood/CSF (Bakkar, Boehringer, & Bowser, 2014). A disadvantage of these biomarkers is that they are only tested from transgenic models unlike the BforSMA, which tested human SMA patients. As with the BforSMA, longitudinal studies are necessary to qualify these findings.

There is significant evidence of genetic and mechanistic interaction between SMA and ALS. Unfortunately the low rate incidence of ALS – approximately 2 in every 100,000 people – has limited the expenditure of resources into the understanding of the pathogenesis and treatment of this disease. Thus any linkage between ALS and the more common disorder of SMA may prove extremely beneficial. With further research on the shared pathways and possible common biomarkers of ALS and SMA, therapies of MNDs may emerge that may combat the devastating consequences of both of these neurodegenerative disorders.

### Future Directions

To further clarify the potential linkage, between SMA and ALS, both the limb- and bulbar- onset types of ALS must be brought under a more uniform classification scheme that recognizes the homogeneity of these diseases which may then be applied to SMA. The elucidation of the genetic make-up of ALS may also lead to a common gene for multiple sites of origin. Moreover, some genetic mutations in ALS may also be shown to augment symptoms common in SMA. The fact that both of these MNDs affect

lower motor neuron function and share a common biochemical pathway in pathogenesis may lead to other insights into the interaction between these MNDs. Biomarker identification will likely prove extremely useful in future clinical trials as a potential possible diagnostic tool.

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# Nanotechnology

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## Abstract

Nanoparticles are gaining immense popularity in the medical world, specifically in its use in drug delivery systems. The objective of this paper is to study, based on available published literature, how nanoparticles are utilized in drug delivery and more importantly to identify the potential toxic effects of nanoparticles. Based on textual research, it is clear that there are benefits to nanoparticle use, but new studies are showing that there are many potential hazards of nanoparticle-like particles. In order to fully determine the toxicity of the hundreds of types of nanoparticles, a clear method to categorize these particles is needed and more research using empty nano-carriers needs to be done.

## Introduction

The U.S. National Nanotechnology Initiative defines Nanomaterial as materials that have at least one dimension in the 1-100 nm range. Despite its size, you can see things with the effects of nanoparticles all around you. They give the sunset its red color, allow birds to navigate, and help geckos stick to trees. All around us, there are nano-sized materials present from volcanoes, forest fires, viral particles, biogenic magnetite and combustion products (Nano.gov). What has taken the scientific world by storm in the last few years is not the naturally existing nanoparticles, but rather, it is the newfound ability to create new materials on a nanoscale. The term "Nanotechnology" by the material science standard refers to the creation of these new particles and its usage. In the physical sciences, nanotechnology is associated with the quantum behavior of subatomic particles in nanoscale structures. In the biomedical sciences, nanotechnology is used in imaging, diagnosing, monitoring diseases, gene delivery, artificial implants, and targeted drug delivery (Nasimi, Haidari 2013). Engineered nanomaterials are useful, specifically in drug delivery, because of their large surface area to mass ratio (Oberdorster 2004). When a drug is encapsulated in a nanoparticle, there is a more accurate delivery to the targeted tissue. Drug permeability will also increase, thereby reducing the dosing frequency. For instance, an intravenously administered hydrophilic drug has poor reabsorption after glomerular filtration, often caused by the rapid renal clearance of the drug; whereas, encapsulating the drug in a nanoparticle reduces renal clearance and allows for better absorption. An orally administered drug has to endure enzymatic degradation in the gastrointestinal tract and a pass through the liver before it enters systemic circulation. By encapsulating the drug in a nanoparticle, the exposures to harsh conditions on the digestive tract are minimized (Kadam, et al 2012).

The key to using nanoparticles in drug delivery systems is ensuring that the drug can be released at the proper time. In that vein, a biodegradable nanoparticle formulation would be needed, as it is the intention to transport and release the drug in order to be effective. However, model studies to the behavior of nanoparticles have largely been conducted with non-degradable particles. Most data concerning the biological behavior and toxicity of particles comes from studies on inhaled nanoparticles. This is part of the unintended release of ultrafine or nanoparticles by

combustion-derived processes such as diesel exhaust particles (Oberdorster, Oberdorster et al 2005). Research has demonstrated that exposure to these combustion derived ultrafine particles/nanoparticles is associated with a wide variety of effects, including vascular thrombosis, peripheral thrombosis, increased plasma fibrinogen levels and cardiovascular effects (Radomski, Jurasz et al 2005; Oberdorster, Oberdorster et al 2005). Since the size for both ultrafine and nanoparticles (100 nm) is relatively the same, many use both terms as equivalents. Based on the similarity of character and size between the two, researchers are speculating that the adverse effects of ultrafine particles, as part of environmental pollution, may be similar to the negative effects of engineered nanoparticles.

## Methods

The NCBI PubMedCentral database, the Touro College library Database, Proquest, and Google Scholar were search engines used to find information. The following key words were searched to obtain research related to this paper: nanotechnology, nanoparticles, nano-medicine, nanotechnology and medical uses, nanotechnology and drug delivery, toxic effect of nanoparticles, negative effects of engineered nanoparticles, toxicity and nanoparticles, applications of nanoparticles, and hazards of nanotechnology. Further sources were found by using appropriate references cited in various journals and reviews.

## Nanoparticles and Drug Delivery

Nanoparticles are formed through natural or human facilitated degeneration of larger structures or by controlled assembly processes. These procedures occur either in the gas phase, in a plasma, in a vacuum phase or in the liquid phase (SCENHIR 2006). Naturally occurring nanoparticles are found in the air in surprisingly high concentrations - approximately 106 to 108 nanoparticles per liter of air depending on conditions. They originate from the oxidation of volatile compounds, diesel and car engines, and photo-oxidation. The most significant concentration of particles and smallest particle size are associated with high-speed road traffic, apparently due to the subtle conditions during concomitant cooling and dilution of the exhaust gases (SCENHIR 2006). Although there are many naturally occurring nanoparticles, the ones that are man-made and biodegradable are the ones that are used in drug delivery systems (Oberdorster 2004).

### Preparation of Nanoparticles

Before choosing a method to prepare nanoparticles for use in drug delivery, many factors have to be taken into account. Some of these factors are the size of the nanoparticle required, the inherent properties of the drug (ex. solubility, stability), surface characteristics and the degree of biodegradability, biocompatibility and toxicity (Mohanraj, Chen 2006). The following are the three main methods used to prepare nanoparticles for drug delivery:

**Dispersion of preformed polymers:** A polymer is dissolved in an organic solvent, which is also used as the solvent for the hydrophobic drug. This mixture is then emulsified in an aqueous solution containing a surfactant. After a stable emulsion is formed, the organic solvent is evaporated. Some of the polymers used in this method include poly-lactic acid, poly(D, L-glycolide), poly(D, L-lactide-co-glycolide) and poly(cyanoacrylate) (Mohanraj, Chen 2006).

