Dear Reader,

In 1997, five undergraduate scientists began to question the inconspicuousness of undergraduate research amongst the scientific communities across the world. In an attempt to showcase more undergraduate research and form an undergraduate scientific community, the Journal of Young Investigators (JYI) was founded with financial help from Duke University, Swarthmore College, and Glaxo Wellmore, Inc. After one conference in Baltimore and a recruiting campaign across the east coast, the first issue of JYI was published in December of 1998 with five research papers. After receiving a grant from the National Science Foundation and help from the newly formed Board of Directors, JYI began publishing peer-reviewed undergraduate research from all across the world along with science news and careers in November of 2001.

Currently, JYI employs nearly one hundred undergraduate staff and ten members of the Board of Directors, who serve as an advising body to the executive board—all of whom act on a completely volunteer basis. Staffs from ten different countries and more than sixty different institutions across the world are represented at JYI, making it one of the only international undergraduate scientific research journals. Our mission here at JYI is not only to provide educational experiences for undergraduate researchers, but also to showcase the incredible research being done by undergraduates all across the world. That is why we have decided to create the first ever “Best of JYI” print issue to showcase the best papers published in JYI during 2014 and 2015.

JYI has truly come a long way since our first issue in 1998 and we will continue to move forward. In this issue, you will find three research papers from 2014 and three from 2015, along with four papers published by our very own Science News and Features and Science Career journalists. If this special issue of JYI interests you, please follow us on Facebook and Twitter to see more science updates or visit us on our website at JYI.org to read more papers, submit your own manuscript, or even apply for an open position. Getting involved with JYI means learning more about the peer review process and joining an international undergraduate science community.

Best,

Daniel Patrick Chapman
Editor-in-Chief, Journal of Young Investigators

Acknowledgements

The Journal of Young Investigators would like to thank the following individuals for their contributions toward putting together JYI’s Best of 2014-2015:

Maya Gosztyla, Managing Editor: Project supervision and layout design
Taylor Landeryou, Director of Publications: Layout design
Sahba Seddighi, Chief Development Officer: Cover design
Matthew Brousil, Former Managing Editor: Project initiation

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The RNA HOTAIR Promotes Chromatin Alteration in Cancer

Elsa Axelsdottir

Originally published in the March 2014 issue of the Journal of Young Investigators

Long non-coding RNA (lncRNA) is an integral part of the transcriptome and provides a regulatory role in the inactivation of genes. By methylating chromatin, lncRNA can cause gene silencing and form heterochromatin. These transcripts can act both in-cis and intrans; however, this review focuses on the specific lncRNA: HOX transcript antisense intergenic RNA (HOTAIR) in-trans. The long RNA sequence of HOTAIR binds to the HOX C locus and consequently silences chromatin at the HOX D locus. This silencing has shown to be indicative of specific cancers and their progression. In this review, HOTAIR’s involvement in cancer is discussed. Here, evidence for the direct relationship between the over expression of HOTAIR and an individual’s tumour progression into metastasis is presented. The abundance of the transcript in cancerous tissues can be used to diagnose the depth and progression of the tumours, as well as the general prognosis of the patient. HOX transcript antisense intergenic RNA’s mechanism of action presents itself as a potential target for cancer therapeutics. Blocking the interaction between the associated proteins and the transcript holds potential for the treatment of the disease. Moreover, further investigation of HOTAIR’s presence and mechanism of action could result in less invasive therapies and diagnostic tests with direct applications in a clinical setting.

INTRODUCTION

The human genome project discovered that only about 20,000 genes encoded protein, while the remaining 99% of the genome was left non-coding and hence non-functional (Lander et al., 2001). The current opinion in genetics, however, suggests the opposite. The functions of non-coding RNA have begun to emerge with compelling evidence of a role in gene regulation. The transcriptome is described as the total RNA transcribed from DNA and includes both coding and non-coding transcripts. Within the non-coding transcript there are two sub divisions: long ncRNA (lncRNA) and short ncRNA (sncRNA), both of which are characterized physically by the sequence length of the transcript. The functional definition of the non-coding transcript is accepted as the genetic information that does not code for protein, the latter being the functional unit of life. Consequently this definition yields little importance to the non-coding portion of the genome. However, it cannot be overlooked that the non-coding transcript comprises 99% of the genomic information. Thus, it is no surprise that these non-coding transcripts do indeed possess functions that are important. The importance of these non-coding transcripts has only recently been elucidated (Dermitzakis et al., 2005).

The first lncRNA to be identified was H19 in 1990. Sedimentation analysis showed that the RNA was not associated with any translational machinery, suggesting it was not involved in coding for protein (Brannan et al., 1990). Soon after the discovery of H19, more lncRNAs were quickly identified including Xist, the X chromosome silencer, (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991) and HOTAIR, a silencer of the HOX D locus (Rinn et al., 2007). Xist was discovered by slot blot analysis and northern blot, initially used to examine the presence of the Xist cDNA sequence present in inactive X chromosomes (Brown et al., 1991). John Rinn at Yale University discovered the unique transcript of RNA, HOTAIR, first by observing them in fibroblasts. Tiling microarray analysis of the HOX gene loci found that HOTAIR associates with HOX C and silences the chromatin on HOX D (Rinn et al., 2007).

When lncRNA was first identified, technologies were limited. However, the technologies for lncRNA identification have since dramatically improved. There are now several ways to identify non-coding transcripts. These include Crosslinking immunoprecipitation (CLIP) and High throughput sequencing of RNA Isolated by crosslinking immunoprecipitation (HITS-CLIP), deep RNA sequencing, tiling microarray analysis, and genomic selex (Li et al., 2012). These have all been modified and developed for the demands of the current era of next generation sequencing. The sequences of these ncRNA are available in databases such as Rfam, RNAdb and Incrnadb, the latter being a database that specifically annotates lncRNA.

Gene regulation can be considered the control of active and inactive expressions of the gene. This review considers the inactivation of genes caused by methylation and the alteration of chromatin states from chromatin to heterochromatin. Both small and long non-coding RNA are involved in gene regulation, either by activating or inactivating genes (Mattick & Makunin, 2006). Some form and associate with protein complexes that specifically methylate chromatin, while others act individually, silencing the gene by specific binding to the sequence. In addition, it is becoming evident that small and lncRNAs regulatory roles may be interdependent (Nana-Sinkam & Croce, 2011). LncRNA, in particular, has demonstrated a gene regulatory role in cancer, controlling the on and off states of tumour suppressor genes. Long non-coding RNAs play a key role in gene regulation and pathology. In particular, HOTAIR plays a role in cancer. This review will investigate the role

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of HOTAIR in four types of cancer: breast, lung, liver, and colon. In addition, the possibility of HOTAIR as a diagnostic tool for cancer progression will be discussed. Furthermore, a mechanistic investigation of HOTAIR could allow for the discovery of future therapeutic cancer targets.

Long non-coding RNA (lncRNA)
Long non-coding RNAs are most often defined as sequences starting from 200 bases to about 100kb (Setoyama et al., 2011). These long transcripts can take many different forms and include long intergenic non-coding RNA (lincRNA) as well as transcribed ultra-conserved regions (T-UCR) (Esteller, 2011). Examples of these and their functions are shown in Table 1. It is important to note that other types of lncRNAs in humans are also present, including ribosomal RNAs and pseudogenes (Gibb et al., 2011).

Long non-coding transcripts are unique in that they can be classified in several different ways including by function, mechanism, loci of origin, and orientation (Ponting et al., 2009; Li et al., 2012). The first classification is by function, that is, whether they are regulatory or not (Ponting et al., 2009). This requires the distinction between regulatory function and transcriptional noise (Ponting et al., 2009). Therefore there must be a distinction between the functional and non-functional sequences. Classification by mechanism defines the different lncRNAs that alter structure by either discrete binding to the DNA sequence itself or by recruiting intermediates. When lncRNA binds directly to the DNA, the mechanism of action is referred to as in-cis. In this event the chromatin structure is directly modified by the presence of the bound lncRNA molecule. When the lncRNA instead recruits intermediate protein complexes to mediate changes in chromatin structure, the mechanism is referred to as in-trans. The process of modification in-trans is known as transvection (Mercer et al., 2009) and will be the focus of this review. Further classification by loci of origin and orientation defines the transcripts by the physical location within the gene i.e. intergenic, partially intronic and totally intronic (Nakaya et al., 2007) and location on either sense or antisense strands (He et al., 2008) respectively. The mechanism of chromatin alteration by lncRNA is governed by the lncRNA molecule’s ability to stabilize protein complexes for the process of chromatin alteration and may also provide a scaffold for chromatin alteration of specific genes (Shamovsky & Nudler, 2006). The stability of lncRNA may also affect the stability of other RNA sequences (Ramaiah et al., 2012). It has been found that in fact antisense sequences may play a role in the regulation of sense sequences and seem to negatively regulate the sense transcripts (Lapidot & Pilpel, 2006; He et al., 2008).

Classifying the transcripts serves to organize newly discovered lncRNA and allows for the grouping of RNAs into families. The respective functions can then be inferred accordingly. Rfam is a database that annotates these sequences (Griffiths-Jones et al., 2003).

Mechanism of chromatin alteration in-cis
There are several well-identified and characterized examples of chromatin alterations in-cis, despite a majority that remain to be discovered. Chromatin modification by non-coding RNA in-cis can be described through the examples of two long non-coding RNAs: Xist and AIR.

Xist is a lncRNA transcript involved in X-chromosome inac-

<table>
<thead>
<tr>
<th>Name</th>
<th>Size</th>
<th>Location</th>
<th>Number in Humans</th>
<th>Functions</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>LineRNA</td>
<td>&gt;200 bp</td>
<td>Widespread loci</td>
<td>&gt;1,000</td>
<td>DNA-chromatin complex formation</td>
<td>HOTAIR, HOTTIP, lincRNA-p21</td>
</tr>
<tr>
<td>T-UCR</td>
<td>&gt;200 bp</td>
<td>Widespread loci</td>
<td>&gt;350</td>
<td>Possibly the regulation of mRNA and miRNA</td>
<td>uc.283+, uc.338, uc160+</td>
</tr>
<tr>
<td>Other</td>
<td>&gt;200 bp</td>
<td>Widespread loci</td>
<td>&gt;3,000</td>
<td>Imprinting, X chromosome inactivation, telomere regulation</td>
<td>XIST, TSIX, TERRAs, p15AS, H19, HYMAI</td>
</tr>
</tbody>
</table>

Table 1. Examples of lncRNAs in humans. (Adapted from Esteller, 2011)
tivation. The X-chromosome inactivation centre (Xic) locus, characterized by a repeated A motif encoding an RNA called RepA, is involved in the initiation of X-chromosome inactivation. Regulatory lncRNAs are present at this centre and mediate the inactivation in-cis (Rastan, 1994). The polycomb repressor group protein, PRC2, is suggested to associate with Xist to help the process of X-chromosome inactivation (Li et al., 2012). With an accumulation of Xist lncRNA, PRC2 proteins are recruited to the X-chromosome with the help of RepA (Figure 1) (Lee, 2010). The binding of Xist in-cis to cause methylation of the chromatin by PRC2 silences transcription and inactivates the X-chromosome (Li et al., 2012).

The lncRNA, Antisense Igf2r RNA (AIR), epigenetically silences the slc22a3 gene in-cis. Antisense Igf2r RNA is an antisense transcript that is associated with chromatin modifying complexes PRC2 and G9a (Nagano et al., 2008). G9a is a chromatin modifying complex that trimethylates lysine 9 (Khalil et al., 2009). AIR is transcribed from the hypomethylated paternal allele on the Insulin like growth factor 2 receptor (IGF2R) gene (Della Vedova & Cone, 2004). AIR directly overlaps the slc22a3 gene in the antisense orientation to induce silencing (Mohammad et al., 2009).

Mechanism of chromatin alteration in-trans
HOX transcript antisense intergenic RNA (HOTAIR) is one of the few well-characterized examples of lncRNA action in-trans. HOX transcript antisense intergenic RNA is an antisense long intergenic non-coding RNA (lincRNA). This lncRNA is classified as a HOX locus RNA, meaning its location of transcription as well as the location where it silences chromatin are both within the HOX loci (Rinn et al., 2007). Its length is 2.2kb and is transcribed from the HOXC locus, located on human chromosome 12, intergenic of HOXC11 and HOXC12. HOTAIR then silences the chromatin of HOXD, 40kbps away on chromosome 2 (Rinn et al., 2007). The activity of the lincRNA between two HOX loci illustrates the mechanism referred to as “in-trans”.

HOX loci are a series of transcription factors that are important for segmental identity and body patterning (Myers, 2008). HOX genes determine positional identity (Chang, 2009). They are antagonistic to the trithorax programming and instead induce the inactive chromatin state by trimethylation of lysine 27 on histone 3, inhibiting acetylation of lysine 4 and hence maintaining the silent state of chromatin (Schuettengruber et al., 2007). The HOX loci may transcribe several lincRNAs, highlighting them as global regulators (Khalil et al., 2009). Many human lincRNAs associate with chromatin-modifying complexes and affect gene expression.

HOTAIR does not associate with DNA alone, but also forms a bridge between protein complexes, forming a molecular scaffold to support chromatin silencing. These complexes of proteins include the Polycomb repressor complex 2 (PRC2), histone lysine demethylase (LSD1), co-repressor for elements-1-silencing transcription factor (CoREST), and repressor for elements-1-silencing transcription factor (REST). LSD1, CoREST, and REST are a complex of three interacting proteins (Tsai et al., 2010). HOTAIR is associated with PRC2 at the 5’ end and LSD1, CoREST and REST at the 3’ end (Figures 3 and 4) (Tsai et al., 2010). LSD1 is a protein that forms a complex with CoREST that then bridges to REST, a neuronal gene silencer, and mediates silencing of the target gene (Figure 3) (Tsai et al., 2010). PRC 2 is made up of protein subunits EZH2, SUZ12, and EED (Kogo et al., 2011). EZH2 is an H3K27 methylase and is phosphorylated by the cyclin dependent kinase CDK1, which then allows HOTAIR to bind to the protein and the rest of the PRC2 complex associates, allowing association with the histone. HOTAIR binds to EZH2 and then recruits and associates with the PRC2 complex (Kaneko et al., 2010). Polycomb grouping proteins are recruited to perform the chromatin alteration (Simon & Kingston, 2009), with most lincRNA associating with PRC2 (Khalil et al., 2009).

It is still unclear whether HOTAIR binds for the purpose of locating the chromatin modifying complexes or if they are also involved in the activation of the protein complexes, exhibiting an effector’s mechanism (Wang et al., 2011). However, it is clear that HOTAIR binds to these protein complexes prior to the binding onto DNA. It is the coordinated protein complex that binds to and associates with the DNA which leads to inductions of methylation and silences the gene, forming heterochromatin (Tsai et al., 2010). Hence, HOTAIR is used as a foundation for polycomb remodelling complexes and histone modification (Prensner & Chinnaiyan, 2011).

HOTAIR helps to induce a specific pattern of methylation, which involves trimethylation as well as demethylation (Croce, 2010). Trimethylation of Histone 3 lysine 27 is mediated by PRC2, while Histone 3 lysine 4 demethylation is mediated by the LSD1 complex (Figure 3) (Croce, 2010). Although the pattern of methylation mediated by HOTAIR is as stated above, the transcription of HOTAIR can also be identified by another specific methylation pattern. This methylation pattern is K4-K36 tri-methylation sites on histone 3 and is an important marker for the presence of the transcription of HOTAIR. This methylation pattern indicates that the transcription of HOTAIR is active and is thus present in the cell. Therefore, it is inferred that a K4-K36 methylated domain is a chromatin signature that indicates the presence of HOTAIR (Khalil et al., 2009).
HOTAIR in Cancer

HOTAIR is a lincRNA that has been found in several forms of cancer. The mechanism of HOTAIR remains consistent across different cancer types, mainly involving altering of the HOX D locus. Here four cancers will be discussed; breast cancer, lung cancer, hepatocellular cancer and colorectal cancer. Although these four present the most compelling evidence for the presence of HOTAIR, it is unlikely that the cancers discussed here construct a comprehensive list. This case study provides evidence for HOTAIR’s ability to promote the localization of the polycomb repressive complex and trimethylation of H3K27 as well as progression into metastasis (Khalil et al., 2009). Evidence for the expression of HOTAIR in tumorigenesis and metastasis is discussed.

It is important to note that HOTAIR has low sequence conservation between species, but conservation is nonetheless still present (Rinn et al., 2007; Prensner & Chinnaiyan, 2011). HOTAIR only exists in mammals and may differ between species (Gupta et al., 2010). It is hypothesised that HOTAIR has gained a functional importance in humans due to a rapid evolution. HOTAIR is a term that is used to refer to the human transcript, while the mouse HOTAIR is denoted as mHOTAIR (Schorderet & Duboule, 2011). Here, the human HOTAIR sequence was assessed in mouse (Gupta et al., 2010).

Breast Cancer

Breast cancer is one of the most common cancers among women and involves the growth of tumours in the breast epithelial tissue (Jemal et al., 2011). Several studies support the role of HOTAIR in the cause and progression of breast cancer (Prensner & Chinnaiyan, 2011). In normal tissues, many non-coding RNAs act as tumour suppressors and protect the cells from cancer development (Croce, 2010). The protective mechanism is through the use of tumour suppressor genes. Some HOX lincRNAs are breast tumour suppressors, such as lncRNA HOX5 (Gupta et al., 2010). The expression of these lincRNAs is deregulated in cancerous tissue (Raman et al., 2000). HOTAIR specifically silences a variety of these tumour suppressor genes namely the protocadherin family such as HOXD10 and PGR, as well as several metastasis suppressor genes including PCDH10, PCDHB5, and JAM2 (Figure 4) (Croce, 2010; Gupta et al., 2010; Kogo et al., 2011). Hence, the cells are not protected against uncontrolled division and cancer can ensue. HOTAIR is upregulated in cancer and this misregulation of the lincRNA has been proven to be detrimental to the prognosis of patients (Gupta et al., 2010). The transformation of breast epithelial cells is also associated with the EZH2 subunit, which is an enhancer of zeste homolog in humans. Its presence is similarly associated with the progression of breast cancer (Kleer et al., 2003).

Additionally, the expression of HOTAIR in cancerous tissues increases the mobility of the cells. The nature of cancer allows the tumorous cells to move and invade other tissues, causing a phenomenon known as metastasis. There is a link between metastasis and over expression of HOTAIR as it is suspected to silence multiple metastasis suppressor genes (Gupta et al., 2010; Kogo et al., 2011). HOTAIR associates with and induces tri-methylation of these metastasis suppressor genes (Gupta et al., 2010). HOTAIR induces H3K27me3 and increases PRC2 expression in breast cancer cells (Gupta et al., 2010; Kogo et al., 2011). It was observed that these cells with the H3K27me3 methylation profile resemble embryonic fibroblast cells (Gupta et al., 2010; Gibb et al., 2011). The nature of this reprogramming causes the metastatic mobile and invasive properties. HOTAIR expression is linked to a poor prognosis of breast cancer patients (Gupta et al., 2010). Although a lot of work has been done on HOTAIR’s presence in breast cancer, it is becoming increasingly evident that this lincRNA plays a regulatory role in a number of other cancers such as lung, hepatocellular, and colon cancer as well.

Lung Cancer

Many cancers metastasize to the lung, but it has been found that cancerous tissues with the over expression of HOTAIR tend to increase their mobility to the lung. In particular, the movement of cancerous cells from breast to the lung is discussed in Gupta’s paper (Gupta et al., 2010). The non-coding RNA MALAT-1 had previously characterized lung cancer, but evidence for the presence of HOTAIR in lung metastasis from the breast is strong, raising the possibility of it playing an important role as well (Gibb et al., 2011). When a non-metastatic cell line was induced with the expression of HOTAIR, the cell line was able to move to and invade the lung (Gupta et al., 2010; Liu et al., 2013).

Hepatocellular Cancer

Hepatocellular carcinoma (HCC) presents itself with over 600,000 cases per year worldwide. One growing problem with HCC is that patients treated with a liver transplant often suffer from tumour relapse and ultimately progression into metastasis. Thus the understanding of metastasis and cancer progression is important. In hepatocellular cancer, again, the over expression of HOTAIR is linked to cancerous tissue. Interestingly, the presence of HOTAIR in the tissue is linked to recurrence and relapse in patients (Yang et al., 2011). This is indicative of the transcripts ability to migrate and invade. HOTAIR expression was lower in non-cancerous tissue than in cancerous tissue (Geng et al., 2011; Yang et al., 2011). The cumulative relapse-free survival of patients with high HOTAIR expression was significantly lower than in patients with low expression of the lincRNA, as analysed by the Kaplan-Meier method and log-rank test (Geng et al., 2011). Additionally, the cancerous tissue was more responsive to chemotherapy and became more sensitive to apoptosis mediated by TNF-a with lower expression of HOTAIR (Yang et al., 2011). The EZH2 subunit of PRC2 is also important in HCC, with high levels of expression in HCC of human cell lines (Sudo et al., 2005). The confirmation of EZH2’s importance in the progression of cancer indicates the importance of the collective interactions between the subunits that allow cancerous properties to progress.

Colorectal Cancer

Figure 3. Mechanism of breast cancer metastasis by HOTAIR. The PRC2 complex contains the subunits EZH2, SUZ12, EED associate with lincRNA HOTAIR and form a bridge with LSD1, CoREST, and REST inducing methylation and heterochromatin formation. This inhibits the transcription of the metastasis suppressor genes, PCDH10, PCDHB5, and JAM2, allowing metastasis to ensue. (Croce, 2010)
In a single study on colorectal cancer (CRC), it was found that the state of the tumour was related to the expression of HOTAIR. The depth of the tumour and differentiation of the cells were also related to the expression of HOTAIR. High HOTAIR expression was associated with metastasis in the liver. This indicates that the expression of HOTAIR and invasiveness of the cancerous cells are directly related. Increasing HOTAIR expression increased invasion, while decreasing HOTAIR expression decreased invasive properties. Therefore, it is likely that HOTAIR may be responsible for increasing the number of undifferentiated cancer cells. The increased expression of HOTAIR was an independently causative variable as invasive properties increased (Kogo et al., 2011). Further study on the invasive properties of colorectal cancer cells will be required to determine the role of HOTAIR as a causative factor in the progression of the disease.

**Diagnosis and Therapy**

Cancer is a misregulation of pathways within the cell and lncRNAs are often associated with this misregulation in the cancerous tissue. There is a clear trend that the levels of expression are linked to cancer progression. Hence there are emerging proposals of the use of lncRNAs as biomarkers for cancer (Tsai et al., 2011). As discussed above, there is a strong relationship between the levels of lncRNAs in the cell and subsequent metastasis. This may have profound clinical implications. As seen in hepatocellular cancer, HOTAIR is not only a biomarker for the progression of cancer but also a marker for the potential relapse, indicating the transcript could be important in cancer risk assessment as well as in the process of classifying patient prognosis. The use of HOTAIR as a biomarker in hepatocellular cancer could prove to be clinically significant, particularly in patients undergoing liver transplants (Yang et al., 2011). Increased levels of HOTAIR can help estimate relapse-free survival in liver cancer patients. Similarly, it would be just as significant to use this relationship to determine the potential for metastasis from breast to lung as well as depth of the tumour in colorectal cancer (Gupta et al., 2010; Kogo et al., 2011). It seems clear that the use of HOTAIR as a biomarker can have immediate benefits if incorporated into clinical practice as a diagnostic tool. The interaction between HOTAIR and the two protein complexes, PRC2 and LSD1, are also instrumental in methylating the chromatin and as a consequence, they could also be used for diagnosing the methylation that occurs. A direct relationship between the HOTAIR associated protein expression and methylation is apparent: a high expression of PRC2 and LSD1 yields a high level of chromatin methylation and gene silencing by HOTAIR (Sudo et al., 2005). This relationship can be used as a diagnostic tool to further clarify the diagnosis.

In addition, breast cancer in particular is known to be highly resistant to chemotherapy and radiotherapy (Mego et al., 2010). This is significant in that there is a need for alternative methods to treat the cancer. It is well characterized that the PRC2 complex plays a fundamental role in the mechanism of HOTAIR mediated chromatin silencing and thus this could be a target for therapy. Exploiting the interaction of PRC2 and HOTAIR may interfere with the functional process of the chromatin silencing. Small molecule interference as well as oligonucleotide interference have been methods of interfering with small ncRNA and could therefore have the potential to work with the interference of HOTAIR and its respective protein subunits. A possible method of this interference could be the introduction of a competitive antagonist. Additionally, fragmenting large ribonucleoprotein complexes would inhibit the formation of the HOTAIR and EZH2 complex (Tsai et al., 2011) and is, thus, a potential therapeutic strategy. In addition, developing a method of reversing the methylation mediated by HOTAIR by introducing demethylase complexes or introducing trithorax group proteins to counteract the effects of the PRC2 protein may re-activate the tumour suppressor genes.

It is significant to note that it is not only the mechanism but also the misregulation of lncRNAs that may be important to cancer progression (Gupta et al., 2010). Therefore, maintaining the function of the regulatory non-coding RNA would be an important task for therapeutics. This could be achieved by suppressing the transcription of HOTAIR potentially by RNA interference (Tsai et al., 2011).

**CONCLUSION**

This study was supported by the Fulbright foundation, University of Cincinnati Research Grant, and The US-Israel Binational Science Foundation.

These four examples present compelling evidence for the importance of HOTAIR in the phenomenon of cancer and it is for this reason that the expression pattern of long non-coding RNA is rapidly becoming accepted as a hallmark of the disease (Gibb et al., 2011). Screening for the over expression of this transcript in patients’ cells can be used to identify cancer progression. Hence, HOTAIR can be used as a biomarker for cancer diagnosis. The current mechanistic understanding gives rise to potential therapies to target the functions of HOTAIR. Applications of such therapies are yet to be implemented, but a movement away from chemotherapy and radiation towards the direct targeting of HOTAIR could potentially have profound impacts on the treatment of cancer and patient survival.

HOX transcript antisense intergenic long non-coding RNA, however, is not the only lncRNA transcribed at the HOX locus. There are still many aspects to the regulatory mechanism of ncRNA yet to be identified. Most lincRNAs use the PRC2 pathway, hence, HOTAIR can be used as a model to help understand the mechanism of other lncRNAs (Khalil et al., 2009).

It would be interesting to identify the metastatic trends between tissue types affected by tumours with cancer. As there are several other non-coding transcripts that are prevalent in cancer, examining the associations and expression profiles of each transcript in cancer would greatly increase the understanding of the importance of lncRNA in the disease. In particular MALAT-1 is known to associate with lung cancer (Gibb et al., 2011) and it would be interesting to see if MALAT-1 was also present in the metastatic lung tissue in Gupta’s breast cancer patients. This could help elucidate the interaction of ncRNAs together, as cancer is a multi-factorial disease.

Additionally, it is possible that the functions of both small and lncRNAs are intertwined (Nana-Sinkam & Croce, 2011). Understanding the interdependence between the transcripts will be important for gaining a comprehensive understanding of the mechanism in which the genes are silenced as well as the potential treatment targets. It is important to the progression of the field that a complete list of ncRNA for bio-marking in cancer be identified (Huarte & Rinn, 2010).

Hox transcript antisense intergenic RNA is becoming increasingly well-known and abundant in several tissue types and it is unlikely to be limited to the tissue and cancer types discussed in this review, therefore further exploration is required to enhance the understanding of the mechanistic action of lncRNA in cancer.
REFERENCES


Association of Music with Stress, Test Anxiety, and Test Grades Among High School Students

Radhika Rastogi*1 & Ellen Silver2
Originally published in the May 2014 issue of the Journal of Young Investigators

Music is an integral part of many adolescents’ lives and has been shown to have anxiety-relieving effects in high-stress settings, such as hospitals. Adolescents also face high levels of stress in academic environments, which have been correlated with poor academic performance, particularly test grades. However, the relationship between stress, academic performance, and music listening among adolescents has not been studied. We hypothesized that students who spent more time listening to music while studying would report lower levels of stress and receive higher test grades. A survey assessing academic stress, test anxiety, and music listening habits was administered in science classes following quarterly testing. Test grades were obtained as objective measures of academic performance. We found that time spent listening to music while studying was positively correlated with test anxiety and academic stress and negatively correlated with test performance. Though girls reported higher levels of stress than boys, they did not have significantly different test grades. Music-listening habits differed between academic levels, with introductory levels reporting more time listening to music, higher levels of stress, and poorer test grades than more advanced levels. When adjusted for these differences, the association of music with test grades was rendered non-significant suggesting that academic rigor and test anxiety mediated the association of music with test grades. Because music was not found to be associated with decreased stress in academic settings, it is possible that it might be distracting in the study environment. The distraction theory, which posits that music helps individuals cope by distracting them from stressful scenarios, has been proposed to explain the pain-relieving nature of music in hospital settings. This may explain the lack of stress-reduction by music in an academic context. These findings may help students create more effective, less stressful study environments.

INTRODUCTION

Given the omnipresence of music, it is relevant to explore its effects on various aspects of adolescents’ lives. Personalization of music listening through MP3 players and online radio stations allows adolescents to listen to music throughout the day (Vogel et al., 2009). Listening to music has also been associated with neurological stimulation and alleviation of anxiety (Fukui & Toyoshima, 2008; Koyama et al., 2009; Wachi et al., 2007). In stressful environments such as operating rooms, emergency rooms, and waiting areas in hospitals, music reduces anxiety associated with pain, both in adults and children (Austin, 2010; Holm & Fitzmaurice, 2008; Klassen et al., 2008; Nilsson, 2008). However, the relationship between music and reduction in other forms of stress, especially academic stress among adolescents, remains unclear.

Academic stress, related to test performance, student-teacher relationships and peer relationships (Abouerie, 1994; Kouzma & Kennedy, 2004), plays an important role in students’ ability to learn and thereby influences school performance and participation. Therefore, it is relevant to investigate patterns of stress in an adolescent population. It is important to note that the academic setting includes not only the physical location of classes and testing but the study environment as well. Test anxiety, a form of academic stress that is characterized by a feeling of nervousness before or during an exam (Sarason & Gordon, 1953), is a particularly salient form of stress among high school students. Test anxiety is advantageous in low levels as it can increase the student’s focus on the test, recollection of key facts, and usage of problem-solving skills (Cassady & Johnson, 2002). However, high levels of test anxiety are associated with poorer recall and decreased ability to focus during exams (Dutke & Stöber, 2001; Eysenck et al., 2007). Highly test-anxious students have been shown to have lower grade-point averages regardless of material knowledge or time spent studying (Chapell et al., 2005; Culler & Holahan, 1980). Delineating levels of test anxiety are associated with an ineffective study environment, so a more conducive environment may help with better preparation (Eysenck et al., 2007), thereby reducing test anxiety. Though music has been shown to decrease perceived levels of stress in clinical settings, whether an equivalent relationship exists among adolescents in the academic setting of the study environment has not been elucidated.

Studies have investigated the association of music with stress in college students and have found beneficial emotional responses, such as decreased levels of stress, associated with the music conditions, which involved listening to music as opposed to sitting in silence (Hirokawa & Ohira, 2003; Labbé et al., 2007). However, these studies were conducted in a controlled environment, where students took cognitive tests and then either listened to music or sat in silence. While this study addressed the effects of music after a stressful experience, the role music plays before a stressful experience, such as during study periods before exams, needs to be researched further. Additionally, in the college student cohorts,

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it was observed that females generally reported higher levels of stress than their male counterparts (Abdulghani et al., 2011; Eum & Rice, 2011). It would therefore be relevant to consider whether similar sex differences are present in a younger high school student population. Thus, this study was conducted to address these gaps in knowledge by administering surveys about music listening habits and test performance to high school students. In order to investigate the relationship between music and stress prior to the testing situation, we asked students about their study environments. It was hypothesized that students who spent more time listening to music would report lower stress levels and receive higher test grades. We further hypothesized that gender would be a significant determinant of stress levels among adolescents. Our findings will help understand what kinds of study environments are associated with lower stress levels and better performance.

MATERIALS AND METHODS

Participants

In this cross-sectional study, students enrolled at Scarsdale High School, a suburban public high school in Scarsdale, NY, completed a survey about music preferences, study habits, test anxiety, and academic stress. The study was conducted between January 2010 and February 2010.

Measure

The students provided demographic details including their age, gender, grade, class and academic level to which they were assigned. The students also indicated the amount of time spent listening to music while studying for their science tests. To assess the students’ perspectives on their test, the survey included items about difficulty of material, time spent studying, and use of extra help provided by the teachers. These items, included in the demographic section, were ultimately not relevant to the research questions and were therefore omitted during analysis.

The survey used two previously validated measures, Sarason’s Test Anxiety Scale (TAS) and Abouerie’s Academic Stress Questionnaire (ASQ) to quantify stress and test anxiety (Abouerie, 1994; Sansgiry & Sail, 2006; Sarason & Gordon, 1953). Both the TAS and the ASQ, designed for college age students, have also been validated among high school students (Kouzma & Kennedy, 2004; Sarason & Gordon, 1953). These measures were chosen because they used simple language and addressed several causes and effects of stress. All individual items of the surveys were included and the scaling model was kept the same. The format of the two tests was true-false for the TAS and a Likert scale for the ASQ. The TAS was divided into two domains: emotional and physical. Similarly, the ASQ was divided into four domains: social, performance, diligence, and time management. These distinct domains allow for the identification of specific aspects of behavior influenced by stress that may have impacted test performance. The survey administered had a total of 44 questions, including 11 demographic questions, 21 questions from the ASQ and 12 questions from the TAS.

Study design

The survey was administered the day following the designated quarterly testing date for science classes. The participants completed the survey in class. Although given the choice to opt out, all the students agreed to complete the survey. The students were blinded to the purpose of the study and were informed that purpose of the survey was to determine the utility of music in creating a more conducive study environment in the common areas in the school building. The teachers then collected the survey and reported the students’ grades from the most recent quarterly exam on the survey itself. Test performance provides one of the most objective measures of the students’ ability to handle the pressure and anxiety (Cassady & Johnson, 2002). Before the surveys were returned to the author, the students’ names were removed to maintain anonymity. The study was approved by the school’s Institutional Review Board.

Statistical analysis

Reliability analysis was done to ensure the internal consistency of the chosen items in each of the scales. Internal consistency refers to the correlation between individual items and the total score of each of the questionnaires to gauge the reliability of the measures. The Cronbach’s alpha values resulting from the reliability analyses were 0.66 for the TAS and 0.87 for the ASQ. The standard cutoff value used to evaluate consistency based on the Cronbach’s alpha is 0.7. While the ASQ meets this standard, the TAS is just below the value, in the acceptable but not ideal range, perhaps due to the smaller number of items in that portion of the survey (Tavakol & Dennick, 2011). The domains for each test were determined using factor analysis after the data was collected. The questions were not separated by domain in the survey that was administered. In the TAS, questions that dealt with physical responses to stress, such as stomach aches or irregular heart rates, were placed in the “physical” domain and items that indicated emotional response to stress, such as forgetfulness of known facts and lack of concentration, were placed in the “emotional” domain. Descriptive analysis was done on the demographic variables. Composite mean scores of the ASQ and TAS as well as means of individual domains were calculated. Time spent listening to music was converted to a discrete variable by assigning the middle value for each choice (i.e. 1.5 hours for the 1-2 hour choice). The mean was then calculated for each group of interest, such as students in a particular academic level or students of a specific gender.

Test grades were the primary outcome of interest. Means of test grades, survey composite scores, those of individual domains, and time spent listening to music were compared between the genders using a t-test. The same variables were compared between multi-level categorical variables (e.g., grade in school and academic level) using the analysis of variance with Bonferroni correction for post-hoc testing to determine which groups were significantly different from one another (α = 0.05). Pearson correlation coefficients were calculated to assess the relationships between test grades as well as with time spent listening to music with each of the survey’s domains. Multivariate analysis using linear regression was performed to identify independent predictors of test grades after adjusting for any confounding variables. An alpha value of 0.05 was used to determine significance of interactions.

RESULTS

Seven hundred and twenty eight students completed the survey (Table 1). These included students from all four high school grades (9th-12th), three levels of academic rigor (regular, honors and advanced placement (AP)) and six sciences (biology, chemistry, physics, earth science, geology and environmental science).
Each student was only enrolled in one science class of a given academic rigor. While participation did not differ by gender, fewer 12th graders than any other grade completed the survey. As fewer students take AP classes, the number of participants in AP classes was smaller than the number of students in honors and regular level classes. Average time taken to complete the survey was 15 minutes.