**Polymerization method:** Monomers are polymerized and form nanoparticles in aqueous solution. The drug is encapsulated either by dissolving it in the polymerization medium or by absorption onto the nanoparticles post-polymerization. The nanoparticles are separated from the suspension by ultra-centrifugation (Reis, et al 2006).

**Coacervation or ionic gelation method:** A significant amount of research has been focused on the preparation of nanoparticles using hydrophilic, biodegradable polymers such as chitosan, gelatin and sodium alginate. A method was developed by P. Calvo, et al. for preparing hydrophilic chitosan nanoparticles by ionic gelation. They mixed two aqueous solutions together- one was the polymer chitosan and the other was the polyanion sodium triphosphate. The positively charged group of the chitosan interacts with the negatively charged triphosphate to form coacervates (an aggregate of colloidal droplets held together by electrostatic attractive forces) with a size in the range of a nanometer (Calvo, et al 1997).

### Drug Loading and Release

The ideal nanoparticle for drug delivery should have a high drug loading capacity, thereby reducing the quantity of matrix materials (Mohanraj, Chen 2006). The two main ways to load a drug into a nanoparticle is by incorporating it at the time of nanoparticle production, or absorbing the drug after formation of the nanoparticles by incubating the nanoparticle carrier with a concentrated drug solution. The efficiency in loading the drug is very much dependent on the solubility of the drug in a solid state into the matrix material or polymer. The solubility is related to the polymer composition, molecular weight, the drug-polymer interactions and the type of functional group present (ester or

carboxyl) (Govender, et al 1999). Once the drug is loaded, the next step is to ensure an opportune release. In general, the release of the drug is dependent on the solubility of the drug, drug diffusion through the nanoparticle matrix and nanoparticle matrix degradation. (Mohanraj, Chen 2006). Very often, the drug is released by interactions between intracellular chemicals and the nanoparticle matrix. For example, the cationic surface of some nanoparticles allows penetration through the cell membrane and the drug is then released after the nanoparticle matrix is triggered by intracellular glutathione. Another example is the reduction of cadmium sulfate or ferric oxide (which are used to cap silica nanoparticles) by thiols that release the molecules inside the nanoparticle (Nasimi, Haidari 2013). An alternate mechanism for release is the use of pH-responsive nanomaterials. A further achievement in the area of drug release was reached when a method was developed to use multifunctional super-magnetic nanoparticles that can be released remotely (Derfus, et al. 2007).

### Passing the Blood Brain Barrier (BBB)

From several standpoints the brain is a challenging organ for drug delivery. First, the occurrence of progressive diseases in the brain will increase with the aging population. Secondly, the blood brain barrier (BBB) is well known as the best gatekeeper in the body toward exogenous substances. Generally pharmaceuticals including most small molecules do not cross the BBB. The BBB is formed by tight junctions between the cerebral endothelial cells, which abolish all aqueous diffusion pathways, and by biochemical systems consisting of enzymes, which specifically metabolize many drugs. However, the barrier properties may be compromised intentionally or unintentionally by allowing the passage of nanoparticles (Kreuter, et al. 2002). Many studies were done that show coating of the nanoparticles with the polysorbate (80) surfactants resulted in transport of drugs across the blood brain barrier (Schroder 1996). It is interesting to note that studies were first done using engineered nanoparticles to cross the BBB; however, with increased research it was discovered and published that natural ultrafine particles can cross the BBB and cause damage.

### Toxicological Hazards of Nanoparticles

#### General concepts

To effectively tap into the potential of Nanotechnology in Nanomedicine, full attention is needed to focus on safety and toxicological issues. For pharmaceuticals, precise drug delivery formulations may be used to increase the so called "therapeutic ratio" which is the margin between the dose needed for clinical efficacy and the dose that would induce adverse side effects (toxicity). Also, for these specific formulations, a toxicological evaluation is needed. The US Food and Drug Administration approval is crucial for clinical applications of nanotechnology, but considerable problems come into account when it comes to

approving nanotechnology-based products. The Food and Drug Administration regulate pharmaceuticals and biological devices differently, and it is not yet clear how emerging nanotherapeutics will be evaluated. It is important to have a safety guide particularly in the applications of nanoparticles for drug delivery. In these applications, particles are brought intentionally into the human body and environment (Buxton, et al 2003). Opinions started to divert when toxicologists claimed that new science, methods and protocols are needed (Nel et al 2006). However, the need for a safety guide is now underlined by several expert reports and more importantly by the following concepts:

1) Nanomaterials are made for their unique surface properties as opposed to the similar properties of bulk materials. Since the surface is the layer that interacts with the body tissue, and an essential factor of particle response, these unique properties need to be investigated from a toxicological perspective. When nanoparticles are used for their distinctive reactive characteristics, it may be probable that these same characteristics also have an impact on the toxicity of such particles. Although existing tests and procedures in drug and device assessment may work to detect many risks related to the use of these nanoparticles, it cannot be presumed that these assays will detect all potential risks. (SCENIHR 2006) The toxicity may differ depending on the type of particles used, i.e., biological versus non-biological origin.

2) Nanoparticles are recognized as having different physical and chemical characteristics from micron-sized particles. This may result in changed body distribution, passage of the blood brain barrier and triggering of blood coagulation pathways. In view of these characteristics, specific emphasis should be on testing and studying the distribution of nanoparticles. What is currently lacking is a basic comprehension of the biological behavior of nanoparticles in relation to the distribution in vivo both at the organ and cellular level.

Using nanoparticles as a drug carrier may reduce the toxicity of the incorporated drug. In general, research focuses on the toxicity of the entire formulation. The results of the nanoparticles itself are not described, so differentiation between drug and nanoparticle toxicity cannot be made. There should be a specific emphasis on the toxicity of the "empty" non-drug loaded particles. This is especially important when slow or non-degradable particles are used for drug delivery. (Oberdorster, Maynard, et al. 2005)

## Evidence for Nanoparticle Toxicity

The largest database on the toxicity of nanoparticles comes from the PM10 literature (particulate matter with a size below 10  $\mu\text{m}$ ), where studies on inhalation and the 'Nanoparticle hypothesis' have proved to be a powerful drive for research (Oberdörster, Oberdörster et al 2005). Therefore it is relevant to discuss this

<b>Table 1</b>	
<b>Particle type</b>	<b>Description</b>
PM10, PM2.5	Particle mass fraction in ambient air with a mean diameter of 10 or 2.5 $\mu\text{m}$ respectively. Basis of current standards for ambient particles in Europe and USA
Coarse particles	The mass fraction of PM10, which is bigger than 2.5 $\mu\text{m}$
Ultrafine particles (PM0.1)	The fraction of PM10 with a size cut-off at 0.1 $\mu\text{m}$ . Contains primary particles and agglomerates smaller than 100 nm
PSP	Poorly soluble particles with low specific toxicity. Maybe be fine or ultrafine. Terminology used in relation to bulk synthetic particles. Examples TiO <sub>2</sub> , carbon blacks, Amorphous silica, Iron oxides (Fe <sub>2</sub> O <sub>3</sub> ), Zinc oxides (ZnO)
CDNP	Combustion derived nanoparticles, such as diesel exhaust particles (DEP)
DEP	Diesel exhaust particles