We found that time spent listening to music while studying had a small but significant negative correlation with test grades \( (p=0.049) \) (Table 2). While the diligence \( (p=0.01) \) and social \( (p=0.01) \) domains of the ASQ and the emotional domain \( (p<0.01) \) of the TAS correlated positively with time spent listening to music, there was a negative correlation between the physical domain of the TAS and time spent listening to music \( (p=0.02) \) (Table 2). All domains in ASQ and TAS were negatively correlated with test grades. In particular, the physical \( (p=0.01) \) and diligence \( (p<0.01) \) domains of the ASQ and both the physical \( (p<0.01) \) and emotional \( (p<0.01) \) domains of the TAS reached statistical significance.

In addition to the correlations between music, stress, and test performance, it was found that both time spent listening to music \( (p=0.04) \) and academic stress \( (p=0.002) \) were negatively associated with academic rigor. Test performance was positively associated with the academic rigor of the science classes (Table 3). Students in the AP level scored significantly higher on their science exams than the students in the regular level \( (p<0.001) \) and the honors level \( (p=0.001) \). Furthermore, students in the AP level listened to significantly less music than students in the regular level \( (p=0.04) \) and scored significantly lower in the ASQ \( (p=0.002) \). There was also a significant difference in the emotional domain of the TAS, with AP students reporting lower levels of emotional stress than regular students \( (p=0.003) \).

Given prior studies of gender differences in reported academic stress, we investigated if these differences develop at the level of high school. While there was no significant difference in the test scores or time spent listening to music \( (p=0.47) \) between male and female students \( (p=0.9) \), females reported significantly higher levels of stress in both the ASQ \( (p<0.001) \) and TAS \( (p<0.001) \) than males (Table 4).

Upon multivariate analysis, it was found that the TAS emotional domain \( (p<0.001) \) and the academic rigor of the classes \( (p=0.003) \) were significant independent predictors of test performance (Table 5). In particular, those in AP classes averaged 3.9 points higher on their tests than those in regular level classes \( (p=0.003) \). In addition, those reporting an emotional response to the testing situation scored 9.1 points lower than those who did not \( (p<0.0001) \). Moreover, the time spent listening to music was significantly different between the three academic levels, although its association with test grades was rendered non-significant \( (p=0.1) \) when adjusted for academic level and emotional response.

**DISCUSSION**

The purpose of this study was to investigate the relationship between music listening habits, academic stress, test anxiety, and test grades among high school students. We hypothesized that students who spent more time listening to music while studying would have higher test grades and report lower levels of stress. Instead, we found that time spent listening to music was associated with higher levels of stress in high school students. In addition, we found a negative association between time spent listening to music and test scores, an association that was mediated by levels of test anxiety as well as by the academic rigor of the classes. Students in higher academic levels reported lower levels of stress but also spent less time listening to music. Together, our findings suggest that there is a positive association between time spent listening to music and test anxiety, both of which are negatively associated with test performance among high school students.

To the authors’ knowledge, this is the first study demonstrating a relationship between music, stress, and academic performance in high school students. Our findings of the negative correlation between stress and performance among high school students extend those reported among college students (Cassady & Johnson, 2002; Sarason, 1984). However, our results on the association of music with stress and test performance differ from studies among college students. Our distinct findings may be due to differences in the study settings. Studies among college students assigned experimental conditions with or without music in which students completed the cognitive tests and reported beneficial effects of music (Hirokawa & Ohira, 2003; Labbé et al., 2007). In contrast, our study investigated the association of music listening during study.

### Table 1. Demographics of the study sample.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boys</td>
<td>375</td>
<td>52.5</td>
</tr>
<tr>
<td>Girls</td>
<td>339</td>
<td>47.5</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9th</td>
<td>191</td>
<td>27</td>
</tr>
<tr>
<td>10th</td>
<td>215</td>
<td>30.4</td>
</tr>
<tr>
<td>11th</td>
<td>174</td>
<td>24.6</td>
</tr>
<tr>
<td>12th</td>
<td>127</td>
<td>18</td>
</tr>
<tr>
<td><strong>Academic Rigor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular</td>
<td>276</td>
<td>38.5</td>
</tr>
<tr>
<td>Honors</td>
<td>369</td>
<td>51.5</td>
</tr>
<tr>
<td>Advanced Placement</td>
<td>72</td>
<td>10</td>
</tr>
<tr>
<td><strong>Test Grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A+</td>
<td>94</td>
<td>13.1</td>
</tr>
<tr>
<td>A</td>
<td>121</td>
<td>16.9</td>
</tr>
<tr>
<td>A-</td>
<td>95</td>
<td>13.2</td>
</tr>
<tr>
<td>B+</td>
<td>75</td>
<td>10.5</td>
</tr>
<tr>
<td>B</td>
<td>103</td>
<td>14.4</td>
</tr>
<tr>
<td>B-</td>
<td>63</td>
<td>8.8</td>
</tr>
<tr>
<td>C+</td>
<td>55</td>
<td>7.7</td>
</tr>
<tr>
<td>C</td>
<td>30</td>
<td>4.2</td>
</tr>
<tr>
<td>C-</td>
<td>24</td>
<td>3.3</td>
</tr>
<tr>
<td>D</td>
<td>34</td>
<td>4.7</td>
</tr>
<tr>
<td>F</td>
<td>21</td>
<td>2.9</td>
</tr>
</tbody>
</table>

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periods before the test was taken. Our results differed from studies investigating music in a pre-testing environment as well. Studies in which classical music was played prior to cognitive testing report increased performance (Rauscher et al., 1995; Rauscher & Shaw, 1998), while we observed a negative relationship between listening to music before an exam and test performance.

The cognitive distraction effect of music during study periods may also explain our results. This distraction theory has been proposed as an explanation for the palliative effect music has in inpatient and oncology wards (Huang, Good et al., 2007; Mitchell et al., 2006; Ruscheweyh et al., 2011) and more recently, in academic situations (Doyle & Furnham, 2012). As per the distraction theory, music may play a different role in stress-mediation depending on its usage in an academic or non-academic environment. Students who listen to music while studying may not focus as effectively on the material. Less effective studying may result in a false sense of preparedness, which has been shown to lead to greater test anxiety, and is strongly correlated with poor performance in our study as well (Cassady & Johnson, 2002; Culler & Holahan, 1980).

Our findings suggest that the influence of music on stress varies by the populations studied. We observed that academic levels mediated this relationship such that the negative association of music with school performance was greater among those at regular academic levels as compared to those in the honors and AP levels. A possible explanation for this finding is that students in more rigorous classes may handle stress differently than students in more introductory courses, as reflected by their disparate test grades and music-listening habits. Test anxiety, the second mediator of test performance, may independently mediate the relationship between music and test performance because students who are test anxious may look to music as a source of stress relief. However, our findings suggest that such a utilization of music to mitigate stress does not lead to either decrease in stress or improved academic performance.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Time Spent Listening to Music</th>
<th>Test Grades</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation</td>
<td>Correlation</td>
</tr>
<tr>
<td></td>
<td>p-value*</td>
<td>p-value*</td>
</tr>
<tr>
<td><strong>Academic stress questionnaire (ASQ)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Composite score</td>
<td>0.002</td>
<td>-0.04</td>
</tr>
<tr>
<td>Performance</td>
<td>-0.02</td>
<td>0.54</td>
</tr>
<tr>
<td>Diligence</td>
<td>0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Time Management</td>
<td>-0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>Social</td>
<td>0.1</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Test Anxiety Survey (TAS)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Composite score</td>
<td>0.01</td>
<td>-0.23</td>
</tr>
<tr>
<td>Physical</td>
<td>-0.09</td>
<td>0.02</td>
</tr>
<tr>
<td>Emotional</td>
<td>0.11</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*significant p-values (α=0.05) are bolded.

Table 2: Association of ASQ and TAS domains with time spent listening to music and test grades

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regular</th>
<th>Honors</th>
<th>Advanced Placement</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test grade</td>
<td>84.87±10.8</td>
<td>85.84±9.65</td>
<td>90.35±7.9</td>
<td>0.002</td>
</tr>
<tr>
<td>Time Spent Listening to Music</td>
<td>2.01±1.25</td>
<td>1.78±1.22</td>
<td>1.73±1.28</td>
<td>0.04</td>
</tr>
<tr>
<td>ASQ Mean</td>
<td>2.47±0.64</td>
<td>2.43±0.75</td>
<td>2.22±0.42</td>
<td>0.002</td>
</tr>
<tr>
<td>ASQ Performance</td>
<td>2.94±0.65</td>
<td>2.94±0.61</td>
<td>2.74±0.71</td>
<td>0.05</td>
</tr>
<tr>
<td>ASQ Diligence</td>
<td>2.35±0.64</td>
<td>2.23±0.61</td>
<td>2.01±0.58</td>
<td>0.0001</td>
</tr>
<tr>
<td>ASQ Time Management</td>
<td>2.88±0.64</td>
<td>2.81±0.62</td>
<td>2.78±0.65</td>
<td>0.29</td>
</tr>
<tr>
<td>ASQ Social</td>
<td>1.77±0.68</td>
<td>1.66±0.56</td>
<td>1.48±0.49</td>
<td>0.0012</td>
</tr>
<tr>
<td>TAS Mean</td>
<td>0.44±0.21</td>
<td>0.43±0.21</td>
<td>0.38±0.21</td>
<td>0.13</td>
</tr>
<tr>
<td>TAS Physical</td>
<td>0.47±0.29</td>
<td>0.50±0.29</td>
<td>0.45±0.28</td>
<td>0.23</td>
</tr>
<tr>
<td>TAS Emotional</td>
<td>0.37±0.26</td>
<td>0.33±0.25</td>
<td>0.26±0.23</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*p-value derived from ANOVA. Significant p-values (α=0.05) are bolded. All values are reported as mean ± standard deviation.

Table 3: Comparisons of test grade, music listening, and ASQ and TAS domains by level of academic rigor.
Table 5: Multivariate Analysis of the Predictors of Test Performance

<table>
<thead>
<tr>
<th>Predictor</th>
<th>β-value</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time spent listening to</td>
<td>-0.44 (-1.1 to 0.2)</td>
<td>0.1</td>
</tr>
<tr>
<td>music</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Academic rigor</td>
<td>3.9 (1.3 to 6.5)</td>
<td><strong>0.003</strong></td>
</tr>
<tr>
<td>ASQ performance domain</td>
<td>0.53 (-0.86 to 1.92)</td>
<td>0.45</td>
</tr>
<tr>
<td>ASQ diligence domain</td>
<td>-0.83 (-2.1 to 0.49)</td>
<td>0.22</td>
</tr>
<tr>
<td>TAS emotional domain</td>
<td>9.14 (-12.4 to -5.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TAS physical domain</td>
<td>-1.41 (-4.4 to 1.57)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

* Significant p-values (α=0.05) are bolded.

Table 4: comparisons of test grades, music listening and ASQ and TAS domain scores by gender.

<table>
<thead>
<tr>
<th>Variable</th>
<th>All participants</th>
<th>Male (375)</th>
<th>Female (339)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test grade</td>
<td>85.92±10.07</td>
<td>85.96±9.83</td>
<td>85.86±10.36</td>
<td>0.90</td>
</tr>
<tr>
<td>Time Spent Listening to</td>
<td>1.86±1.24</td>
<td>1.83±1.22</td>
<td>1.90±1.27</td>
<td>0.47</td>
</tr>
<tr>
<td>Music (hours)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASQ Mean</td>
<td>2.43±0.68</td>
<td>2.33±0.61</td>
<td>2.53±0.74</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ASQ Performance</td>
<td>2.92±0.64</td>
<td>2.80±0.03</td>
<td>3.05±0.03</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ASQ Diligence</td>
<td>2.25±0.63</td>
<td>2.21±0.63</td>
<td>2.30±0.62</td>
<td>0.05</td>
</tr>
<tr>
<td>ASQ Time Management</td>
<td>2.84±0.63</td>
<td>2.71±0.67</td>
<td>2.98±0.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ASQ Social</td>
<td>1.69±0.61</td>
<td>1.65±0.60</td>
<td>1.74±0.61</td>
<td>0.05</td>
</tr>
<tr>
<td>TAS Mean</td>
<td>0.43±0.21</td>
<td>0.37±0.21</td>
<td>0.48±0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TAS Physical</td>
<td>0.49±0.29</td>
<td>0.41±0.28</td>
<td>0.57±0.27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TAS Emotional</td>
<td>0.34±0.25</td>
<td>0.31±0.24</td>
<td>0.38±0.26</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Comparison between genders. Significant p-values (α=0.05) are bolded. All values are reported as mean ± standard deviation.

In summary, we found that that time spent listening to music was negatively associated with school performance as measured by test grades among high school students. This relationship was mediated by the level of academic rigor since adolescents in more challenging academic levels listened to less music, suggesting that they may have alternate stress coping mechanisms. Given the growing role of music and media in teenagers’ lives, the study is important as it links performance with anxiety in a particular kind of study environment in which the student chooses to listen to music. The results can be explained in part by using the distraction theory, lending credence to one mechanism that explains the relationship between music and stress. These results can help students make informed decisions about their study environment as well as their preparation techniques for their exams since there is substantial indication that study environments without music are associated with less stress and better grades. Moreover, the findings may provide insight into the mechanism by which music and stress are related in a cognitive setting. Overall, this study highlights the complex interaction between study environment, anxiety levels, academic material, and students’ performance.

ACKNOWLEDGEMENT

The authors would like the acknowledge the Science Department and the Science Research teachers, Ms. Beth Schoenbrun and Mr. Jeremy Szerlip, at Scarsdale High School, without whom the work would not have been possible.
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A mathematical model of cancer networks with radiation therapy

Olivia Manley

INTRODUCTION

Cancer is typically thought to be the result of one or more mutations in a cell’s genes that cause uncontrolled cell division (Evan & Vousden, 2001). Werner (2011) proposed a new way to explain the growth and development of cancer. Rather than focusing on genetic mutations, he suggested that cancer is caused by specific mutations in developmental control networks, which are like instructions for growth and development that are carried out by the cells. Not all networks are cancerous. Developmental networks describe how normal, noncancerous cells divide and differentiate. Developmental networks can be activated by signals from inside the cell, from other cells, and from the cell’s environment. These cues are how normal cells are prompted to divide and differentiate. Specific mutations change the way a cell is told to divide and differentiate and cause the network and cell to become cancerous. Rather than random, uncontrolled growth, Werner described cancer as a highly regulated process in which the new instructions tell the cell to grow abnormally.

Linear Cancer Networks

Werner (2011) presented various types of developmental control networks that, if mutated, can direct a cell to produce cancer. Our research focused on linear cancer networks. A linear cancer network is a type of network where the number of cells grows linearly with respect to time. They are described as having a slower growth rate than other cancerous network types.

Linear cancer networks begin with one type of cancerous cell (hereafter A cell). When A cells divide into two cells, they produce another A cell and an A cell that differentiates into another type of cell (hereafter B cell). The A cells are cancer stem cells. The B cells are terminal, which means they do not divide. This asymmetrical division results in the number of A cells staying constant and the number of B cells growing linearly. In this case, A cells are the cells that are cancerous because they are responsible for producing the unlimited growth of B cells, and the B cells are not necessarily cancerous because they do not produce growth. This results in a tumor that consists of mostly B cells but is sustained by the A cells. These linear networks are not limited to only two cell types. There can be many different types of cell that divide and differentiate into other types of cells, but for the simplicity of the models, the only network considered was the linear network consisting of cells of type A and type B.

Cancer Stem Cells

Werner suggested that the A cell is a cancer stem cell. Normal stem cells are unique cells in that they have the ability to undergo self-renewal, where they create more of themselves, and they can also differentiate, where they turn into other types of cells after cell division. They are unspecialized cells that are responsible for maintaining the equilibrium number of cells of different types in the human body, replacing normal cells lost from injury or apoptosis, scheduled cell death. They do this by differentiating from their unspecialized forms to more specific types of normal cells found throughout the body. They are often tissue specific, meaning they differentiate into different types of cells that contribute to one type of tissue (Li & Xie, 2005). Normal cells undergo apoptosis after they have been dividing for long periods of time. The more a cell divides, the more likely it is to lose parts of its DNA or create defective cells.

Normal cells cannot proliferate forever. Stem cells, however, can. Stem cells have larger amounts of a specific enzyme that prevents their DNA from being damaged during cell division. For this reason, stem cells are able to live and divide indefinitely (Soltysova, Altanerova, Altaner, 2005). If stem cells become damaged and...
die, some of the other stem cells will stop differentiating and will produce more stem cells to replace those lost to return to the appropriate number of cells (Li & Xie, 2005).

It has been hypothesized that a cancer stem cell may be the result of either a mutated stem cell or a mutated normal cell that causes it to reproduce abnormally. There is a growing amount of evidence that supports the existence of cancer stem cells in several types of cancer, including Dingli and Michor (2006) and Milas and Hittelman (2009). Cancer stem cells can differentiate into other cells, just like noncancerous stem cells (Soltysova, Altanerova, Altaner, 2005). This agrees with Werner’s assertion (2011) that the A cells are cancerous stem cells. They differentiate into noncancerous B tumor cells that make up the majority of the tumor. However, in Werner’s description of a linear cancer network, he did not allow for the possibility that cancer stem cells may divide without differentiating, meaning they may increase the population of A cells.

Cancer Treatments

Radiation therapy is a form of cancer treatment that delivers photons to a tumor, and the photons release energy to break apart a cell’s DNA. Once its DNA is damaged, the cell can no longer reproduce and will eventually die (Sachs, Hlatky, Hahnfeld, 2001). A downside to radiation therapy is that it penetrates all the way through the body and deposits radiation in all of the cells it passes. This means the beam harms healthy tissues, even past the tumor. Photons can travel through the tumor and continue destroying tissue (Suit, 2003). But, the strength of the beam decreases exponentially as it travels. Radiation works best for tumors near the surface of the body where it still has the most energy, and deeper tumors are problematic to treat (Castellucci, 1998). Although it is an effective method to treat cancer, radiation therapy has many drawbacks. Proton therapy is similar to traditional radiation therapy in how it destroys the cell. It is a form of radiation that shoots a beam of protons at the tumor, rather than photons like in traditional radiation therapy (Castellucci, 1998).