*Various denominations of particles in inhalation toxicology and drug delivery in relation to their source (ambient, bulk, engineered)*

evidence with the expectation that it will shed light on the toxicity of engineered nanoparticles. The adverse health effects of particulate matter (PM) are measurable as causes of respiratory disease and deaths as well as hospitalizations and deaths from respiratory and cardiovascular disease. Many laboratory studies have been done to investigate the effects of ultrafine and PM particles. It was found that some nanoparticles might have the extra potential of affecting cardiovascular disease directly. Vascular function was impaired after inhalation of diesel exhaust particles. However, data to date is limited and not all studies of nanoparticles have shown significant translocation from lung to the blood. Understanding clearance kinetics of inhaled ambient air nanoparticles will also be important in understanding the potential for adverse effects. (Oberdorster, Maynard, et al. 2005)

The current standard in particle toxicology is that ultrafine ambient air particles have the potential of affecting cardiovascular disease both indirectly via pulmonary inflammation and directly through particle distribution. Although significant, this property of redistribution has yet to be demonstrated for nanoparticles present in real PM<sub>10</sub>'s. It should be noted that there are several mechanisms whereby nanoparticles could lead to inflammatory effects, as is the case for larger particles. These mechanisms are either based on the large surface area of a particle core or on soluble components released by the nanoparticles (Schins, et al. 2004). Several toxicological studies support the argument that nanoparticles in PM<sub>10</sub>'s could drive inflammatory effects. There are a number of components of PM<sub>10</sub>'s that contribute to the mass but have little toxicity, including salts such as sulfates, chlorides and ammonium salts and nitrates. In fact, within PM<sub>10</sub>'s there are only few components that toxicologists would identify as likely causes of adverse effects – i.e., particle surfaces, organics, metals and endotoxins. A large surface area, organics and metals are all characteristic of combustion-derived particles and so these have attracted considerable toxicological attention (Donaldson, et al. 2005).

### **Effects of Nanoparticle Toxicity**

Many physicochemical factors can influence the potential biological interactions and toxicity of nanoparticles. Therefore, it is important to consider the extent to which the physicochemical properties of nanoparticles have been characterized in any given study. Without sufficient characterization, it is extremely challenging to interpret the results of individual studies and virtually impossible to compare the results of different studies, even in cases where the same nanoparticle has been investigated. As a result, the ability to identify parameters that might influence toxicity is hampered. Although there is not yet a universally accepted standard of parameters that is deemed necessary for nanoparticle characterization, recent reports have highlighted several key physicochemical elements for which it is strongly recommended that data be

reported (Oberdorster, Maynard, et al. 2005). These limitations include method of synthesis, size, size distribution, shape, composition, crystal structure, aggregation and agglomeration status, dissolution, purity, surface area, and other surface characteristics. Classification of nanoparticles in the context of the experimental contact media (cell culture media, dosing solution, aerosol, etc.) is also of substantial importance, as some physicochemical parameters are likely to differ depending on whether they are determined in the experimental media or in the bulk (i.e., "as received") state. Unfortunately, the inclusion of all these parameters in publications describing nanoparticle toxicity studies appears to be rare.

### **Nanoparticles in The Lungs**

When nanoparticles enter the respiratory system, they are thought to cause damage that results primarily from lung particle overload. This is due to the inability of alveolar macrophages to recognize and/or clear particles of this size, resulting in a particle build up, chronic inflammation, fibrosis, and tumor-genesis. However, many studies have not shown a correlation between nanoparticles and inflammation (Card, et al, 2008). Many studies also track whether the particles translocate from the pulmonary system into systemic circulation. One of the studies reported to date indicate that inhaled 99mtechnetium-labeled carbon nanoparticles, which are man-made, are not detected outside of the lungs in significant quantities after inhalation. However, as mentioned to by Mills et al., these findings do not indicate that other nanoparticles will behave in the same manner, nor do they rule out the possibility that nanoparticles may interact with and influence the vascular system in the lungs. Moreover, the studies conducted to date have used a single inhalation exposure protocol, and it is possible that repeated exposures may result in greater pulmonary accumulation and transfer of significant quantities of nanoparticles to the circulation (Mills, et al. 2006).

Fibrosis is a condition that many researchers believe is caused by nanoparticle exposure. Many experiments were done using animals to test the effects of primarily carbon nanotubes, carbon black, fullerenes, silica, and metal-based nanoparticles including titanium dioxide, silver, and nickel. Though it is known that the pathogenic mechanisms underlying animal models of lung fibrosis and human lung fibrosis are not necessarily the same, increased collagen deposits and structural deviations to the lungs can result in changed respiratory mechanics that are common features of both. Fibrosis in animal models is defined by increased collagen content and/or histopathological evidence of structural alterations to the lung that are consistent with fibrosis (Card, et al, 2008). It was found that the type of carbon nanotubes, their length, and the way specific fibers interact, all have varying effects on pulmonary inflammation and fibrosis. Studies have found that the longer the nanotube fiber length, the greater the toxicity, and the more likely it is to cause fibrosis and cancer (Donaldson, et al. 2006).



### Effects on Blood and Cardiovascular System

In a study by Radomski, et al, (2005) the effects of various nanoparticles on platelet function were studied. In vitro studies were done using human blood samples, and then in vivo studies were done in rats to confirm the effects of platelet- aggregation found in the human blood. Engineered nanoparticles were found to cause activation and aggregation of human platelets. The efficacy of the nanoparticles in blood aggregation in vitro was matched by the same results in the rats. Treatment with nanoparticles caused rat vascular thrombosis. The data shows that not all nanomaterials act similar in this test, and that surface area is not the only factor playing a role here. The data also validates the idea that mainly cationic species have an effect on blood clotting. Interestingly, this was the first study that allows bridging of data, since a PM10 sample (SRM1648) was included in the test-series, in combination with nanoparticles. The PM sample actually showed a lower effect on platelet aggregation compared to the carbon nanotubes (Radomski, Jurasz et al 2005). Another study shows that repeated exposure to PM10's causes a systemic inflammatory response, including bone marrow stimulation, and is related to the progression of atherosclerosis in the coronary arteries and aorta (Suwa, et al. 2002).