Proton therapy has been shown to be more effective than other forms of radiation. In a study by Mu et al. (2005), the mean dose of radiation absorbed by surrounding organs was about 0.0 Gy, which was significantly lower than other forms of radiation treatment. Proton therapy delivers a larger dose of radiation to the tumor and harms the surrounding healthy organs and tissues less than photon radiation that uses X-rays. The greatest dosage is delivered at the end of the beam (Chen et al., 2012). Since protons have mass, they only travel a specific distance into the body and do not travel all the way through. The depth of the beam may be controlled by how much energy the protons start with, and that can be easily calculated and controlled. The maximum dosage of radiation can be administered to the tumor itself, rather than wasted on the surface of the skin (Castellucci, 1998). Proton therapy is more effective in killing cancer cells than traditional photon therapy because it can deliver more radiation to the tumor itself and less to the nearby healthy cells. Suit (2003) predicted proton therapy would largely replace photon therapy over the next 10 to 20 years.

The goal of this research was to mathematically model a linear cancer network as described by Werner (2011). Once the model of this network was established, the effects of radiation treatment and how it interacted with the network was explored, and mathematical models that show the effects of treatment on cancer stem cells, tumor cells, and healthy cells were created.

PREVIOUS MODELS

There are many mathematical models of radiation treatment for cancer, including Sachs et al. (2001), Dingli and Michor (2006), Belostotski and Freedman (2005), Freedman and Belostotski (2009), and Freedman and Pinho (2009).

Sachs, Hlatky, and Hahnfeld (2001) presented a linear-quadratic model of radiation therapy that focused on how the cell is damaged. Logistic growth was used with the rationale that tumor growth slows over time as the tumor becomes larger. Tumors rarely produce exponential growth. They grow rapidly in their initial stages but slow over time (Skehan, 1986), which supports the assumption of logistic growth.

In the work by Dingli and Michor (2006), a model was proposed that includes cancer stem cells. They described normal healthy cells, healthy stem cells, normal cancerous cells, and cancerous stem cells, and they modeled several forms of treatment. However, radiation therapy was not included in their models. It was concluded that one of the most important factors in treating cancer is destroying the cancer stem cells. If these cancer stem cells remain in the body, the tumor can grow back rapidly. In two works, Freedman and Belostotski (Belostotski & Freedman, 2005; Freedman & Belostotski, 2009) developed a model for radiation treatment using a system of two differential equations, where one considered healthy cells and the other accounted for cancerous cells. They assumed each cell population grows logistically and cancer cells and healthy cells each inhibit the growth of the other. They used four different methods of describing how the radiation dose was administered— as a constant, proportional to the number of cancer cells, proportional to the ratio of cancer cells to healthy cells, and periodically.

The model proposed in this paper expanded upon these previous models by incorporating the idea of cancer stem cells and cancerous developmental control networks. It drew support from these models in its depiction of radiation and cell division.

MODELS

Basic Linear Cancer Network Model

For a linear cancer network, Werner (2011) described a system where the population of A cells stayed constant and the population of B cells increased at a rate proportional to the number of A cells. B cells were terminal, so they did not divide, and they were produced indefinitely. Also, all the cancer cells lived forever. This can be modeled by the system of differential equations,

\[
\frac{dA}{dt} = 0 \quad \frac{dB}{dt} = k_1 A
\]

where

- A represents the number of A cancer stem cells,
- B represents the numbers of B tumor cells, and
- \(k_1\) is the rate that A stem cells are dividing.

Refined Linear Cancer Network Model

The previous system of Eq. 1-2 was slightly changed to make it more realistic. The new assumption was made that all cells grow logistically. Works by Skehan (1986) and Laird (1964) supported this assumption, as a tumor’s growth rate was shown to slow as...
it grows larger, and it was in accordance with many other models of cancer growth, such as Sachs et al. (2001), Belostotski and Freedman (2005), Freedman and Belostotski (2009), Freedman and Pinho (2009), and Dingli et al. (2006). Also included were healthy cells (H). These were only the healthy cells adjacent to the tumor that were close enough to be vulnerable to radiation. Although healthy cells are not part of a linear cancer network, they were included because the effect of radiation on these cells is important to monitor. It was assumed that they do not interact with the cancerous cells and vice versa for the simplicity of an analysis.

Our refined model is as follows:

$$\frac{dA}{dt} = k_1A(1 - \frac{A}{S})$$
$$\frac{dB}{dt} = k_1A(\frac{A}{S})(1 - \frac{B}{M_1}) - dB$$
$$\frac{dH}{dt} = k_2H(1 - \frac{H}{M_2})$$

where

- A represents the number of A cancer stem cells,
- B represents the numbers of B tumor cells,
- H represents the number of healthy cells vulnerable to radiation,
- $k_1$ is the rate that A stem cells are dividing,
- $k_2$ is the rate that H cells are growing,
- S is the desired number of A stem cells in the tumor,
- A/S is the fraction of A cell divisions producing B cells,
- M$_{1,2}$ are the carrying capacities of B and H, respectively, and
- d is the death rate of B cells.

Werner (2011) suggested that cancer starts off as one mutated cell, which is the first A cell, and that the A cell always divides asymmetrically, producing one A cell and one B cell, exemplified in the system of Eq. 1-2. It was then assumed in our model that the first A cell produced more A cells before differentiating into B cells, similar to how normal stem cells behave (Soltysova, Altanerova, Altaner, 2005). In the system of Eq. 3-5, A cells grew to a specific number by dividing to produce two daughter A cells rather than one A cell and one B cell until they reached the desired number (S). The fraction A/S in the dB/dt equation represents the fraction of A cell divisions that differentiate into B cells. If A<S, some A cells produce more A cells and some produce B cells. If A=S, all of the A cells produce B cells. It was assumed that A will never exceed S because A cells will not produce more A cells if they are at their capacity.

A death rate was incorporated in Eq. 4. While $k_2$, the growth rate of H, accounted for the birth rate and death rate of H cells, $k_1$ only described the division rate of A cells, which was also the birth rate of B cells. Therefore, it was important to include the - dB term in the equation for B cells to account for their natural death over time. Many types of cancer cells cease to undergo apoptosis, but there are other proposed mechanisms by which the cells can die (Brown & Attardi, 2005). Some oncogenes actually promote cell death (Lowe & Lin, 2000). Other causes for cancer cell death include natural immune responses to the cancer (Usman, Jackson, Cunningham, 2009). However, these occurrences would be relatively slow, so d was assumed to be a very small number.

To depict the growth of a tumor from its initiation, the initial conditions, which were denoted by A(0)= A$_0$, B(0)= B$_0$, and H(0)= H$_0$, described the tumor at the point immediately after the first A cell was created. This meant initial conditions A$_0$=1, B$_0$=0, and H$_0$=M$_2$.

**Radiation Treatment Model**

The goal of this research was to describe the effect of radiation therapy on these cancer networks with a mathematical model. The model proposed here considered only radiation delivery as a constant in its differential equations, as proposed in the work of Freedman and Belostotski (2009) and Freedman and Pinho (2009). The model is as follows:

$$\frac{dA}{dt} = k_1A(1 - \frac{A}{S}) - r_1,$$
$$\frac{dB}{dt} = k_1A(\frac{A}{S})(1 - \frac{B}{M_1}) - dB - r_2,$$
$$\frac{dH}{dt} = k_2H(1 - \frac{H}{M_2}) - r_3,$$

where $r_{1,2,3}$ are the respective effects of radiation.

It is important to note the initial conditions of the treatment models may not start at A$_0$=1, B$_0$=0, and H$_0$=M$_2$. Starting at these initial conditions would imply that a cancer cell is being treated immediately after it is formed, which is unreasonable. The cancer must grow large enough to be noticed before treatment may begin. It is more reasonable for treatment to begin when the populations are beginning to reach their carrying capacities. The simulations start with each cell population at carrying capacity.

**Dimensionless Form**

The model was nondimensionalized to reduce the number of parameters and simplify the calculation of equilibrium points. The dimensionless model is shown below.

$$\frac{dx}{d\tau} = x(1 - x) - Q_1$$
$$\frac{dy}{d\tau} = \alpha y^2(1 - y) - \delta y - Q_2$$
$$\frac{dz}{d\tau} = \beta z(1 - z) - Q_3$$

where

- $y = \frac{B}{M_1}, \quad \beta = \frac{k_2}{k_1}$,
- $z = \frac{H}{M_2}, \quad Q_1 = \frac{r_1}{k_1S}$,
- $\tau = k_1\tau, \quad Q_2 = \frac{r_1}{k_1M_1}$,
- $\alpha = \frac{S}{M_1}, \quad Q_3 = \frac{r_1}{k_1M_2}$. 

It is important to note that the dimensionless system produces the same behavior as the original treatment system (Eq. 6-8). Also, the parameters are all still positive. For more about nondimensionalization, see *A First Course in Mathematical Modeling* (Giordano, Fox, Horton, 2013).

**ANALYSIS**

**Equilibrium Points**

Equilibrium points were calculated by setting the growth rate equations in the dimensionless form (Eq. 9-11) equal to zero and solving in Mathematica 8. The equilibria helped in predicting the long-term behavior of the model. This produced four equilibrium points $E_n$ of the form:

$$E_1 = (x_1, y_1, z_1)$$
$$E_2 = (x_2, y_2, z_2)$$
$$E_3 = (x_3, y_3, z_3)$$
$$E_4 = (x_4, y_4, z_4)$$

where

$$x^* = \frac{1}{2}(1 - \sqrt{1 - 4\rho_1}), y^* = \frac{a(x^*)^2 - q_2}{a(x^*)^2 + b}, z^* = \frac{b\sqrt{\beta^2 - 4\beta_3}}{2\beta}$$

**Positivity of Equilibria**

It is interesting to establish conditions for the parameters of the model that determine whether the equilibrium points are positive or negative. This model did not include an equilibrium point where any of the populations are zero, which is typically thought of as a population dying. Whenever a population approaches a negative equilibrium value, it will die in a finite amount of time. When such a population reached zero, it was understood that it would not decrease further.

Let $R^+ = \{x \in \mathbb{R} : x > 0\}$. If it was assumed that $\rho_1 \leq 1/4$ and $\rho_3 \leq \beta/4$, it was clear that $x^*, z^* \in R^+$. Note that $A$ cell and healthy cell ($x$ and $z$) populations were positive only if they are real-valued. However, the positivity of $y^*$ was less clear than the other two populations.

The denominator of $y^*$ was clearly positive, but the numerator may not be. To eventually kill all the $B$ cells, the numerator must be negative, so it must be true that $\rho_2 > a(x^*)^2$. That meant for $E_1$ and $E_2$, it must be true that $\rho_2 > a(x^*)^2$, and for $E_3$ and $E_4$, it must be true that $\rho_2 > a(x^*)^2$ for the $B$ cell population to die out in the long term. The effects of this inequality will be further examined at a later point in this paper.

**Jacobian Matrix**

To analyze the stability of each equilibrium point, the Jacobian matrix of the model was calculated by taking the partial derivatives of the system of Eq. 9-11 in Mathematica 8.

$$J = \begin{bmatrix}
1 - 2x & 0 & 0 \\
2ax - 2axy & -ax^2 - \delta & 0 \\
0 & \beta - \beta z & 0
\end{bmatrix}$$

**Stability**

Upon substituting each equilibrium point into the Jacobian matrix in Mathematica 8, the positive eigenvalues produced from $E_1$, $E_2$, and $E_4$ revealed that these equilibrium points were always unstable. $E_3$ was the only stable equilibrium point, as eigenvalues of this equilibrium point substituted into the Jacobian matrix are all negative. This stable equilibrium point was then further examined.

**NUMERICAL STIMULATIONS**

**Low Radiation Effect on Nondividing Cells**

All cell types will reach an equilibrium at the stable equilibrium point, $E_3$, when the inequalities $\rho_1 \leq 1/4$, $\rho_3 \leq \beta/4$, and $\rho_2 \leq a(x^*)^2$ are satisfied. It was assumed that $\rho_2 \leq a(x^*)^2$ because radiation affects cells that are nondividing less than cells that are quickly dividing (Santini, Rainaldi, Indovina, 2000). However, it is interesting to consider the behavior of the system when one or both of the inequalities $\rho_1 \leq 1/4$ and $\rho_3 \leq \beta/4$ are violated.

Consider $\sqrt{(1 - 4\rho_1)}$ in $x_1$ and $y_1$, and $\sqrt{(\beta - 4\beta_3)}$ in $z_1$. If $\rho_1 > 1/4$, then $x_1, y_1 \notin R$. Likewise, if $\rho_3 > \beta/4$, then $z_1 \notin R$. To investigate the effect these inequalities have on the behavior of the model, four cases were developed (Figure 1):

- **Case 1**: $x_1, y_1, z_1 \in R$
- **Case 2**: $x_1, y_1 \in R$ and $z_1 \notin R$
- **Case 3**: $z_1 \in R$ and $x_1, y_1 \notin R$
- **Case 4**: $x_1, y_1, z_1 \notin R$

The dimensionless parameters were used to find numerical values for the original parameters that fit each case (Table 1). They were chosen arbitrarily, simply to describe each case. More realistic numbers may be acquired through biological study.

The rate of division of $A$ cells was arbitrarily chosen. For Cases 1 and 3, the growth rate of the healthy cells was chosen to be similar to the division rate of the cancerous cells. This was because studies have shown that individual cancer cells usually do not appear to divide more quickly than healthy cells (Santini, Rainaldi, Indovina, 2000). This meant that $B$ cells and $H$ cells were likely produced at the same rate, so the death rate of $B$ cells was chosen to bring the overall growth rate closer to the overall growth rate of $H$ cells. For Cases 2 and 4, the value of $k_2$ was chosen simply to display the desired behavior of each case.

In our simulations, all populations started at their carrying cap...
Each case was graphed in Mathematica 8 using the appropriate parameters shown in Table 1 to visualize the behavior the various real or nonreal elements of the equilibrium points created (Figures 2-5).

These graphs revealed that an equilibrium containing a non-real number resulted in the population reaching a negative number, though it was understood that the population would remain zero when it is reached. The number of B cells in Case 3 (Figure 4) and both B cells and healthy cells in Case 4 (Figure 5) would continue to decline until they reach zero. However, the number of A cells reached zero first, and the graphs cannot continue when the A cell population becomes negative because the negative population values produced unrealistic behavior.

### High Radiation Effect on Nondividing Cells

Each case’s behavior can vary further. Above, it was assumed that \( \rho_2 < \alpha(x+)^2 \). This meant that the radiation had very little effect on the B cells, since the B cells are terminal (Santini, Rainaldi, Indovina, 2000). While this could still be true for some types of cancer, investigating the effects of \( \rho_2 < \alpha(x+)^2 \) produced more interesting and biologically relevant behavior. This meant assuming radiation has a slightly larger effect on the B cells than before, which was highly plausible.

The following graphs (Figures 6-9) for each of the cases with large \( \rho_2 (\rho_2 > \alpha(x+)^2) \) were developed in Mathematica 8. The only difference between the parameters used for these graphs and the previous (Figures 2-5) was that \( \rho_2 \) was increased, as shown in Table 2. Figures 6-9 were generated by plotting Eq. 6-8 with the initial populations, each at their equilibrium. When the population of B cells reached zero, the simulation was stopped and Eq. 7 was replaced with dB/dt = 0, to keep the B cell population from continuing into negative values. Then, the graph was allowed to continue until the next population reached zero or a nonzero equilibrium. Each revised case resembled the previous graphs of each original case (Figures 2-5), except the B cells died out much more quickly. These graphs showed that it is possible for B cells to die out before Acells do.

Changing \( \rho_2 \) did not affect the previously discussed inequalities \( \rho_1 \leq 1/4 \) and \( \rho_2 \leq \beta/4 \). Changing \( \rho_2 \) merely affected the rate at which B cells died off. Although increasing \( \rho_2 \) even slightly had a dramatic effect on the behavior of the B cell population, there was no effect on the overall survival of the entire tumor. The difference was that A cells could then outlive the B cells, and if the A cells were not killed, they would repopulate the B cells and the tumor would grow back, which describes recurrence.

### DISCUSSION

#### Implications

The model predicted many possible outcomes of radiation treatment, including a cure, failed treatment, and cancer recurrence. The dimensionless parameter values for \( \rho_1 \) and \( \rho_2 \) that correspond to the outcome of each treatment is shown in Figure 10.

Three important outcomes of treatment explained by the model – tumor survival, tumor death, and recurrence – are summarized in Figure 10. Too small a value of \( \rho_1 \) resulted in the tumor surviving. This was exemplified in Case 1 (Figures 2 and 6) and Case 2 (Figures 3 and 7). In Case 2, realistically, the radiation would be stopped before the healthy cell population reached zero because the patient would be unable to physically withstand any...
The number of cells in each population on an arbitrary scale.

**Figure 2.** Case 1 is shown in this plot, where all equilibria are real numbers. $\rho_1 < 1/4$, $\rho_3 < \beta/4$. **Figure 3.** Case 2 is shown in this plot, where $x$ and $y$ are real, $z$ is nonreal. $\rho_1 < 1/4$, $\rho_3 > \beta/4$. **Figure 4.** Case 3 is shown in this plot, where $x$ and $y$ are nonreal, $z$ is real. $\rho_1 > 1/4$, $\rho_3 < \beta/4$. **Figure 5.** Case 4 is shown in this plot, where all equilibria are nonreal numbers. $\rho_1 > 1/4$, $\rho_3 > \beta/4$. **Figure 6.** Case 1 revisited is shown in this plot, where all equilibria are real numbers. $\rho_1 < 1/4$, $\rho_3 < \beta/4$, and $\rho_2 > \alpha(x)^2$. **Figure 7.** Case 2 revisited is shown in this plot, where $x$ and $y$ are real, $z$ is nonreal. $\rho_1 < 1/4$, $\rho_3 > \beta/4$, and $\rho_2 > \alpha(x)^2$. **Figure 8.** Case 3 revisited is shown in this plot, where $x$ and $y$ are nonreal, $z$ is real. $\rho_1 > 1/4$, $\rho_3 < \beta/4$, and $\rho_2 > \alpha(x)^2$. **Figure 9.** Case 4 revisited is shown in this plot, where all equilibria are nonreal numbers. $\rho_1 > 1/4$, $\rho_3 > \beta/4$, and $\rho_2 > \alpha(x)^2$. 
more treatment. Biologically, in Case 1 and 2, the dose of radiation delivered to the A cells was not great enough. The radiation decreased the population of A cells but did not kill them all. Also, the effect of radiation on the B cells, \( \rho_1 \), was not great enough to reduce the B cell population to zero. Radiation in this range merely shrunk the tumor, or lowered all populations’ equilibria without decreasing them to zero. If radiation were stopped, the remaining A cells would regenerate and the tumor would grow back to its pre-treatment size or larger.

Another possible outcome of the treatment the model can predict is recurrence. This is when there is not enough radiation delivered to the A cells, allowing them to survive, but the B cells receive enough radiation and are killed. This was shown in revisited Cases 1-4 (Figures 6-9), where the B cells died out before the A cells. Since there was such a massive number of B cells relative to the number of A cells in the tumor prior to treatment, if all of the B cells were killed by the treatment, the tumor would be too small to identify. This may be misidentified as a cure state, and radiation would be discontinued. The remaining A cells could regrow the tumor, and the cancer would return, as the model suggested. Even in revisited Cases 3 and 4 (Figures 8 and 9), where the A cells would eventually be killed, radiation may likely be halted before the A cells have time to die off because the B cells, the visible bulk of the tumor, will die first. The course of recurrence is exemplified in Figure 11, where treatment is ceased at time 30. The remaining cancer stem cells were able to quickly regenerate the tumor. Supporting this idea of recurrence, Werner (2011) stated that the goal of treatment is to remove all the cells that are actually dividing. It is important to kill the A cells, since they are the driving force behind the tumor and responsible for its growth and survival.

To prevent the previous two outcomes, radiation must be delivered so that the previously discussed inequality \( \rho_1 > 1/4 \) is true, demonstrated by original Case 3 and Case 4 (Figures 4 and 5) and revisited Case 3 and Case 4 (Figures 8 and 9), as long as radiation is continued for an adequate amount of time to kill the A and B cells. The ideal case was Case 3, where the tumor cells were killed by the radiation but the healthy cells survived. Converting the dimensionless inequalities back to the original, biologically meaningful form resulted in the following Case 3 inequalities:

\[
\begin{align*}
\rho_1 &> \frac{1}{4} \\
\frac{r_1}{k_5S} &> \frac{1}{4} \\
\frac{r_1}{k_5M_2} &\leq \frac{k_5}{4k_1} \\
r_3 &> \frac{k_5}{4} \\
r_3 &\leq \frac{k_5M_2}{4}
\end{align*}
\]

The first inequality (\( \rho_1 > 1/4 \)) suggests that in order to kill the cancer, the dose of radiation must exceed one-fourth of the growth rate times the carrying capacity of A cells. This makes sense biologically because increasing the size of the tumor (increasing \( S \)) or increasing the rate at which the tumor is growing (increasing \( k_5 \)) will require a larger amount of radiation. The maximum number of A cells and the rate at which they are dividing are what determine the size and growth of the tumor. A large \( S \) means that there are many A cells to produce B cells and that the tumor will grow faster and larger. Similarly, a large \( k_5 \) indicates that A cells can produce B cells and reproduce other A cells very rapidly. The dose of radiation (\( r_1 \)) must be large enough to combat those factors to result in the cure state.

The second inequality (\( \rho_3 \leq \beta/4 \)) suggests that the dose of radiation may not exceed one-fourth of the growth rate times the carrying capacity of healthy cells in order for the healthy cells to live. Increasing the total number of healthy cells (increasing \( M_2 \), because the initial population of healthy cells will be at the carrying capacity) or increasing the rate at which the cells can recover (increasing \( k_5 \), the rate they divide and regenerate) will result in a population of healthy cells that can withstand and recover from larger amounts of radiation. This inequality being true protects the healthy cells from dying. However, a cure state can be reached even when the inequality \( \rho_3 \leq \beta/4 \) is not true as long as \( \rho_1 > 1/4 \) is satisfied. This was exemplified by the original and revisited Case 4 (Figures 5 and 9). If radiation can be stopped after the cancerous A cells are killed but before too many of the healthy cells are, then a successful cure state will be reached.

**Figure 10.** A graph of \( \rho_1 \) vs. \( \rho_2 \) describing the result of treatment based upon the numerical values of the parameters. **Figure 11.** A plot of the number of cells in each population over time depicting the course of recurrence. After all of the B cells are killed, the tumor is too small to detect, and treatment is stopped at time 30. The A cells are able to quickly regrow the tumor.
Proton Therapy

Proton therapy does not greatly differ from typical radiation therapy in terms of the mathematical model, but there are differences in the parameters that distinguish the two from each other. Since proton therapy is much more selective toward the tumor (Chen et al., 2012), the value of $r_1$ would be significantly lower when modeling proton therapy than when modeling radiation therapy. The corresponding $r_2$ and $r_3$ could be much greater since a larger dose of radiation can be delivered to the tumor while affecting the healthy tissues less when treated with proton therapy (Suit, 2003).

The predicted benefits of proton therapy for the model are that the inequality $r_1 \leq \beta/4$ will be violated less frequently and the population of healthy cells will be less affected. Cases 2 and 4 (Figures 3, 5, 7, 9), where the healthy cells are greatly damaged, could be avoided. This would result in more successful treatments that would not have to be stopped because too many of the patient’s healthy tissues were being harmed. The patient is predicted to undergo less physical stress on his or her body with proton therapy. More realistic use of the model may show that it is difficult to kill the cancer stem cells without greatly harming the healthy cells with radiation. Proton therapy has been shown to greatly reduce the toxicity to healthy cells and may be much more effective at targeting cancer stem cells (Milas & Hittelman, 2006). Overall, proton therapy is much more effective than radiation therapy.

Strengths of the Model

A major strength of the model is that it uses the idea of cancer stem cells and developmental control networks. Few models have used the idea of cancer stem cells. One of the few mathematical models of cancer stem cells is Dingli and Michor (2006). But, our proposed model incorporated the idea of developmental control networks. Developmental control networks are a new idea on which very little research has been conducted, but they offer many explanations for the behavior of cancer (Werner, 2011). This is important because it allows the model to predict recurrence. Providing a mathematical explanation of this phenomenon is a great contribution from cancer control networks.

The treatment model (Eq. 6-8) and the related inequalities may also be used to predict the time required to kill all tumor cells and prevent tumor recurrence. This will be of great help to the medical field, because recurrence is a major problem for many types of tumors (Lin, 1999). Several measurements of a patient’s tumor progression over time may provide realistic, individualized parameters to be used in this model. Using realistic and accurate parameters, the time it takes to kill all of the tumor cells, including the cancerous stem cells, may be calculated, and radiation can be delivered to a patient accordingly. Also, if it is shown that the A cells die before the B cells, treatment may be stopped after killing all of the A cells, since the B cells will eventually die out. This could save a patient money and the stress of undergoing unnecessary radiation treatments.

Limitations of the Model

Although the model explained many new concepts, Werner proposed many types of cancer networks, such as linear, exponential, geometric, stochastic, and more. There are even expansions of the basic linear cancer network, called linear mixed cell networks, where there are more than A and B cells (Werner, 2011). These other types of cancer networks may be better predictors of actual cancerous behavior than the most basic linear network.

The model became unrealistic once any population reached zero. Realistically, the treatment would cease to affect a population of cells once that population reached zero. Instead, the model continued to subtract $r_{1,2,3}$ from the negative cell populations. This behavior was not biologically meaningful.

Another factor limiting the model was how the radiation is administered. In reality, radiation is not a one-time occurrence. It is delivered periodically, with breaks between treatments to allow the patient to recover from the previous treatment. Unfortunately, the model did not take this into account. The model depicted radiation as a singular treatment that either killed or failed to kill the cancer over time. The model was still very effective, because it described an overall, average effect of radiation in the long term, but it would be more realistic for a model to describe a periodic administration of treatment.

Also, there are different characteristics of cancer that can be modeled. One is competition between the cancerous cells and the healthy cells, like in Sachs et al. (2001), Belostotski and Freedman (2005), Freedman and Belostotski (2009), and Freedman and Pinho (2009), which can explain cancerous and healthy cells fighting for resources, healthy cells being negatively affected by byproducts of the cancerous cells, and more. The terms that model these interactions can get incredibly complicated and nonlinear. Unfortunately, competition was ignored for the sake of the analysis.

Future Work

There is much to expand on with this basic model of cancer networks and cancer treatment. First, the proposed model was of the simplest type of cancer network – a linear cancer network. As mentioned above, there are a seemingly endless number of networks that could all be modeled by differential equations. In the future, these could each be mathematically modeled.

As previously mentioned, the one-time administration of radiation depicted by the model was not entirely realistic. Though still meaningful, the model could be made more realistic if radiation was delivered periodically, similar to the fourth control in the work of Freedman and Belostotski (2009). This would be an interesting direction to take this research in the future.

However, Werner (2011) stated that radiation may not be the best way to treat a cancer that fits into the category of a linear cancer network because the radiation may mutate B cells into A cells instead of killing them. This is a common fear of radiation therapy. Radiation has been shown to sometimes mutate healthy cells into cancerous cells and cause a secondary cancer (Hall 2006). The model did not account for this mutation into A cells, and developing a model that does may be of interest. Although proton therapy reduces the risk of mutating other cells into cancerous cells, investigating and modeling other treatments and how they affect healthy cells, cancer stem cells, and developmental control networks could be beneficial.

CONCLUSION

The model was an effective depiction of a linear cancer network and radiation treatment, and it provided a mathematical explanation for recurrence, resulting from the incorporation of cancerous developmental control networks in the model. The model also can be used to predict the time and dose of radiation necessary to prevent negative results of treatment. This will be a great aid to
medical professionals, but to properly utilize this model, realistic numerical values for the parameters must be found. Also, there is much more to investigate about these developmental control networks, like other types of networks, cells, cell interactions, and cancer treatments. Ideally, this research is a launching pad for future study.

ACKNOWLEDGEMENTS
I would like to thank my research mentor, Dr. Zachary Abernathy, and the program director, Dr. Joseph Rusinko. This research was conducted during the 2013 NREUP at Winthrop University. I would like to thank the MAA for creating this program, which is funded by the NSA (grant H98230-13-1-0270) and the NSF (grant DMS-1156582).

All computations are available from the author upon request.

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Supercomputers and Atoms: The career of a computational chemist

Aiman Faruqi, University of Michigan

In the late seventeenth century, Isaac Newton laid down the foundation for classical physics with his laws of motion and gravity. His work remained the central pillar of the physical sciences until the early twentieth century, when an entirely new set of physical laws—quantum mechanics—was in its infancy. The development of quantum mechanics was motivated by the discovery of the atom and its constituent subatomic particles; although the concept of an atom—a fundamental unit of matter that cannot be split—dates back to ancient Greece, the first pieces of scientific evidence supporting its existence emerged in the nineteenth century.

“Since the discovery of the atom, chemistry has tried to explain phenomena at the atomic level. This motivated the development of theories that could predict molecular structure and behavior. Due to the immensely small size of atoms, however, experimental techniques have trouble seeing these details—thus computational chemistry stepped in to directly simulate the properties of very small things,” says Dr. Paul Zimmerman, a computational chemist at the University of Michigan.

Indeed, the properties of the subatomic realm are drastically different from our intuition of the macroscopic world. Under the rules of quantum mechanics, the states of particles are described in probabilities rather than definite states. Additionally, as the macroscopic systems become more complex, from single atoms to large molecules, the math needed to describe them becomes completely intractable. These formidable challenges of the quantum world necessitated the use of a new tool—the digital computer—to make any progress on our understanding of this new frontier.

Although computational chemistry emerged from the singular development of quantum mechanics, its modern applications are quite diverse.

As Dr. Zimmerman explains, “the field extends from energy sciences and materials for solar cells to medicinal synthesis and biological systems and beyond. In my opinion, there are a huge number of applications to computational chemistry to be excited about.”

In one recent study, a team of biochemists at Duke University and the University of Hong Kong used computational methods to determine the chemical mechanism by which bacterial pathogens bind to human cells during the infection process, a task that would have proven tremendously difficult by experimental approaches. Although computer models are unlikely to replace traditional approaches in other fields, they have nonetheless shown to be useful new tools.

In addition to its novel utility in a wide variety of fields, computational chemistry also demonstrates the synergy between theory and experiment, an often-contentious topic within the scientific community.

Dr. Zimmerman notes, “Theory and experiment are highly complementary to one another. The most successful science employs both—experiment leads to hypotheses that can be modeled by theory, and theory returns the favor by allowing predictions of the outcome of new experiments.”

Experimental labs often collaborate with computational labs to explain interesting results or guide experimental procedures, and professionals often participate in both types of research settings to ensure the validity of their findings. One of Dr. Zimmerman’s current collaborations is with an experimental catalysis lab.

“Our group is working with the McNeil group at Michigan to develop catalysts that can switch their functionality. Normal catalysts perform a single task very well, for instance synthesis of a specific plastic from petroleum resources. The collaboration with McNeil’s group is to design and test catalysts that can perform more than one task, which should yield not only new basic science for catalysis, but also abilities to synthesize new types of materials.”

The interdisciplinary nature of computational chemistry provides professionals with tremendous flexibility. An added advantage of using computational resources either to predict experimental results or confirm empirical findings is the tremendous computational power of modern supercomputers; these machines can run thousands of complex mathematical calculations and provide accurate simulations of chemical systems in a matter of hours, all without the need for advanced expertise.

The day-to-day routine of a computational chemist at a university is nothing out of the ordinary: “Typical days are spent meeting and advising students, preparing and teaching classes, as well as writing grant applications,” though Dr. Zimmerman also notes that “graduate students working in this area are able to spend the majority of their time preparing and analyzing simulations.”

Unlike many other fields however—where the scope of required knowledge is relatively narrow—computational chemistry demands proficiency in multiple fields: “Programming and mathematics [are] necessary tools for computational chemistry,” Dr. Zimmerman explains. “These skills, however, are most useful when applied using chemical ideas. I spend the majority of my time thinking chemically, and then apply these chemical principles to simulation using whatever aspects of math and programming that are needed.”

The central role of modern technology in computational chemistry makes the field an excellent career choice for students of the 21st century. Traditionally, science students with an interest in math would pursue physics or engineering; computational chemistry provides an additional opportunity for the mathematically minded.

Additionally, computer science students who are interested in the physical sciences may wish to consider the field. Dr. Zimmerman also suggests that “students who are interested in how physical properties lead to big picture outcomes might love computational chemistry.” Perhaps most significantly, the field’s diverse array of potential applications—from materials engineering to drug design—outfits students to become indispensable professionals in an economy where strong STEM skills are sorely needed.
The ‘Steaks’ Are High: In vitro meat moves from the petri dish to the palate

Ria Foye-Edwards, Syracuse University


Over the past century and a half the world has witnessed some of the most innovative ideas come to life; some of these ideas were stumbled upon by accident and others arose from pure inquiry. Take, for instance, the creation of the potato chip and the discovery of the antibiotic, penicillin. These inventions have proven successful over time and are still being produced for the public today.

However, great creations are seldom the result of trickery and accident (as was the potato chip and penicillin), but instead the outcome of years of hard work done by researchers who aim to fix worldwide problems.

One issue that has attracted much concern is the rapid use of Earth’s natural resources and the strain that humans are inflicting on the environment in order to support a growing population. As a result, the world’s population of 7 billion people requires and uses massive amounts of energy and land - resources which are not easily replenished.

For millennia, farming livestock for meat has proven a dependable method of providing food for many. In recent years, however, as researchers from around the globe become more aware of the changing climate, livestock have been determined as one of the main sources of methane, a major greenhouse gas.

One of the most creative attempts to reinvent a food system that is environmentally friendly and capable of keeping up with rising demand is the use of a tissue engineering method to create in vitro meat.

Producing in vitro meat involves extracting muscle cells from an animal, putting them into a growth medium, and letting the cells proliferate and develop into muscle tissue - which is the main component of meat we consume. This method, if performed on a large scale, could supplement increasing meat demands and use fewer animals, supporters say.

Dr. Mark Post, a researcher at Maastricht University in the Netherlands, is currently at the forefront of research to produce in vitro beef, also called cultured beef. Post first became involved with the idea back in 2008 when he taught tissue engineering at Eindhoven University of Technology.

“I coincidentally came across people who were interested in the idea and specifically an older gentleman in Amsterdam who had been obsessed with the notion of creating meat from stem cells through cell culture,” Post recalls.

It wasn’t by coincidence, but rather hard work that six years later Post and his team have successfully made the first in vitro hamburger.

“It’s a traditional cell culture method so you use culture medium containing amino acids, sugars, minerals and vitamins with the addition of fetal bovine serum to grow the cells...it takes about eight to nine weeks and is pretty intensive, for one hamburger you typically grow about twenty billion cells,” exclaims Post.

The cultured burger has been sampled and the tasters agreed that it had a texture similar to a farmed beef burger, but that it lacked the fat that is responsible for additional flavor and fatty acids. As a result, research is currently being undertaken by Post to culture fat cells.

“Currently we are culturing fat cells separately,” he says. “We could co-culture them with muscle cells, however it’s more complex, and that would be the next phase.”

Post and his team are experimenting with new ideas in order to enhance the burger’s appearance and taste. “Another thing we are working on is trying to boost the expression of myoglobin, which is a protein in the muscle that carries iron and oxygen...I have a feeling that the iron contributes to the taste and definitely adds to the color of the meat through its oxygen carrying. We hope that helps recreate the taste,” Post adds.

With the current method, this process remains rather time consuming and production must be scaled up in order to reduce the price of the product (which is currently £250,000, that’s $400,145!) and make it readily available to the public. However, there is much concern from researchers and those advocating for in vitro meat about general acceptance.

Post says, “Are people generally going to accept this as an alternative for meat? As for the vegan and vegetarian communities some are very receptive and welcome this idea while others are more skeptical and feel it’s still an animal-derived product.”

Alanna Wagy, a College Campaign Assistant for PETA, shared several reasons why PETA is among those in the vegan/vegetarian community who are supportive of in vitro meat. In fact, PETA is offering a one million dollar reward for the first researcher to create in vitro chicken.

“Since we announced the contest, which actually ended on the fourth of May, more research than ever has been done in terms of in vitro meat in the U.S. and internationally,” shares Wagy.

But, unlike Dr. Post’s motive for securing the welfare of the environment, PETA has concerns rooted more strongly in the protection of the animals that are being used for food sources.

Wagy claims, “We decided to get involved because of the number of chickens that are killed. Roughly one million chickens are killed in an hour in the U.S and we realized that, while it’s somewhat easy to transition to a vegan diet and live plant based, there are always going to be people who are set in their dietary habits. We wanted to explore every possible alternative to stop animal cruelty at the source.”