### Uptake and Effects of Nanoparticles in The Brain

Nanoparticles can gain access to the brain by two different mechanisms. (1) Trans-synaptic transport after inhalation through the olfactory epithelium. (2) Uptake through the blood-brain barrier. The first pathway has been studied primarily with model particles such as carbon, Au and MnO<sub>2</sub> in experimental inhalation models in rats (Oberdörster et al 2004; Oberdörster, Oberdörster et al 2005). The pathway via the BBB has been the topic of research for a while, especially for drug delivery. Studies suggest that the physiological barrier may hinder the distribution of some proteins and viral particles after trans-vascular delivery to the brain, suggesting that the healthy BBB contains defense mechanisms protecting it from blood-borne nanoparticle exposure. When nanoparticles with different surface characteristics were evaluated, neutral nanoparticles and low concentrations of anionic nanoparticles were found to have no effect on the BBB, whereas high concentrations of anionic nanoparticles and cationic nanoparticles were toxic for the BBB (Nel et al 2006). Fullerenes and C60 nanoparticles have been shown to induce the production of reactive oxygen species, oxidative stress, and rapid brain lipid peroxidation in marine species. Because of all the negative effects on the brain by nanoparticles, further tests would need to be done before using fullerenes and C60 for human and industrial use (Oberdorster 2004).

### Conclusion

Although there is a considerable amount of data on the toxicity of nanoparticles, this data is mainly based on a small sampling

and the assumption that a lot of effects by particulate matter are driven by the ultrafine particle fraction in it (Oberdörster, Oberdörster et al 2005). This small sampling doesn't really give enough information to adequately determine the potential hazards. Although hazard identification is the general approach for safety evaluation of healthcare products, it is recommended to add testing driven by the anticipated application and classification by risk. Some engineered nanoparticles that are airborne will pose inhalation and cardiovascular hazards, while cosmetics with nanoparticles provide dermal exposures. Each nanoparticle formulation should be tested on a case-by-case basis in the requisite ways focusing on their method of entry. In this respect the potential adverse effects of empty particles should also be considered. In developing testing procedures and protocols a number of basic issues need to be considered:

- 1) It needs to be determined whether noticed effects are caused only by nanoparticles, or are the adverse effects caused by something else and only aggravated by nanoparticles. It is clear from research that both PM and ultrafine particles can cause inflammation, cancer, etc., but these new and smaller nanoparticles may cause different effects.
- 2) Most of the conclusions drawn about nanoparticles are based on correlations made between the behavior of ultrafine particles and PM's to that of nanoparticles. The question is how much of a correlation is permitted to be drawn, how many assumptions can be made, as manufactured nanoparticles and ultrafine/ PM particles are not identical.
- 3) The scientific world is dealing with a increasingly growing number of nanomaterials. All have the potential to create a new toxic effect that has never been studied before. The current testing and classification system for nanoparticles does not seem sufficient to fully identify and quantify the toxicological effects of these new nanoparticles.

For years pharmaceutical sciences have been using nanoparticles to reduce toxicity and the side effects of drugs. Up to recently it was not realized that these drug carrier systems themselves may cause risks to the patient. The type of hazards that are introduced by using nanoparticles for drug delivery are beyond what is posed by conventional hazards imposed by chemicals. However, as of current data, the scientific example for the possible toxic and adverse reactivity of nanoparticles is lacking and we have little understanding of the basics of the interaction of nanoparticles with living cells, organs and organisms. A conceptual understanding of biological responses to nanomaterials is needed in order to develop and apply safe nanomaterials in drug delivery in the future. Furthermore, a close partnership between those working in drug delivery and those working in particle toxicology is necessary for

the exchange of concepts, methods and to establish a common system for identifying the potential dangers of nanoparticles.

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# Should Sports Drinks be Given to Children and Adolescents Engaged in Athletics as an Effective Source of Hydration?

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## Abstract

Dehydration in children and adolescents is a major concern for caretakers. Often children are not drinking enough, particularly while participating in physical activity. Utilizing data drawn from pediatricians, nutritionists, and bio-scientists, this paper will examine whether sports drinks are a good source of fluids for the exercising child. The results of the research indicate that the advantages generally associated with sports drinks, to achieve higher body water absorption levels, prevent electrolyte deficits, and maintain proper electrolyte/water concentrations, are reserved for unique circumstances. Sports drink intake should be encouraged only when the child is participating in prolonged physical activity, in intense exercise with short break intervals, or in hot/humid environments. In these instances, sports drinks adequately resupply the lost fluids and electrolytes. Furthermore, the carbohydrates contained in sports drinks replenish energy supply. However, under normal conditions, sports drinks are associated with health risks, primarily due to their excess of sugar. Care should be taken to encourage children to avoid sports drinks and instead encourage them to consume plain water at a rate equal to sweat loss. The research establishes that health risks involved with sports drink intake outweighs the benefit of increased voluntary consumption.

## Introduction

### *The Importance of Hydration*

Water is an essential nutrient required for life. It is the most abundant compound found in the human body and is responsible for moving nutrients along the body's pathways. Water is also involved in biochemical reactions as a solvent and reagent; its physical properties affect reaction rates and mechanisms of various processes. Dehydration, an imbalance between water intake and body water loss, which can be caused by sweating, respiratory process, and/or urine production, is generally defined as a loss of 1-2% of body weight (Kliener, 1999). This level of dehydration increases the core body temperature (Ganio et al., 2007), impairs aerobic capacity, decreases plasma volume, and increases cardiovascular strain (Hill et al., 2008). Furthermore, studies indicate a correlation between insufficient fluid intake and different forms of cancers (Kleiner, 1999). Even when the water concentration deficit is less than 1% there can be negative effects, such as headaches, digestive problems, lack of concentration, and dry skin (The Expert Group on Hydration, 2005).

"Sports drinks are beverages that may contain carbohydrates, minerals, electrolytes, and flavoring and are intended to replenish water and electrolytes lost through sweating during exercise" (Pediatrics, 2011).

### Children

The average daily total water recommendation for children aged 6 to 11 is 1.5 liters for girls and 1.7 liters for boys (National Academy of Sciences, 2005). Children aged 11 and above should be drinking approximately 2 liters per day. Special care needs to be taken to ensure that children and adolescents are properly hydrated. They are less likely to take the time to drink regularly, especially when in school where decisions are made for them (The Expert Group on Hydration, 2005). Due to their smaller

size, children are at greater risk of the effects of poor hydration (Pediatrics, 2000).

Data from the U.K.'s National Diet and Nutrition Survey of 2000 are cited to demonstrate that most children are not drinking enough and most parents are not aware of this issue. In the study, 40% of 11-18 year olds were consuming less than the Food Standards Agency's minimum of 1.2 liters. In schools, 38% of students were not offered sufficient fluids even after physical education (The Expert Group on Hydration, 2005), despite the fact that the effects of dehydration can be especially severe when children exercise. They generate more metabolic heat per mass unit than adults do during exercise, even when just walking or running (Pediatrics, 2000).