This attempt to stop animal cruelty at its root could possibly cause livestock farms and meat manufacturing companies to dwindle. That is, If ever in vitro meat becomes the dominant form of meat production, this could spell trouble for existing producers. However, these companies could perhaps transition to new meth-
ods that will keep their businesses thriving. But how would in vitro meat impact local farmers? Would they be able to transition and produce in vitro meat like larger livestock manufacturers? Dr. Post describes such a scenario:

“There might be incentives to doing this [producing in vitro meat] on a smaller scale. Take, for instance, a small town with a farm with a few animals where locals townsmen feed them, tend to them and even give them names—and once in a while you take a sample from them for the stem cells and, in a building adjacent to the farm, you grow the meat for the entire community. Then you won’t have a lot of transport issues, people realize where their meat is coming from, and it would become more idealistic.”

If in vitro meat catches on, it will likely take some time before seeing in the “beef” aisle at the grocery store. But what’s clear is that much progress has been made since the idea was first introduced. With researchers like Dr. Post and organizations like PETA promoting a more sustainable and animal friendly alternative, who knows if researchers will invent even more foods in vitro.

That being said, there is still plenty of time for the public to form their opinion and either accept or reject this new form of meat production. But for those working towards an in vitro food system they can only hope for one thing: for society to remain open to this alternative method.

This feature was written under the guidance of Science Writing Mentor Brian Howard.
INTRODUCTION

Humans encounter a variety of microorganisms every day, many of which generally do not cause disease (Belkaid & Segre, 2014; Macpherson & Harris, 2004; Zanetti, de Luca, Leoni, & Sacchetti, 2014). Delftia spp. is one such genus of microorganisms identified in human samples (DiGiulio et al., 2008; Hagiya et al., 2013; Kawamura et al., 2011; Khan, Sistla, Dhodapkar, & Parija, 2012; Peraneva et al., 2013; Preiswerk, Ulrich, Speich, Bloemberg, & Hombach, 2011). Delftia spp. are rod-shaped, gram-negative, aerobic, flagellated bacteria that have been isolated from Tibetan glaciers (Zhang, Yang, Wang, & Hou, 2010) as well as public and private water filtration devices in Italy (Zanetti et al., 2014). Delftia spp. are also noted for involvement in the formation of gold nuggets, for the capacity to produce antifungal nanoparticles, and for their identification in the water system of the International Space Station (Johnston et al., 2013; Kumar & Poornachandra, 2015; La Duc, Sumner, Pierson, Venkat, & Venkateswaran, 2004).

Recent case reports indicate that Delftia spp. are resistant to antibiotics from multiple classes, including aminoglycosides (Hagiya et al., 2013; Khan et al., 2012). Because Delftia spp. are generally considered non-pathogenic and are resistant to commonly used antibiotics, patients frequently suffer delays in effective treatment due to the time required to identify Delftia spp. infection and assess antibiotic susceptibility. An example of this is when a 46-year-old patient developed Delftia acidovorans-associated bacteremia as the result of organophosphorus poisoning (Hagiya et al., 2013). This patient was administered ineffective antibiotics for eight days prior to identification of D. acidovorans (Hagiya et al., 2013).

Many clinical case reports link Delftia spp. infection with intravenous or indwelling catheters. For example, D. acidovorans-associated bacteremia was found in a healthy four-year-old female with an intercostal drainage tube (Khan et al., 2012). In a different case, an eleven-year-old immunocompromised female suffered recurring D. acidovorans-mediated bacteremia as a result of a contaminated indwelling catheter (Kawamura et al., 2011). Despite increasing reports of infection by Delftia spp., the pro-inflammatory response elicited by this bacterium has not been determined in previous studies. Given that isolates of this bacterium are resistant to many commonly used antibiotics, understanding the immune response is essential to identifying novel anti-inflammatory targets to prevent the development of sepsis in patients infected with Delftia spp.

During sepsis, bacterial antigens, such as lipopolysaccharide (LPS) and flagellin, initiate a robust and dysregulated systemic inflammatory response by activating innate immune receptors, such as toll-like receptors (TLRs) (Opal et al., 1999; Ramos, Rumbo, & Sirard, 2004). Herein we investigate the pro-inflammatory immune response to Delftia spp. infection by stimulating THP-1 cells with two heat-killed species: D. acidovorans ATCC 13751 and Delftia sp. Cs1-4. THP-1 cells, a human monocytic cell line, robustly respond to bacterial antigens, such as LPS, which is a component of gram-negative cell walls. THP-1 monocytes are a good model for studying sepsis, given that monocytes are blood mononuclear cells that readily become activated upon stimulation with bacterial antigens.

In this novel study, we stimulate monocytes with Delftia spp. strains at different multiplicities of infection (MOI), or the amount of bacteria relative to the amount of monocytes in each sample, to...
study the affect that the dose of bacteria may play on initiation of the pro-inflammatory response. We evaluate THP-1 mortality via a trypan blue and automatic cell counting approach using a cellometer. We also evaluate the expression of three molecules involved in the pro-inflammatory immune response: cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF), and myristoylated alanine-rich c-kinase substrate (MARCKS). The expression of these mediators was determined by Western blot and enzyme-linked immunosorbent assay (ELISA). COX-2 is a pro-inflammatory enzyme involved in the production of prostaglandin E2, which acts as a vasodilator and activates immune cells (van Ryn, Trummlitz, & Pairet, 2000). TNF is an acute phase, pro-inflammatory cytokine that also serves as a vasodilator and promotes the inflammatory response (Bradley, 2008). While COX-2 and TNF are necessary for normal inflammatory responses, during sepsis the expression of these mediators becomes dysregulated, resulting in septic shock, organ failure, and if not properly treated, death. MARCKS is an actin-binding protein that is upregulated by LPS stimulation and has been shown to negatively regulate LPS-mediated signaling (Mancek-Keber et al., 2012; Rose, Byers, Morash, Fedoroff, & Cook, 1996; Sunohara, Ridgway, Cook, & Byers, 2001). MARCKS also regulates the migration of immune cells toward sites of inflammation as well as the production of cytokines and inflammatory mediators (Eckert, Neuder, Park, Adler, & Jones, 2010; Green et al., 2012; Li et al., 2013; Mancek-Keber et al., 2012).

Our overall hypothesis for this study was that stimulation of monocytes with *Delftia* spp. would result in an increased pro-inflammatory response, as measured by elevated COX-2, MARCKS, and TNF protein expression. Our data presented herein supports this hypothesis and demonstrates that stimulation of monocytes with *Delftia* spp. results in increased mortality and expression of pro-inflammatory mediators that are associated with sepsis. The results of this study will raise awareness within the medical and public health communities about the *Delftia* genus and its modulation of the pro-inflammatory response.

**MATERIALS AND METHODS**

**Preparation of heat-killed *Delftia* spp.**

*Delftia* strain Cs1-4 was obtained from the laboratory of Dr. William Hickey at the University of Wisconsin-Madison. *Delftia acidovorans* strain ATCC 13751 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cultures were plated and grown in order to prepare sufficient amount of bacteria for the stimulation experiments. *Delftia* cultures were propagated from -80°C freezer stocks by plating on duplicate Tryptic Soy Agar (TSA) plates. TSA plates were incubated for 72hr at room temperature. Tryptic Soy Broth (TSB) cultures (2.5mL) were inoculated in duplicate with isolated colonies of each strain and incubated overnight in a shaking incubator at 30°C and 200rpm. So that an equivalent amount of bacteria was used in each experiment, the cultures were each normalized to an OD600 absorbance value of 1.0 in phosphate-buffered saline (PBS). One hundred microliters of each culture was set aside for determination of colony forming units (CFU)/mL per OD600 unit. The remainder of each normalized culture was incubated at 60°C for 1hr in order to kill the bacteria. An aliquot of the heat-killed cultures was plated in duplicate on TSA plates and incubated for 3d at 30°C to test cell viability after heat exposure. The remainder of the heat-killed cultures were stored at -80°C until use.

**THP-1 cell culture and stimulation**

THP-1 cells (TIB-202, ATCC) were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) with 10% heat-inactivated fetal bovine serum, penicillin (100U/mL), streptomycin (100μg/mL), amphotericin B (250ng/mL), and β-mercaptoethanol (0.1%). This medium provided optimum growth conditions with antibiotics to prevent any bacterial growth. Viable cells were counted by trypan blue exclusion using a Nexcelom Bioscience Auto T4 Cellometer (Lawrence, MA) so that equivalent numbers of viable monocytes were used in each stimulation. 1x106 viable cells were added to six wells of a 24-well plate. One mL of sterile PBS was added to the empty wells. Each well was stimulated as follows: (1) PBS, (2) 100ng/mL LPS (Sigma-Aldrich, St. Louis, MO), (3) *Delftia acidovorans* ATCC 13751 at a multiplicity of infection (MOI) of 0.6, (4) *D. acidovorans* ATCC 13751 MOI of 6.0, (5) *Delftia* sp. Cs1-4 MOI of 0.8, and (6) *Delftia* sp. Cs1-4 MOI of 8.0. The plate was incubated for 24hr at 37°C with 5% CO₂.

**Lysate Preparation**

Samples were harvested (with aliquots reserved for mortality assays) and centrifuged at 3,000rpm for 5min at 4°C. The supernatant was removed and stored at -80°C for the enzyme-linked immunosorbent assay (ELISA). The remaining cell pellets were lysed in order to later measure the protein contained within. The cell pellets were resuspended in 100μL ice-cold (RIPA) buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150mM NaCl, 50mM Tris-HCl) to denature the cell membranes and was supplemented with a Roche complete mini protease inhibitor cocktail tablet (Indianapolis, IN). One tablet was added to 10mL of RIPA buffer. Each sample was sonicated three times for 15sec with ice incubation between sonication steps. Lysates were clarified by centrifugation at 13000rpm for 10min.

**SDS-PAGE, Western blot, and densitometry**

To ensure that the same amount of protein from each sample was used in the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), protein concentrations of the different samples were calculated two different ways. Protein concentrations of the samples from the first three experiments were determined using the DC protein assay (Bio-Rad, Hercules, CA). The working reagent was prepared using a 50:1 ratio of Reagent A to Reagent S. In each well of a non-treated 96-well plate, 5μL of sample was mixed with 25μL of working reagent, and 200μL of Reagent B was added. The plate was incubated at room temperature for 15min and absorbance was measured at 750nm on a BioTek Synergy HT plate reader (Winooski, VT). The fourth experiment used a Pierce™ BCA Protein Assay Kit (Rockford, IL). A working reagent was prepared by mixing 50:1 ratio of Reagent A to Reagent B. This working reagent was added to each sample at a ratio of 20:1 working reagent to sample in a non-treated 96-well plate. The plate was incubated at 37°C for 30min and absorbance was measured at 562nm on a plate reader.

SDS-PAGE was used to separate all proteins in the lysate isolated from the cells. Lysates were resuspended in 5X Laemmli sample buffer and boiled for 5min at 95°C prior to loading equal protein levels onto an SDS-PAGE gel (10% NuSep nUVview pre-cast gels, New South Wales, Australia). Each sample was loaded...
onto two separate SDS-PAGE gels along with a Precision Plus Kaleidoscope molecular weight marker (Bio-Rad) and gels were run at 60mA in Tris-Glycine-SDS running buffer. Total protein was transferred to a nitrocellulose membrane using an iBlot semi-dry transfer system (Invitrogen, Carlsbad, CA) so that the proteins became fixed on the membrane to allow for antibody-mediated probing of specific proteins. The gel was placed on a nitrocellulose membrane between the anode and cathode stacks and incubated in the iBlot for 7min on the pre-set Program 3 (P3) to complete the transfer.

Both membranes were cut at 50kDa in order to probe for cytochrome-c oxidase (COX-2; 70kDa) and myristoylated alanine-rich c-kinase substrate (MARCKS; 75kDa) at the same time as the glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 35kDa) loading control. The membranes were first blocked to prevent non-specific binding of the antibodies to the membrane. Membranes were blocked in 5% non-fat dry milk (NFDM; suspended in PBS containing 0.05% Tween (PBS/T)) for 1hr at room temperature, shaking. The membranes were then incubated in primary antibodies specific to the proteins being probed. The COX-2 (D5H5) and MARCKS (D88D11) antibodies (1:1000 dilutions) were purchased from Cell Signaling Technology (Beverly, MA); the GAPDH antibody (1:5000 dilution) was purchased from Sigma (clone GAPDH-71.1). Antibody dilutions were prepared in 5% bovine serum albumin (BSA) in PBS/T with 0.2% sodium azide and incubated overnight at 4°C while shaking. Membranes were washed in PBS/T five times for 5min each.

Next the membranes were incubated in secondary antibodies, which served two purposes: (1) binding of the protein-specific primary antibody and (2) detection of the protein of interest through an enzyme-substrate reaction. The GAPDH blots were incubated in goat anti-mouse horseradish peroxidase (HRP, enzyme); E3113; Santa Cruz Biotechnology, Dallas, TX) at a 1:5000 dilution. The COX-2 blot was incubated in anti-rabbit HRP (7074S, Cell Signaling Technology) at a 1:3000 dilution for the first experiment and a 1:4000 for the last three. The MARCKS blot was incubated in anti-rabbit HRP (7074S, Cell Signaling Technology) at a 1:3000 dilution. Secondary antibody dilutions were made in 5% BSA in PBS/T and incubated at room temperature shaking; GAPDH incubated for 1hr while COX-2 and MARCKS incubated for 2hr. Membranes were washed as described above and exposed to the Thermo Scientific Pierce enhanced chemiluminescence (ECL) Western Blotting Substrate for 1min. This substrate reacted to the HRP conjugated to the secondary antibodies, allowing for visualization of the proteins of interest. Membranes were separately imaged in the Bio-Rad Molecular Imager ChemiDoc XRS+ Imaging System using Image Lab software (Version 4.1; Bio-Rad, 2012). A 600sec exposure was used with images taken every 10sec.

The blots were analyzed using densitometry, which is a measurement of the intensity of the substrate signal of a sample. Densitometry of the membranes was performed using Image Lab software (Version 4.1; Bio-Rad, 2012). Density values of each band were recorded and analyzed in Microsoft Excel. The COX-2 and MARCKS bands were normalized to their corresponding GAPDH bands and fold-change relative to the PBS sample was calculated.

**Mortality**

Cell viability was performed using trypan blue exclusion on an automated cellometer with the aliquots saved during the lysate preparation. Percent mortality was calculated by dividing the number of dead cells (trypan blue positive) by the total number of cells counted and multiplying by 100.

**TNF ELISA**

Production of tumor necrosis factor (TNF) was determined using the eBioscience human TNF alpha ELISA Ready-SET-Go! Kit (San Diego, CA). In this assay, a specific protein (i.e., TNF) binds to a series of antibodies in a 96-well plate, with an enzyme-substrate reaction-mediated color change as the readout for protein presence that is measured by a plate reader. The greater the color change of the enzyme-substrate reaction, the greater amount of the specific protein present. All solutions used were diluted as specified on the certificate of analysis and the provided experimental procedure was followed. The plate was incubated with the capture antibody overnight at 4°C and blocked for 1hr at room temperature before the samples (diluted 1:10) and standard were added to the plate in duplicate. The standards were prepared by serial dilutions of a 500pg/mL stock, resulting in the following standard curve points (pg/mL): 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 0. The standard curve of known concentrations was used to calculate unknown protein concentrations in the sample. The plate was incubated for 2hr and washed five times in the provided wash buffer before the 1hr detection antibody incubation. The plate was again washed five times before incubation with Avidin-HRP for 30min. The plate was washed five times again and substrate solution was added and incubated for 10min before the stop solution of 1M H3PO4 was applied to halt the reaction so that the samples could be analyzed by the plate reader. The plate was read at 450nm on a BioTek Synergy HT plate reader. The equation of the standard curve was used to calculate protein concentration in pg/mL from the absorbance readings.

**Statistical Analysis**

Statistical analysis was performed using the GraphPad Prism software package (Version 6; Graph Pad Software, Inc., 2014) and a two-tailed t-test, with a p-value of less than .05 considered significant.

**RESULTS**

A statistically significant increase in mortality was observed in lipopolysaccharide (LPS) and Cs1-4 multiplicity of infection (MOI) of 8.0 treatment groups as compared to phosphate-buffered saline (PBS) when stimulated for 24 hours (Figure 1). The remaining groups (ATCC MOI of 0.6, ATCC MOI of 6.0, Cs1-4 MOI of 0.8) resulted in a modest increase in cell mortality compared to PBS control. The PBS group resulted in about 5% monocyte mortality, which may be attributed to the natural mortality rate of the cells.

Stimulation with the higher MOI of *Delftia* spp. (ATCC MOI of 6.0 and Cs1-4 MOI of 8.0) revealed dramatically increased cytochrome-c oxidase (COX-2) expression as compared to both PBS and LPS stimulated groups, with the Cs1-4 MOI group being statistically increased (p < .05) compared to PBS (Figure 2). A modest elevation of COX-2 protein expression compared to the baseline protein expression of the PBS group was observed in the LPS, ATCC MOI of 0.6, and Cs1-4 MOI of 0.8 treatment groups. The LPS stimulated group was not significantly increased.
Figure 1. Stimulation of THP-1 monocytes with *Delftia* sp. Cs1-4 (MOI of 8.0) resulted in significantly increased mortality. Percent mortality was determined by automated trypan blue exclusion. Significance (\(p < .05\)) relative to the PBS group is denoted by an asterisk (*). Data is representative of four independent experiments.

Figure 2. THP-1 monocytes treated with the higher MOI of *Delftia* spp. strains (high and low MOI) and the LPS group significantly increased tumor necrosis factor (TNF) production in THP-1 cells as compared to PBS (Figure 4).

**DISCUSSION**

Our experiments focused on the human immune response to *Delftia* spp. using two commonly-used strains: *Delftia* sp. Cs1-4 and *Delftia acidovorans* ATCC 13751. Upon stimulation of monocytes with several different multiplicities of infection (MOI), our results consistently showed an increased inflammatory response with regards to cell mortality, cyclooxygenase-2 (COX-2) and myristoylated alanine-rich c-kinase substrate (MARCKS) protein expression, and tumor necrosis factor (TNF) production.