Due to the high risk of dehydration, should sports drinks be given to children and adolescents engaged in athletics as an effective source of hydration?

## Method

Critical analyses of research articles were utilized to gather this information. These articles were collected from online databases and journals, such as, Touro College's online databases, PubMed, and Google Scholar.

### Sport Drinks: A Source of Hydration

Many experts assert that sports drinks are an invaluable source of hydration, as they maintain body electrolyte concentrations and lead to greater fluid consumption.

### Electrolytes

Excessive intake of sodium-free drinks, such as plain water, can lead to hyponatremia, a rapid fall in serum sodium concentration and serum osmolality (Maughan, et al., 1993). Even replacing large

fluid losses with equal amounts of pure water may dilute the plasma sodium level (Ganio et al., 2007). Water alone cannot replace electrolytes lost through sweat. Therefore, it has been suggested that electrolytes and water should be replaced with sports drinks that contain the proper amounts and ratios of these elements (Maughan, et al., 1993, Pediatrics, 2000).

### Water Absorption

Dr. R. J. Maughan, a Fellow of the American College of Sports Medicine, and his colleagues maintain that electrolytes contained in sports drinks enhance fluid absorption in the body. Sports drinks manufacturers frequently use this assertion when advancing their products and also claim that sports drinks are scientifically formulated to tackle dehydration. Lucozade®, a drink manufacturer states, “Adding both carbohydrate and sodium to a sports drink, helps your body absorb fluids more effectively” (Lucozade, 2015).

### Taste

Naturally, people, especially children, tend to consume fluids that they enjoy. The effect of taste is found even within various sources of water. One survey found that out of 124 respondents, 43% said taste was the primary reason they chose bottled water over tap water (Kliener, 1999). Surely, the palatability of cool, pleasant-tasting sports drinks greatly enhance fluid intake. More specifically, sodium chloride, commonly found in sports drinks, has been shown to increase voluntary fluid intake by 90% compared to unflavored drinks (Pediatrics, 2000). Additionally, the carbohydrates that sports drinks contain, to various degrees, may influence palatability (Maughan, et al., 1993). Therefore, while water is an easily available drink, flavored beverages, sports drinks, are often the beverage of choice to increase fluid intake, specifically, in the case of an exercising child, where the need for rehydration and sensitivity to flavor are especially significant.

### Thirst Inhibiter

The body's natural reaction to dehydration is thirst. The hypothalamus senses fluid loss when the concentration of sodium in the body rises. It responds by increasing renal sodium and water conservation, which stimulates the sensation of thirst (Noakes, 2012). Drinking plain water could lead to a rapid fall in sodium concentration which could alter the chain of reaction, ultimately inhibiting thirst and causing a reduction of fluid intake. Sports drinks are preferred, since they have accurate water-sodium ratios and do not inhibit thirst.

### Refuting the Claims in Favor of Sports Drinks

#### Taste

While tap water does not have a favorable taste and can lead to children not drinking enough fluids, palatability can be achieved in ways other than sports drinks. For example, parents and

caregivers can supply children with filtered, purified, and/or bottled water. If this does not supply the desired fluid intake, taste can be enhanced by adding a minimal amount of flavor, such as lemon slices, to the water (Ahmad, 2008).

### Electrolyte

Sweating is the most efficient method that the body uses to dissipate excess heat, either a result of byproducts of muscular work or from a hot environment (Burke, 1997). The water contained in sweat decreases the core body temperature through evaporative cooling at the skin surface. Generally, the sweat produced by the average exercising child or adolescent is a relatively dilute plasma secretion containing far more water than electrolytes. Since the main fluid lost is water, the main replacement should be water, not sports drinks which contain electrolytes (Ahmad, 2008). Nevertheless, to avoid incorrect electrolyte/water ratios, care should be taken not to drink disproportionate amounts of water (Robert Wood Johnson Foundation, 2012, Pediatrics, 2011). Most children's and adolescents' daily electrolyte requirements are met sufficiently by a healthy balanced diet, while sports drinks offer little to no advantage over plain water. Furthermore, sports drinks can be harmful due to excess caloric, fat, and protein intake and the potential nutritional imbalance that can ensue.

In addition, hyponatremia has been observed in cases of dehydration, maintained hydration, and over hydration. Therefore, excessive water intake cannot be correlated to hyponatremia; the cause may be multi-faceted and circumstantial and further studies need to be done before conclusions can be drawn (Ganio, Casa, Armstrong et al, 2007).

### Energy Source

Despite the above arguments, some maintain that sports drinks are still advantageous for energy resupply (Maughan, et al., 1993). However, since the average child athlete does not need quick energy supplements, there is little need for energy-containing beverages. The recommended daily intake of fruit juices, low fat milk and other daily diet food supply the appropriate amounts of carbohydrates and proteins that children need (Pediatrics, 2011).

### Water Absorption

The advantage of increased total water absorption attributed to sports drinks is only under conditions where further bouts of exercise occur after short recovery periods. However, during rest or low intensity exercise, sports drinks do not improve the overall water absorption rate or the amount consumed by means of plain water. This assertion is maintained by an innovative experiment using a deuterium dilution technique to compare the hydration ability of commercially available sports drinks versus water (Hill, 2008). Scientists provided three different sports drinks solutions, as well as plain water, ingested with an isotope



tracer, to exercising subjects (aged 18-35) at set intervals over the course of sixty minutes. The team then analyzed their saliva samples; the appearance of deuterium in saliva indicated the uptake of water in the body's tissues and enabled the team to calculate the kinetics of the water absorption. With the aid of proven mathematical modeling, the kinetics data provided the researchers with estimates for the maximum absorption rate, the time at which absorption was complete, and the percentage of solution absorbed, at any given time. This innovative approach measured the gastric emptying rate and the intestinal absorption, two major factors that affect absorption of the solution's water content. It is important to note that the exercise was not intensive, consisting of walking on a treadmill at 55% of heart rate maximum for an hour. The results of the experiment clearly indicated that for the speed of rehydration, the maximum rate of absorption was achieved with sports drinks rather than with water. Nonetheless, by half of the total time of absorption there was no difference in the rate of absorption between the sports drinks and water. Furthermore, by the end of the total time, the sports drinks and water solutions accomplished the same total absorption volumes. Therefore, the study determines, "under conditions where recovery periods occur between further bouts of exercise, sports drinks may be favored due to faster speed at which they reached their max absorption rate." However, for normal, non-intensive, conditions, since there is no advantage in total water absorption and no need for quick rates of rehydration, there is no hydration advantage in providing sports drinks instead of plain water.

## **Sports Drinks – A Bad Choice For Hydration**

### ***Excessive Sports Drink Consumption***

Sports drinks contain sugars and recent studies show that most children today consume far more added sugars than the maximum daily recommendation (Johnson RK et al., 2009). Beverage patterns and trends among school-aged children in the United States indicate that, between 1989 and 2008, the percentage of American children aged 6 to 11 consuming sports drinks increased from 2 percent to 12 percent (Lasater et al., 2011). A study by a group of nutritionists measured US children and adolescents per-capita daily caloric intake from sugar-sweetened beverages (SSB) and 100% fruit juice. The authors found that SSB consumption increased from 242 kcal/day in the years 1988-1994 to 270 kcal/day in the years 1999-2004. One hundred percent fruit juice intake increased from 38 to 48 kcal/day. The largest increases occurred among children aged 6 to 11 years (Wang et al., 2008). In another study, researchers asked adolescents why they drank sports drinks. No one claimed they did so to maintain hydration (O'dea, 2003).

Excessive sugar intake causes weight gain, poor nutrition, displacement of healthy ingestion, obesity, and poor dental health. In

addition, SSBs lead to type 2 diabetes and cardiovascular disease (Robert Wood Johnson Foundation, 2012). Additionally, the gratuitous amounts of sodium found in sports drinks displace necessary nutrients and results in excessive caloric intake (Pediatrics, 2011). Therefore, to achieve optimal health, sports drinks should not be used as a source for hydration.

## **The Value of Sports Drinks**

In the event that the child is playing in a hot and/ or humid environment maintaining hydration is even more crucial, as children do not adapt to extreme climatic changes as well as adults do. "A child may need as many as 8 to 10 exposures (30 to 45 minutes each) to the new climate to acclimate sufficiently." Hot air temperatures and high humidity levels, even with relatively normal air temperature, may lead to heat stress. Furthermore, children have a large area-to-body mass ratio which results in significant heat gain. Moreover, children's sweating capacity is considerably lower than adults, which diminishes their ability to dissipate body heat via evaporation. Consequently, children exercising in hot and humid environments are at greater risk from the effects of dehydration (Pediatrics, 2000). More specifically, children playing in hot and/or humid conditions for more than one hour lose excessive amounts of electrolytes, such as sodium, potassium, and chloride, which cannot be replaced sufficiently by plain water (Ahmad, 2008). Under these conditions, "[s]ports drinks have been shown to decrease fatigue and replace electrolytes lost in sweat" (Robert Wood Johnson Foundation, 2012).

Likewise, sports drinks are appropriate for children and adolescents who engage in prolonged vigorous physical activity, as the amount of energy and electrolytes lost cannot be regained through plain water alone (Robert Wood Johnson Foundation, 2012). To prevent fatigue and maintain performance it is necessary to resupply muscle glycogen stores through ongoing intake of carbohydrates. The carbohydrates and electrolytes contained in sports drinks restore the loss, and provide the necessary nutrients in the proper ratios.

## **Discussion**

In view of the above, for the average child, water is the best form of hydration. Sports drinks are detrimental, in large part because they provide children with excess sugars. Furthermore, their added ingredients can negatively affect the proper amount and relative proportion needed for a healthy diet. Cohen (2012) affirms that after 38 years of research there is still no scientific proof for sports drinks providing higher fluid intake or better absorption than water. Sports drinks were designed for committed athletes, and they should be restricted to them.

In addition, while sports drinks may be advantageous because of their greater palatability in comparison to tap water, which will

lead to children consuming more fluids, parents and caregivers should keep in mind that the bad health effects far outweigh these benefits. Therefore, water should be the beverage of choice, and if the child is not drinking enough, a parent should seek methods to encourage voluntary water consumption, such as by providing cool, filtered water and promoting the health benefits of water (Loughridge and Barratt, 2005).

Nutritionists offer water drinking guidelines to maintain hydration. In the case of a child participating in normal physical activity, for less than three hours and in normal air/humidity conditions, water intake should occur every 15-20 minutes (Ahmad, 2008). The main body fluid lost is water, therefore, water is the only substance needed to be replenished. Still, care should be taken to replace water at a rate equal to, not greater than, the sweat rate (Ganio et al., 2007). In events of intensive physical activity, water and a balanced diet, for example, soup, fruits and vegetables, are enough to replace the water, carbohydrates and electrolytes lost during exercise, provided the activity does not last more than an hour (Robert Wood Johnson Foundation, 2012). In the event of prolonged physical exercise, children should be well hydrated before and during the activity. Periodic drinking should be enforced throughout, even if the child does not feel thirsty (Pediatrics, 2000). Also, children and adolescent athletes should drink water even after the activity is over (Pediatrics, 2011).

In cases of hot or humid conditions and during intense exercise lasting over an hour, since there is a greater loss of electrolytes (sodium, potassium, and chloride) and energy, water alone is not enough to rehydrate and replenish energy, and sports drinks should be provided (Robert Wood Johnson Foundation, 2012). Ahmad (2008) advises that every 15-20 minutes the child and adolescent should be provided with five ounces of electrolyte-replacing fluids such as water mixed with juice or sports drinks.

Also, sports drinks are commendable in situations where quick rehydration is critical. It is important to note that the various amounts of sodium, potassium, and magnesium, found in the different sports drinks all achieve quicker retention rates over a short period of time, in relation to plain water (Hill et al., 2008).

### Thirst as an Indicator for Hydration

In general, as mentioned in the paper, exercise raises the core body temperature which causes the body to sweat out the heat and to replenish fluids lost in the process by the conscious feeling of thirst (Noakes, 2012). Over the course of the research, however, it was evident that scholars debate whether thirst is a good indicator for fluid replacement in children and adolescents. Some argue that thirst is not a good indicator and relying on it for fluid intake may lead to severe dehydration. Firstly, thirst often-times lags behind changes in hydration (Ganio, et al., 2007).

The threshold of the indication of thirst occurs at a point when a person is already dehydrated to a level of .8 % to 2% loss of body weight (Kliener, 2009). Secondly, drinking water creates a reduction in plasma osmolality and reduces the drive to drink, even before sufficient fluid has been consumed to replace loss (Maughan RJ et al., 1993). Thirdly, children frequently do not feel the need to drink enough to replenish fluid loss during prolonged exercise, and if they find plain water non-appealing, they may not drink even if they are feeling thirsty (Pediatrics, 2000). Lastly, environments may alter the thirst mechanism. For example, swimmers may have virtually no thirst response during immersion (Kliener, 2009). Therefore, these researchers conclude that children should be given to drink even if they are not thirsty. The risk of relying on just thirst to identify when to provide fluids is greater in hot/humid environments, where the potential for hypo-hydration in children and adolescent athletes is even greater.

However, it can be argued that thirst is a reliable indicator; it is a natural biological response to dehydration and children rehydrate voluntarily when fluid is available (Kliener, 2009). Cohen (2012) questions the scientific validity of studies that claim thirst to be unreliable, calling it a "war on thirst". The article states that scientists who invalidate thirst as an indicator of dehydration are associated with the sports drinks industry and their results are biased. Instead, Cohen's article maintains that thirst is a good indicator of when to drink. In addition, humans do not need to immediately replace all the fluids lost during routine exercise; humans are delayed drinkers. Individuals can rely on the next meal for most fluid replacement. Thirst is a dependable indicator of water and electrolyte deficits that need to be imminently rectified (Noakes, 2012).