In comparison to the phosphate-buffered saline (PBS) treatment group, a trend for increased cell mortality was observed in all treatment groups, and this included a statistically significant increase in the lipopolysaccharide (LPS) and Cs1-4 MOI of 8.0 groups. It should be noted that a 5% mortality rate was observed in the PBS group, which most likely indicates the baseline mortality rate of the cells. It is also important to note that the bacteria were heat-killed prior to stimulation, which may have resulted in decreased monocyte mortality compared to stimulation with live *Delftia* spp. Regardless, the significance of the Cs1-4 MOI of 8.0 treatment group demonstrates that *Delftia* spp. negatively affect the viability of immune cells. The response caused by Cs1-4 MOI of 8.0 is significant throughout our experiment, as significantly increased MARCKS and COX-2 expression as well as TNF production were also observed in cells stimulated with this treatment.

A dramatic increase in COX-2 protein expression was observed in the higher MOI *Delftia* spp. treatment groups compared to the PBS control. The ATCC MOI of 6.0 treatment group was nearly significant (\(p = 0.050\)) and the Cs1-4 MOI of 8.0 treatment was statistically significant. The tenfold increase in MOI in the ATCC and Cs1-4 strains resulted in about a five-fold increase in COX-2 protein expression compared to the PBS control. It is likely that this induction is dependent on the multiplicity of infection and future experiments should include a dose response with *Delftia* spp. strains.

Low COX-2 protein expression was observed in the LPS, ATCC MOI of 0.6, and Cs1-4 MOI of 0.8 treatment groups, which is contrasted to the significant and nearly significant values of the higher MOI *Delftia* treatment groups. The lack of significance in the LPS group was unexpected, as LPS is shown to increase COX-2 expression (Barrios-Rodiles, Tiraloche, Chadee, 1999). This could be explained several ways. First, an LPS dose response could be performed to determine the appropriate LPS concentra-
Figure 3. MARCKS protein expression was significantly increased in THP-1 monocytes stimulated with both strains of Delftia spp. A: Representative Western blot of MARCKS and GAPDH loading control. B: Protein densitometry of MARCKS normalized to GAPDH with fold-change relative to PBS treatment group. Significance ($p < .05$) relative to the PBS group is denoted by an asterisk (*). Data is representative of four independent experiments.

Figure 4. THP-1 monocytes stimulated with both strains of Delftia spp. had significantly increased TNF protein production. TNF protein production (pg/mL) in culture supernatants was measured by ELISA. Significance ($p < .05$) relative to the PBS group is denoted by an asterisk (*). Data is representative of four independent experiments.

Our data suggest that patients who are infected with Delftia spp. in either a local or systemic manner likely have elevated COX-2 protein expression. Given that COX-2 is involved in the genesis of the potent vasodilator prostaglandin E2 (PGE2) (Norberg et al., 2013; Ricciotti & FitzGerald, 2011), we hypothesize that elevated PGE2 is also observed in Delftia spp. infections. Additionally, these data suggest the use of either selective or non-selective COX-2 inhibitors to control inflammation associated with Delftia spp. infection. Examples of selective COX-2 inhibitors include cavidine (Niu et al., 2014) and esculentic acid (Niu et al., 2014), while the non-steroidal anti-inflammatory drug (NSAID) diclofenac is an example of a non-selective COX-2 inhibitor (Kida et al., 2014). Further investigation is needed to confirm that these selective and non-selective inhibitors decrease COX-2 expression and subsequent prostaglandin E2 production upon Delftia spp. stimulation. In our studies, a statistically significant increase in MARCKS protein expression occurred in ATCC MOI of 6.0 and both Cs1-4 groups relative to the PBS treated group. This suggests that different strains of Delftia spp. may differentially regulate gene expression in monocytes, which could be tested through use of a variety of clinical Delftia spp. isolates in future experiments.

To date, LPS is the only toll-like receptor ligand that has been shown to upregulate MARCKS expression. We observed a similar trend in that LPS stimulation of THP-1 monocytes resulted in a non-significant increase in MARCKS protein expression. As mentioned with the COX-2 data, this could be due to an inadequate LPS dosage or the fact that THP-1 cells are being used in this study, which may not elicit as robust of a response as other cell types. This warrants future investigation into the role of LPS in the upregulation of MARCKS in THP-1 monocytes. Regardless, the presence of MARCKS protein in Delftia spp. stimulated cells suggests that Delftia spp. is stimulating an immune response in THP-1 monocytes. The exact role of MARCKS in LPS signaling is not fully understood. Mancek-Keber et al. (2012) have shown that MARCKS-mediated negative regulation of LPS signaling is associated with decreased TNF production. Thus, it is possible that MARCKS may help regulate the pro-inflammatory response by turning off LPS-mediated signaling, with further investigation needed to confirm this hypothesis.

TNF production was significantly increased in all treatment groups as compared to PBS. The observed TNF production in the PBS group is likely due to normal low-level production of the cytokine in healthy, untreated cells. The increase in the Delftia spp. stimulation groups was comparable to LPS stimulation, indicating a strong inflammatory response by the monocytes. TNF has been
shown to be upregulated by both LPS and flagellin, both of which are expressed by Delftia spp. and signal through toll-like receptor 4 (TLR-4) and toll-like receptor 5 (TLR-5), respectively (Bradley, 2008; Uchida et al., 2014). Investigating the contribution of TLR-4 and TLR-5 on Delftia spp. mediated TNF production could provide insight into the mechanisms by which Delftia spp. interact with immune cells. Further, investigation into whether commonly used anti-inflamatory drugs decrease Delftia spp. stimulated TNF production in monocytes is needed. Examples of anti-inflamatory drugs that have been shown to decrease TNF production include glucocorticoids, such as dexamethasone, and SB203580, a p38 mitogen-activated protein kinase (MAPK) inhibitor (Cuenda et al., 1995; Neuder, Keener, Eckert, Trujillo, & Jones, 2009).

The data collected here serve as a starting point for the improved understanding of the immune response in patients infected with Delftia spp. The antibiotic resistance found in Delftia species (Hagiya et al., 2013; Khan et al., 2012) prevents effective treatment, which can lead to the development of sepsis. With greater knowledge of the human immune response to Delftia spp., effective treatments can be administered. Further, prevention of infection is also of critical importance as case studies have regularly found Delftia spp. at sites for intravenous catheters and other indwelling medical devices. Interestingly, we found that Delftia spp. is not easily killed by sonication (data not shown), as thoroughly sonicated bacteria still grew when plated on Tryptic Soy Agar (TSA) media. Heat exposure, however, was sufficient to kill the Delftia spp. strains for use in the experiments presented herein. If DNA of Delftia spp. has been found in sterile DNA extraction kits (Salter et al., 2014), it is also possible that Delftia spp. are present on medical devices before use. Further investigation as to whether Delftia spp. are killed by irradiation, bactericidal UV light, or other sanitizing methods would be useful to help understand how patients are becoming infected with these bacteria.

It is also possible that Delftia spp. are members of the human microbiome, as Delftia acidovorans DNA has been found in the blood of healthy patients (Peraneva et al., 2013) as well as in the oral microbiome (Chen et al., 2015). Inadequate sanitation of patients’ skin prior to the use of indwelling catheters could have resulted in infection. This highlights the need for medical staff working with immunocompromised patients to take extra precautions in order to prevent Delftia spp. infections.

The data presented herein comprise a pilot study, with further investigation needed to better understand the pro-inflammatory response elicited by Delftia spp. Our study focuses on only two species of Delftia, providing little breadth for the characterization of the pro-inflammatory response to this genus as a whole. Additionally, the species we investigated were environmental, not clinical, isolates. Clinical isolates could elicit immune responses distinct from the environmental isolates used herein. Thus, future research will investigate pro-inflammatory responses to additional species of Delftia, including clinical isolates of Delftia tsuruhatensis (Presiswerk et al., 2011).

Our current data are also limited by the use of heat-killed Delftia spp., which were used in this study to decrease the possibility of contaminating our tissue culture facility. In a clinical situation, however, live Delftia spp. would perform biological processes that heat-killed cells do not, such as secreting chemical mediators that may alter the inflammatory response. Furthermore, in vivo bacte-


Invitrogen. iBlot semi-dry gel transfer system. Carlsbad, CA.


Nxcelon Bioscience. Auto T4 Cellometer. Lawrence, MA.


NuSeq. 10% nView precast SDS-PAGE gels. New South Wales, Australia.


Roche. Complete mini protease inhibitor cocktail tablet. Indianapolis, IN.


Santa Cruz Biotechnology. Goat anti-mouse HRP-linked antibody E3113. Dallas, TX.

Sigma-Aldrich. Lipopolysaccharides from *Escherichia coli*. St. Louis, MO.

Sigma-Aldrich. Monoclonal Anti-GAPDH, Clone GAPDH71. J. St. Louis, MO.


Thermo Scientific Pierce. *Bicinchoninic Protein Assay Kit*. Rockford, IL.

Thermo Scientific Pierce. Enhanced chemiluminescence Western Blotting Substrate. Rockford, IL.


Violent Video Games May Kill Your Short-Term Focus: Violent video games may negatively affect a player’s attention and concentration on a short-term basis after brief exposure

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Originally published in the September 2015 issue of the Journal of Young Investigators

This study examined the effects of brief exposure to a violent video game on a test of attention and concentration in 12- to 14-year-old males with low-volume video game play histories. We hypothesized that subjects who played 45 minutes of a violent video game would perform significantly worse on a widely-used neuropsychological test when compared to their baseline performance. Participants were given two versions of Digit Span Forward (DSF), a neuropsychological test that measures attention and concentration. Each subject was tested with DSF Version 1 at baseline and then again with DSF Version 2, immediately following 45 minutes of playing a violent video game. Results revealed that subjects performed significantly worse after brief exposure to a violent video game. Previous studies have linked video game playing for extended and intermediate periods of time to attention problems in both high-volume and low-volume players. This study demonstrates that brief exposure to violent video games may also have a negative effect on attention and concentration.

INTRODUCTION

Every day, millions of children and adolescents in the United States play video games (Granic, Lobel & Engels, 2014). A Pew Poll study found in the United States that 99% of boys and 94% of girls play video games (Lenhart, Kahne, Middaugh, Macgill, Evans & Vitak, 2008). In a 2009 Harris Poll, researchers found that American youth, aged 8 to 18, averaged 13 hours a week of video game play (Gentile, 2009).

In a 2007 study with 1254 middle school students, aged 12-14, researchers at Massachusetts General Hospital found that one quarter of girls and two-thirds of boys reported that they played at least one M-rated game “a lot in the past six months” (Olson et al., 2007). The boys in this study reported that two out of their favorite three games were rated M; girls in the study reported that a rated M game was number two in their top three favorites. More than 50% of the boys in the study endorsed the statement “I play electronic games because I like guns and weapons” (Olson et al., 2007).

Additionally, a recent Pew Poll study reports that 50% of boys, aged 12 to 17, state that their favorite games were rated M (mature) or AO (adults only) (Lenhart et al., 2008).

Both non-violent and violent video game playing has been associated with concentration and attention problems, such as having difficulty maintaining focus on less exciting tasks, like schoolwork, and having a shorter attention span. In a 2010 study, researchers found that children playing non-violent video games between 2 ½ and 3 hours a day were 67% more likely to have attention and concentration problems (Swing, Gentile, Anderson & Walsh, 2010). In 2012, researchers found that children and adolescents that spent more time playing video games had more attention problems, even when pre-existing attention difficulties were statistically controlled for in the analysis (Gentile, Swing, Lim & Khoo, 2012). Bailey, West, and Anderson (2010) also found attention and concentration deficits in young adult male subjects who played violent video games for 40 hours a week or more.

There is also some evidence that suggests violent video games have a more harmful effect on attention and concentration ability than non-violent video games. In 2014, Anderson presented data at the International Society for Research on Aggression Symposium from a recent Iowa State University (ISU) study with 210 student subjects. He found that video game players who play first-person-shooter (FPS) games self-reported more attentional difficulties than subjects that played third-person games, action games, real-time strategy games, and other types of video games (Anderson, 2014).

In another 2014 study, also presented at the International Society for Research on Aggression Symposium, Anderson assessed the impact of intermediate exposure to violent and non-violent video game play on proactive executive functioning in 62 low-volume players. In his presentation, Anderson defines “proactive executive functioning” as the “ability to keep context information active” and is “conceptually… most similar to ‘attention’ in the context of real world attention problems.” The study found that subjects with minimal video game use histories experienced a significant reduction in proactive executive control when playing FPS video games for 10 sessions in 50-minute intervals. These same subjects, when playing SIMS 2 (Electronic Arts, Inc., 2002), a non-violent video game, for the same period of exposure time, experienced an

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increase in proactive executive control.

Given indications that extended and intermediate level exposure to both violent and non-violent video games are associated with attentional difficulties in high-volume and low-volume youth players, we chose to explore the impact of brief exposure time to video games on attention abilities. Because FPS video games are associated with a significant reduction in “real world” attention ability, we focused specifically on the impact of FPS video games on attention abilities. This is the first study that examines the impact of 45 minutes of exposure to FPS video game play on attention and concentration immediately after video game immersion, and the first study to examine brief exposure to any kind of video game play on minimal-use players. We hypothesized that young, low-volume video game players would perform significantly worse than their baseline scores on a neuropsychological measure of attention and concentration after playing an FPS video game for 45 minutes.

MATERIALS AND METHODS

Participants

The subjects for this study were 12 seventh-grade boys aged 12 to 14. All subjects had low-volume video game usage histories, averaging 6 hours or less of video game play per week. All of the subjects had played Halo Reach at least once before enrolling in my study. Participants were recruited from four local middle schools. The subjects were all academic high-achievers from upper-middle-class and upper-class families (all annual family incomes over $150,000). Subjects were white, African-American, and half-Asian/half-white. Subjects came from families with one to three children. All but one subject resided in two-parent households, with one subject residing in a single-parent household (Table 1). Authorities at Flintridge Preparatory School approved the study, and informed consent was obtained from the adolescents’ legal guardians.

Attention and Concentration

Attention and concentration scores were measured using two different, but equivalent, versions of a neuropsychological test called Digit Span Forward (DSF). DSF is a measure of concentration and attention (Lezak, 1983). It is one of 15 sub-tests that make up the Wechsler Intelligence Scale for Children (WISC-IV). Subjects received a scaled score for their performance on this task to control for any age effects.

On Day 1, subjects were given Version 1 of DSF by an examiner who had been given training in standardized test administration by a licensed clinical psychologist. Subjects were verbally presented with a series of digits at a rate of approximately one digit per second. After each series, subjects were asked to immediately recall the digits in the order that they were given. A scaled score was derived based on the number of digit strings that were accurately recalled. On Day 2, subjects were given Version 2 of DSF, and a score was derived in the same way. Higher scores indicate better performance on the DSF.

Violent Video Game Exposure

The video game used during the exposure period in this study is called Halo Reach (Microsoft Game Studios, 2010). It is a FPS game rated M for violence. Halo Reach meets the operational definition for a violent video game according to a California law written by California state senator Leland Yee: “... a video game in which the range of options available to a player includes killing, maiming, dismembering, or sexually assaulting an image of a human being.”

Procedures

Subjects completed the protocol in this study on two different days. On Day 1, subjects were evaluated after school on a day when they had not played video games for at least 18 hours. Subjects consumed a protein bar before testing began to ensure that hunger and low energy levels did not interfere with subjects’ performance during the protocol. Version 1 of DSF was given to subjects on Day 1 in order to establish a baseline measure of subjects’ attention and concentration.

On Day 2, subjects were again evaluated after school on a day when they had not played any video games for at least 18 hours prior to their testing session. Subjects once again consumed a protein bar before they were exposed to video game play. The subjects played Halo Reach for 45 minutes and immediately after, were given Version 2 of DSF.

RESULTS

Table 1 provides participant demographics. On average, the subjects performed significantly worse on DSF after playing Halo Reach for 45 minutes compared to their baseline scores. The mean baseline scaled score before exposure to violent video game play was 15.50 (SD = 2.20). After playing the violent video game, the...
mean score decreased to 11.83 (SD = 3.16). Figure 1 provides the DSF scaled scores for all 12 subjects at baseline and post-exposure.

A paired sample t-test was used to analyze the differences between subjects’ DSF performances before and after violent video game play. A significant difference was found ($p < 0.0001$).

**DISCUSSION**

The results of the present study support the hypothesis that brief exposure to violent video game play would negatively affect attention and concentration on a short-term basis in male teen players. The present study provides an important source of new information regarding the impact of brief exposure to FPS violent video games on academically high-achieving video game players. To date, no study has examined this particular variable when evaluating the impact of violent video game play on attention and concentration. Future investigations could explore whether intellectual functioning plays a role in the way FPS violent video games impact a player’s attention. Since all 12 of the present study’s subjects are academic high-achievers, it would be beneficial to explore whether players with lower levels of intellectual functioning or academic success are less, more, or equally vulnerable to brief exposure to FPS violent video game play.

This study also provides an important source of new information regarding the impact of brief exposure to FPS violent video games on players with minimal-use histories. Another important future area of study should include exploring whether minimal-use players are more vulnerable than heavy-use players to the impacts of brief violent video game play on attention and concentration. Even though the American Academy of Pediatrics has recommended two hours or less of screen activities (television and video games) per day for teens and children (AAP, 2001; AAP 2009), the present study demonstrates that two hours of violent video games per day may be harmful to a player’s attention and concentration ability on a short-term basis. Playing violent video games for less than an hour, especially before starting homework or studying for a test, may in fact, contribute to attention and concentration difficulties on a short-term basis. Further research into how long attention and concentration difficulties are retained after violent video game exposure should also be conducted. Given that male youth prefer violent video games over non-violent video games, they may be especially vulnerable to the negative neuropsychological consequences of even brief violent video game play.

Our results are in contradiction to another study that also evaluated the effect of brief exposure to violent video games. An Indiana University School of Medicine study (Mathews, Wang, Kalnin, Mosier, Dunn & Kronenberger, 2006) found that subjects who played violent video games had less activity in the pre-frontal cortex, the part of the brain that is associated with concentration. However, this study did not find any differences in actual concentration ability scores when they compared teens who played violent video games for 30 minutes to teens who played non-violent video games for 30 minutes. Our study differs in an important design aspect that may explain the difference between the studies. We added 15 additional minutes to the brief exposure time, which may suggest that there is a threshold time length needed to observe an adverse effect of violent video games on short-term attention and concentration at the brief exposure level. The lack of activity in the pre-frontal cortex found in the 2006 study suggests the possibility that the subjects were being negatively impacted by the violent video game exposure, but just not enough to push them over the threshold needed to translate into diminished performance on an attention task.
Though the present study provides useful evidence for violent video game effects on attention and concentration on a short-term basis, there are three primary limitations worth noting. There is no separate control group used in this study; rather, the subjects serve as their own controls in the repeated measure design. A good way to utilize a separate control group in a future study would be to test attention ability in middle school children after they play a non-violent video game for 45 minutes, allowing us to separate the impact of violence from the impact of playing a video game. It should be noted, however, that DSF scores in other repeated measure designs, with no interference, have been shown to improve in non-significant trends (Woods et al., 2012). The findings in the study conducted by Woods et al. (2012) highlight the significance of the decline in subjects’ DSF post-exposure scores in the present study.