### Conclusion

The negative effects of sports drinks render them unfit to properly hydrate children and adolescents; instead plain or flavored water should be the primary beverage. However, if an exercising child is not drinking enough, is playing for more than three hours in normal conditions, is engaging in repeated bouts of exercise interspersed with short breaks, or is exercising in hot, humid conditions for over one hour, then sports drinks would be the best source of hydration.

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# Viability of SiRNA as a Clinical Treatment

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## Abstract

The purpose of this paper is to better understand the methods problems and some solutions for siRNA treatments. The benefits of this novel medical treatment are explored and its benefits are expounded on by comparing it to other more complex and futuristic treatments. The exact process of siRNA silencing and down regulation is unknown. Some hypotheses of how it may work are discussed giving precedence to the most widely accepted hypothesis. Although siRNA treatments are not yet used on a major scale for many diseases, different possible treatment options are compared and explained. Particular care was taken to give a broad range of illnesses in order to show the vast possibilities of siRNA therapy. The common problems and some methods to overcome them are arranged in an orderly manner starting from the time the siRNA treatment is administered, through its delivery and subsequent potency, followed finally by its degradation. SiRNAs viability as a treatment is then analyzed primarily on its large interest in many fields, as well as the enormous progress it has made since its discovery just a short while ago.

## Viability of SiRNA as a Clinical Treatment

The future of medicine is heading closer and closer to perfecting gene therapy. While research is bringing the option to alter genes closer to fruition, there is still a long way until it will be able to be implemented. Simpler therapies albeit with their own set of problems, seem to be a more realistic option for the foreseeable future. One in particular that has seen some progress is the use of small interfering RNA (siRNA). "Since its discovery by Fire and Mello in 1998, siRNA has proven to be a powerful tool for modulating the expression of almost any gene in various species." (Nechaev, et al, 2013) During Göran Hansson's speech after receiving the noble prize for the discovery of a form of RNA silencing, he said, referring to RNAi (more general term for siRNA), that it "has added a new dimension to our understanding of life and provided new tools for medicine." (Eggleston, 2009).

It can have many applications and doesn't necessarily require the alteration of DNA. It allows the DNA to remain in its state of disarray however it won't allow the problematic gene to be translated. This allows for control of the gene without having to alter the DNA. The applications for this are countless. SiRNA can treat many otherwise untreatable diseases ranging from viral infections to complex genetic disorders (Endo-Takahashi, et al. 2012). Although the concept of siRNA is not a new one, there are some kinks in how to use it as a treatment. One major setback is that although it is now possible to create this aforementioned silencing RNA (siRNA), its method of delivery is problematic. The siRNA, if delivered indiscriminately, can wreak havoc in cells it wasn't intended for. It can stop crucial growth and functional elements of important life sustaining cells. It is therefore important to deliver these potentially harmful substances only to their intended targets. This is just one of a few issues that can be a major hindrance in siRNAs future as a treatment. It is imperative to understand how siRNA works before an approach can be found to tackle the issues that can arise in using siRNA as a treatment.

SiRNA silences the expression of specific genes allowing for particular cell functions to seize. SiRNA begins its life as a double stranded RNA molecule (dsRNA). The dsRNA is cleaved into

shorter siRNAs. This process requires dicer to cleave the large dsRNA. The siRNA is incorporated into a protein complex referred to as RNA inducing silencing complex (RISC). The still double stranded siRNA is unwound by the protein complex via multiple steps. The now single strand of siRNA leads the RISC to the target RNA destroying it (Schwarz, et al, 2014). The destroyed mRNA is no longer able to perform the function it was coded for. This allows for a cell to continue with its other functions as long as the specified protein isn't crucial for the cells normal function. The siRNA does not interact with the genome directly allowing for less risk of creating mutant cells.

## Possible Applications of siRNA

SiRNA has been studied largely for its application as a cancer suppressor. It has however, many applications beyond that. Whether in its ability to lower circulating cholesterol (Tep, et al, 2012) by inhibiting the translation of a specific protein, or as a method of controlling HIV-1 (Zhou, et al, 2008) as well as possibly reducing any other potentially harmful protein synthesis. SiRNA can have other non-clinical applications as well. Although the same basic ideas and methods are used for all potential treatments associated with siRNA the broad range of diseases it can potentially treat allows for some unique applications.

One study on the effects that siRNA can play in treating ovarian cancer used a pretty classical method of using siRNA. The surface protein cd44 is necessary in activating signal pathways necessary for cancer, specifically metastasized, to continue its growth and destruction. These pathways instruct the cell to continue transcribing proteins necessary for cell growth, as well as other factors that allow the cancer cells to live and multiply. The cd44 protein is also only found to be expressed by metastasized cancer cells, thus negating many of the issues associated with siRNA delivery that will be discussed later. The study used siRNA as well as an anticancer drug to see the combined effects they could have on metastasized ovarian cancer. The siRNA was specific to mRNA coding for cd44. It was carried out in vitro as well as in vivo with similarly successful outcomes; the cancer cells growth was slowed dramatically in both tests. Compared to just the use

of the cancer drug and other chemotherapies, it was deemed more efficient and aggressive. This is a truly great step forward in finding a new cure with this rarely used method of treatment. (Shah, et al, 2013).

SiRNAs use as a treatment for HIV is also being considered. "HIV-1 gene expression, during productive and chronic infection, is essentially dependent upon the early regulatory genes tat and rev." (Caputo, et al, 1997) If a method to stop these regulatory genes from being expressed can be found it can be used to virtually control HIV. Although there have been many improvements in controlling and preventing new outbreaks of HIV and the associated life altering conditions, HIV is still a still prolific disease (Centers for Disease Control, 2013).

Working on this premise, researchers created a siRNA delivery method that is specific to the HIV strain they were interested in controlling. The siRNA was manufactured to be specific to the genes that coded for tat and rev. In stopping the expression of these genes that are crucial for the viability of HIV-1 they were in essence stopping the disease. Through methods including quantitative real time PCR, they were able to confirm that the genes associated with rev and tat were significantly down regulated (Zhou, et al, 2008).

The extensive range of treatments that can be possible with a treatment involving siRNA doesn't just apply to cases of cancer and the HIV virus. It is being evaluated for its potential as a method of treating conditions involving an increased level of low density lipoprotein (LDL) cholesterol. "In individuals with 5-year risk of major vascular events lower than 10%, each 1 mmol/L reduction in LDL cholesterol produced an absolute reduction in major vascular events of about 11 per 1000 over 5 years" (Cholesterol Treatment Trialists' (CTT) Collaborators. 2012). A reduction in ones LDL levels, specifically in the amount found in one's blood stream, has proven to reduce the risk of cardiovascular disease (Manninen, et al, 1992), and might be a factor in lowering the risk of those who have had a stroke previously from having another (Sacco, et al, 2006).

SiRNA can be used indirectly as a way to lower LDL cholesterol levels. "A novel therapeutic approach to lower LDL-c that is currently in clinical development involves blocking VLDL assembly and secretion by inhibiting the microsomal triglyceride transfer protein (Mtp)" (Tep, et al, 2012). By using siRNA to inhibit translation of the gene responsible for Mtp, they were able to reduce the levels of LDL cholesterol found in the blood stream. This is especially useful for those who don't react well to the current protocol set to reduce LDL levels by prescribing statins.

The broad range of application in a clinical sense for the use of

siRNAs is apparent from the few studies mentioned. There are many other applications that can be utilized, but those mentioned here show how broad its ability as a drug can be. Most of these studies however, are very theoretical. There are many hurdles that must be overcome before being able to use it as a treatment. The main hurdles being its delivery to a specific cell and stopping a specific protein without affecting others.

### Problems and Their Potential Answers

Delivery of SiRNA to cells has been a major problem for researchers. There are quite a few reasons that it is difficult to deliver molecules of siRNA, or more specifically its precursor dsRNA, to cells. The first is its really short lifespan. Even if the dsRNA won't degrade before reaching its desired location, the trouble of getting it into the cytoplasm of the cell is a hurdle in itself. However the biggest issue seems to be how to get these crucial siRNAs to a specific cell. Once in the correct cell, problems may still arise. SiRNA can be finicky at times and down regulate proteins that it wasn't intended for. Basically the siRNA has major issues with its specificity on the cellular level as well as intercellular.

"Unmodified, naked siRNAs are relatively unstable in blood and serum, as they are rapidly degraded by endo- and exonucleases, meaning that they have short half-lives in vivo." (Akhtar, Benter, 2007). This limits the ability to administer siRNAs in their "naked" already modified form. However, chemically modifying the siRNAs seems to prevent their untimely degradation. Studies were done comparing siRNA in its naked, already modified form, to siRNA that was chemically modified either by way of caging, nanoparticles, or PEGylation. When this comparison was done under conditions that simulated living in the blood stream, the siRNA that had been modified showed a significant increase in life span. Chemically modifying siRNA seems to be the way to allow for the siRNA to survive its journey to the cell. (Shah, et al 2013)

Surviving its journey to the cell is just the first of many hurdles for this important new treatment. The next major stumbling block to overcome is how to get it to the cell of choice. Although siRNA is selective for specific proteins it can cause havoc in a cell it wasn't intended for. For example, even if a cell doesn't have the specific protein that this siRNA codes for it can cause unwanted mRNA degradation due to off targeting (B. Scagianti, et al, 2011), a complication that will be discussed in a more general sense in the coming paragraphs.

Some methods are being studied as ways to allow the delivery to be more specific. The use of aptamers has been relatively successful in allowing the siRNA to reach specific cells, although it has its own unique set of issues (Liu, Gao, 2013). Another method



that has been used is connecting the siRNA enclosed in nanoparticles to an existing delivery system. This has shown promising results especially when the intended drug is also used (Shah, et al, 2013). Other methods are showing promising results as well. This leads to the next problem of the siRNA being delivered across the cell membrane.

SiRNA can be especially difficult to deliver through the cell membrane due to its charge. The cell membrane has an affinity to the siRNAs inherent charge causing it to be repelled. There are several methods being studied to find the most efficient way of allowing molecules of siRNA to penetrate the cell membrane. The first is the physical or mechanical method. Using electroporation or ultrasound, for example, to allow for cellular uptake. Another method being looked at is the use of chemicals either to neutralize the negative charge of siRNA or deliver it through other methods. (Zhou, et al, 2008)

With passage of siRNA through the cell now possible, off targeting, the last issue of delivery needs to be resolved. Although siRNA is coded uniquely to suppress the desired protein, it isn't always so specific. If a similar protein is also produced in the cell that the siRNA is delivered to, it can occasionally down regulate that protein as well. A specific protein is one that has a similar genetic code even though it can serve a very different and sometimes crucial role in the viability of the specified cell.

A few methods have been used to negate this off target silencing. The methods mainly use modifications to the siRNA or dsRNA starting material. By modifying the backbone of the strand, improved specificity was able to be reached. Although useful the modifications tend to make the siRNA less efficient against the target mRNA as well. There is one particularly successful option that doesn't affect the down regulation of the target mRNA as much as many of the previous methods tried. This process requires adding a one nucleotide bulge on the antisense strand specifically at the second position. The modified siRNA was found to be more specific to the intended mRNA producing less mistaken down regulation while still properly down regulating its intended target. (Dua, et al, 2011)

With its intended target found, the siRNA can now efficiently down regulate it. However, the stable dsRNA that the RISC now unwound to use as a mRNA inhibitor, is no longer as stable. Due to its less stable state when joining the RISC, "Transfected synthetic siRNA works for only a few days in mammalian cells." (Sioud 2004) While multiple treatments may reduce the issue of short term effectiveness, a better solution may be available. Using retroviral drugs the siRNA can be integrated into the genome more specifically as hairpin RNA. This is like the naturally occurring non synthetic siRNA. Using siRNA the cell can regulate

itself post transcriptionally. By integrating the codons to produce siRNA the time frame for siRNA longevity becomes indefinite. This is not by it increasing in stability rather it allows for a constant dose without further intervention. (Stewart, et al, 2003)

### Conclusion

The concept of using siRNA as a treatment for humans is one that excites and can open many previously unavailable options to those suffering many debilitating diseases. It is so promising that any stumbling block that has arisen along the way hasn't deterred researchers from their final goal of using siRNA in the clinical setting. They have attacked it from every angle and found solutions for many problems and ideas for others. The main issue of delivery primarily affects reaching specific cells or tissues with specificity. There are clinical trials mainly focused on using siRNA as a topical treatment. Although this method of treatment has been proven successful, by eliminating many of the issues associated with delivery, it only works on areas that are able to react to topical treatments. Topical siRNA treatments have seen much progress and continue to pass multiple phases of their testing; some have even been approved for clinical use. These treatments bypass many of the issues discussed, and show the ability of siRNA as a potential treatment for other diseases that need a more complex delivery system.

The extensive opportunities for siRNA use are driving many to discover new and improved methods. The way in which siRNA has turned from a discovery into clinical trials in a mere fifteen years attests to its great potential and likely future as a viable treatment. There are many areas that are still unclear especially regarding the methods in which siRNA works. With more discovery and new technology the methods of delivery and treatments will likely improve and start the important work siRNA was intended for.

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## Notes

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