Additionally, our sample is very homogenous, made up of high-achieving adolescent males from upper-middle-class and upper-class backgrounds. This homogeneity prevented us from examining the likely effects of brief exposure to violent video games on other intellectual, socioeconomic, or ethnic backgrounds, and prevented us from evaluating response differences between genders. It is worth noting that a previous study found that video game play did not predict attention difficulties or academic performance in children from a largely Latino population, with family income playing a much larger role (Ferguson, 2011). Finally, our study is also limited by small sample size, reducing the power to detect small effect sizes. Despite these limitations, the significant results between the baseline and post-exposure DSF scores are an important finding that is worth expanding upon in a future study with a larger sample size.

ACKNOWLEDGEMENTS
The authors would like to thank all subjects for their participation in the study. Additionally, thanks to Leanne Watt, Ph.D., for her assistance with research design, literature review, and editing.

REFERENCES
Analyzing Anomalies in the Ionosphere Above Haiti Surrounding the 2010 Earthquake

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Originally published in the November 2015 issue of the Journal of Young Investigators

Earthquakes pose a significant threat to human life, especially when they affect major centers of population. However, there is currently no method of predicting seismic activity that gives enough time to prepare for a major catastrophe. This paper presents a method for predicting earthquakes months before an actual seismic event occurs. A major earthquake, which occurred near Haiti’s capitol in early 2010, is investigated using the Detection of Electro-Magnetic Emissions Transmitted from Earthquake Regions (DEMETER) satellite. Electron density in the ionosphere is measured through DEMETER. The electron density measurement is then compared to predictions by the Parameterized Ionospheric Model (PIM), a computer model capable of predicting the electron density at a given point in the atmosphere. This comparison reveals a fluctuation in ionospheric electron density which was not predicted by the model and is not accounted for by other solar or magnetic sources. This data demonstrates a correlation between electron density in the ionosphere and major seismic activity, which can be observed months before the seismic event occurs. With further research, it may be possible to predict when and where earthquakes will strike long before they actually occur.

INTRODUCTION

The connection between the ionosphere and earthquakes has come to light recently with research into how the ionosphere can be used to predict seismic activity. Being part of the upper atmosphere, the ionosphere ranges from approximately 85 to 900km above the Earth’s surface and is filled with electrons and other charged particles. One way to characterize the ionosphere is to measure its electron density, which is most often affected by solar and magnetic activity. Seemingly unrelated, earthquakes usually occur at the fault line between two tectonic plates within the earth’s crust. Although tectonic plates typically move very slowly, it is possible for the plates to experience a sudden jolt, resulting in an earthquake. Earthquakes can be devastating, yet there is currently no reliable method for predicting earthquakes.

The first significant advances in connecting the ionosphere to seismic activity were made by Freund (2002; 2010). There are several contending theories concerning the exact mechanics responsible for the seismic indicators in the ionosphere, but the basic premise is fairly consistent. Prior to an earthquake, due to the seismic forces in the Earth’s crust, a thin layer of particles forms as ions originating in the crust travel to the Earth’s surface and begin radiating outwards due to strong electric fields (Namgaladze, Klimenko, Klimenko, & Zakharenkova, 2009). These ions continue propagating away from the Earth’s surface and eventually enter the ionosphere, where they cause a measurable disturbance in electron density (Park & Dejnakaarintra, 1973).

The present study focuses on the Haiti earthquake, which occurred on January 12, 2010. We hypothesize that there will be measurable variations in the electron density of the ionosphere during the months prior to this earthquake. The electron density data reported in this paper is taken from the Detection of Electro-Magnetic Emissions Transmitted from Earthquake Regions (DEMETER) satellite orbiting above Haiti. DEMETER carries many instruments, including an Instrument Sonde de Langmuir (ISL) probe. This ISL probe allows us to measure the electron density of the plasma and the electron temperature in the ionosphere, from which we can obtain the relative ion and electron density along the orbit of the DEMETER satellite.

Once electron density measurements are obtained, they are compared to an atmospheric model called the Parameterized Ionospheric Model (PIM) (Daniell et al., 1995). PIM draws upon several regional theoretical ionospheric models, allowing it to be computationally fast while maintaining accurate physics. It can produce electron densities for altitudes between 90 and 25,000km and is able to output the predicted electron density over time based on solar and geomagnetic parameters as well as latitude, longitude, and altitude data. However, sudden electron density variations caused by seismic activity are not represented in PIM models (Cornely, 2003), so according to our hypothesis, any significant difference between outputs provided by PIM and measurements from DEMETER which cannot be accounted for by solar or magnetic sources should be the result of seismic activity.

Having the ability to use the ionosphere as an indicator for seismic activity may allow scientists to predict earthquakes months ahead of time. This type advanced warning would make it possible to minimize damages and loss of life resulting from significant earthquakes.

METHODS

Figure 1 presents a synthetic plot that illustrates our method for observing electron density variations. If our hypothesis is correct, there will be an observable difference in the two predictions. That difference will be the seismic indicator we are looking for.
this phenomenon could be observed by looking at electron density as a function of both latitude and longitude, only latitude data is presented here for the sake of simplicity.

The Detection of Electro-Magnetic Emissions Transmitted from Earthquake Regions (DEMETER) satellite orbits the earth roughly twice in every 24hr period. We obtained the electron density data from the satellite by downloading data files from the DEMETER website. Data from each orbit is contained in two files: a “burst” file and a “survey” file. The burst file contains data taken at a high sampling rate over a small distance, and the survey file contains the rest of the data taken at a lower sampling rate. This raw data is stored as binary information, and in order to make the data more user friendly we ran each file through IDL Virtual Machine (Exelis Visual Information Solutions, Boulder, CO), a computer program which converts binary files into ASCII files for easier processing.

Once in ASCII format, we processed the data files using MATLAB (The MathWorks Inc., Natick, MA, 2013). Electron density, latitude, longitude, and altitude data were extracted from each burst and survey file. The data set from each file was then combined to provide the electron density as a function of latitude and longitude as shown in Figure 2.

We then used another MATLAB script to process multiple DEMETER data files collected over a given period of time and displayed data from multiple orbits on a single graph. Using this ensemble method, we can observe general trends in the data for each of the months leading up to the earthquake and one month immediately after. Parameterized Ionospheric Model (PIM), using its inputs of solar and geomagnetic parameters, as well as latitude, longitude, and altitude, was used to predict the electron densities along the same path as the DEMETER orbit. An ensemble method was also used with the PIM data sets, so each graph contains outputs from multiple PIM predictions over the course of each month. In order to simplify the graphs and better observe potential electron density disturbances, electron density is presented only as a function of latitude.

Any variations between PIM prediction and values measured through the DEMETER satellite are not necessarily the result of seismic activity. Typically, other sources of ionospheric disturbances can emanate from solar and magnetic field effects. In order to isolate seismic activity as the primordial cause of the observed ionospheric disturbances, certain parameters need to be checked such as the sun spot number (SSN), the storm time index (Dst), the geomagnetic index (Kp), and the solar irradiance (or solar flux, F10.7). Using the National Oceanic and Atmospheric Administration’s SPIDR (SPIDR, 2012) website resources, for each month of interest, each parameter was checked to ensure it did not create any significant effects in the electron density in the months leading up to the Haiti earthquake.

RESULTS
When the Detection of Electro-Magnetic Emissions Transmitted from Earthquake Regions (DEMETER) satellite measurements are compared to Parameterized Ionospheric Model (PIM) predictions during the months leading up to the earthquake, they appeared to have the same general spatial features everywhere except at around 20° latitude. For example, during two and three months before the earthquake (Figure 3a and 3b), both PIM and DEMETER data generally agree on the distribution of the electron density over Haiti at all latitudes. However, one month before the

![Figure 1. Electron density in the ionosphere predicted by PIM and DEMETER data of electron density in the ionosphere plotted as a function of latitude. This is a simulated curve illustrates what we expect to see in the months leading up to the Haiti earthquake. The red curve simulates the electron density predicted by PIM, the blue curve simulates the data obtained from the DEMETER satellite, the green bar at the bottom corresponds to the location of Haiti, and the dotted line pinpoints the epicenter of the earthquake. The oval highlights the variation we expect to see when comparing the two curves. The existence of this kind of variation is the seismic indicator we are looking for.](image-url)
earthquake (Figure 3c), a small, yet definite, variation emerges. During the month prior to the earthquake (Figure 3d), the difference between PIM and DEMETER data is even more obvious. One month after the earthquake (Figure 3e), electron density returns to the behavior observed two and three months prior to the earthquake. These results demonstrate the electron density around 20° latitude was higher than expected over Haiti during the months leading up to the earthquake and higher than at any other time period, which suggests a correlation between seismic activity and electron density in the ionosphere.

In order to isolate seismic activity as the source of this variation in electron density, other common solar and magnetic parameters were also considered. These parameters include sun spot number (SSN), storm time index (Dst), geomagnetic index (Kp), and solar irradiance (or solar flux, F10.7). The solar flux and sun spot number are proxies for the solar extreme ultraviolet (EUV) flux (Chakrabarty et al., 2012).

Solar EUV flux is the radiation that ionizes the neutral atmosphere to produce the ionosphere. These two indices correlate with the overall magnitude of the electron density in the ionosphere, but not with quiet or active conditions. SSN >80 and F10.7 >150 correspond to a higher than normal electron density. Generally, Kp <4 corresponds to quiet conditions, Kp >6 corresponds to active conditions, which are usually associated with geomagnetic sub-storms. When Kp is between four and six, auroral activity is moderate and the effects do not usually extend into mid-latitudes. High Kp usually means that the aurora extends in an equatorial direction and the ionosphere is usually not significantly affected. For example, a Kp of eight or nine is typically associated with full geomagnetic storms. The presence of a geomagnetic storm is indicated by Dst, which is typically a negative number and indicates how much the quiet time geomagnetic field is reduced by the ring current in the magnetosphere. If Dst is more negative than -100nT, it will typically create observable effects on the mid-latitude ionosphere. If it is more positive than -50nT, ionospheric effects are negligible. When Dst is between -50nT and -100nT, ionospheric effects will be highly variable depending on the time of day. Looking at these parameters using the SPIDR website, Dst, Kp, F10.7 and SSN were all within quiet or normal ranges during the time period of interest leading up to the earthquake, indicating that they had no significant effect on the ionospheric electron density.

**DISCUSSION**

The work presented in this paper addresses the problem of earthquake prediction by analyzing the electron density in the ionosphere during the months leading up to the 2010 Haiti earthquake. Comparing predicted electron densities from the Parameterized Ionospheric Model (PIM) and the measured electron densities collected by the Detection of Electro-Magnetic Emissions Transmitted from Earthquake Regions (DEMETER) satellite, there is an observable variation one and two months before the earthquake. Ruling out other common sources of electron density disturbance leaves us with seismic activity as the most likely source of this observed variation.

While the results of this work are promising there are also some limitations to this study. Using the DEMETER satellite for characterizing the ionospheric electron density is accurate, but it only provides information for the exact path of the satellite, leaving
Figure 3. Electron density in the ionosphere predicted by PIM and electron density measured by DEMETER plotted as a function of latitude for the months leading up to the Haiti earthquake. The blue data points represent the measurements taken by the DEMETER satellite. DEMETER data for each month is displayed on the same plot as an ensemble of data points in order to observe the overall trend of the electron density for each month. The outputs from PIM are displayed in red. Several predicted curves from PIM are also displayed on the same plot as an ensemble of data points. From each graph, general trends can be established, and any major variations from these trends can be observed. The location of Haiti is indicated by a green bar. (A) Three months before the earthquake; (B) two months before the earthquake; (C) one month before the earthquake; (D) the month leading up to the earthquake; (E) the month after the earthquake.
electron densities in other parts of the ionosphere unknown. Additionally, the DEMETER scientific mission ended in late 2010, so current DEMETER measurements are no longer available. While past DEMETER data can still be used for data analysis of seismic behaviors and their potential correlations with ionospheric disturbances, more ionospheric electron density data is required to study the real behavior of the background ionosphere near fault lines over earthquake prone regions such as Haiti.

Although additional work is required in order to establish a definitive system for earthquake prediction, the methods described open the potential for a predictor of seismic activity which could be effective as early as one or two months before the actual event occurs. Such a predictor would be of significant utility to earthquake prone countries.

ACKNOWLEDGEMENTS
This investigation is based on observations with the IAP experiment conducted on the DEMETER satellite, launched by Centre National D’études Spatiales (CNES). The authors thank J.J. Berthelier, the Principal Investigator of IAP, for the use of the data.

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The Tough Choice of a Life Scientist: Industry vs. Academia

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The most obvious career choice for life sciences students is research, and the essential dilemma of aspiring researchers is the choice between industry and academia. There are certainly other career options—government positions and scientific consulting, for instance—but industry versus academia remains the popular set of choices for many biology students. This article provides a comparative overview of these different career paths.

Money
Money is certainly an important factor in choosing a career, and compared to academic researchers, industrial scientists have an advantage salary-wise. According to The Scientist’s 2014 Life Sciences Salary Survey results, American, Canadian, and European scientists working in industry earned approximately 30% more than their counterparts in academia.

However, according to the same survey, the overall trend for scientists is increasing average salaries with professional experience, meaning that persistence and hard work in a science career seem to pay off regardless of the particular branch one chooses to pursue.

Another concern is research funding. In academia, scientists need to constantly apply for grants to fund their projects, which often takes up a considerable amount of time; in contrast, industry funding is generally less of a concern, though this added security comes with price as we shall see. Time

Academic researchers have a lot more time flexibility than their peers in industry. In academia, researchers set their own research schedules as long as they fulfill their teaching and departmental obligations. In industry, the work hours are strictly set, and the employee will be expected to be present at work from nine to five, Monday to Friday. Similarly, vacation time is closely monitored in a company setting, while academic researchers often have more latitude and can go on longer holidays, especially in summers when they do not need to teach. The difference stems from the fact that academic researchers are their own bosses, while industrial researchers have to report to their superiors.

However, greater scheduling flexibility does not mean that scientists in academia work fewer hours. On the contrary, professors work an average of 61 hours a week. This is not surprising given the range of obligations they have: teaching, research, applying for grants, student mentoring, and department meetings. Among these, only research and grant applications are directly related to career advancement. In contrast, industrial researchers spend most of their time doing research and attending meetings, which are immediately relevant to advancement. The broad range of responsibilities in academia can be considered advantageous as it allows scientists to develop skills in many different areas; however, for those interested in pursuing only scientific research with no distractions, industry may be a better choice.

Freedom
Academic researchers benefit from significant freedom when choosing their research topics, approaches, and collaborators, while industrial scientists have significantly less say in the direction of their work. However, with freedom comes responsibility—the viability of academic projects depends on researchers’ ability to obtain grant funding. On the other hand, funding in industry often comes from within the company and is assigned to a specific project destined to produce a commercially viable product. Thus, it is easier for industrial scientists to obtain funding for their work, but as a result they are much more restricted in their topic.

Impact
As a result of the difference in the extent of freedom offered to researchers in industry and academia, there is also significant difference in their relative impacts of work. From the very beginning, everything done in a company is directed at creating a product or service for sale, so there is always a tangible result in the end: in biomedicine it could be a drug, a therapy or a new technology. In contrast, in academia, although research results still carry incredible value and may inspire future groundbreaking discoveries, at the time of publication, they may only be of use and interest to a few other scientists working in the same highly specialized field.

Independence
In academic research, there are considerably more opportunities for independent work compared to industrial science. Although academic scientists form collaborations, these are rarely long-term, as there is significant emphasis on building personal portfolios in academia; in most research labs, each member works on a distinct project independently. In industry scientists work in teams to maximize productivity and efficiency. However, industrial scientists usually receive less credit for their work, and their discoveries will belong to the company. Academic scientists get credit and ownership for all of their intellectual outputs.

As evidenced above, there are certainly pros and cons for research careers in both academia and industry. The preference of industry versus academia will depend on personality and priorities: some people need quick results with immediate impact, while others value freedom of choosing the research topic and credit for their discoveries. However, the comparison offered in this article is quite general, and in reality everything is not so black and white. Scientists’ experience in industry will vary greatly depending on whether they are in start-ups or in multinational corporations; similarly, in the academic setting, experience will be different for researchers in small schools compared to those in large research institutions. Given the diversity of options, there will certainly be a perfect job for anyone, in either career path. For some, it may not be necessary to make the choice at all—when motivated by an interest in a particular research topic, one will recognize and seize the best opportunity, whether it be in an academic or an industrial setting.
We all know that diet is important to physical well-being, but is it possible that the food and drink we consume also have an effect on our mental health?

A growing body of evidence suggests that this might be the case. Some studies indicate that certain nutrient-based prescriptions may help in managing mental disorders. These nutrients include omega-3 fatty acids, iron, zinc, magnesium, choline, B vitamins, and amino acids. According to many reviews and meta-analyses, this association is consistent across countries, cultures, and populations.

Recently, the results of a longitudinal study of more than 15,000 individuals regarding the effect of diet on mental health were published in the journal BMC Medicine. The study, led by Almudena Sanchez-Villegas, was designed to assess the relationship between particular dietary scores and depression. These dietary scores included the Mediterranean diet, the Pro-vegetarian Dietary Pattern, and Alternative Healthy Eating Index-2010.

“We wanted to understand what role nutrition plays in mental health, as we believe certain dietary patterns could protect our minds. These diets are all associated with physical health benefits and now we find that they could have a positive effect on our mental health,” says Dr. Sanchez-Villegas.

All participants were a part of the Seguimiento Universidad de Navarra (SUN) Project, a dynamic cohort established in 1999 to study the effect of lifestyle and diet on obesity, coronary heart disease, depression, and hypertension risk. Additionally, all participants were free of depression from the onset of the study.

Baseline assessment and follow-up information were gathered from the subjects via mail or online questionnaires every two years. After eight years, 1051 cases of clinical depression were reported. The results of the study indicate that moderate level of adherence to any of the aforementioned diets could be effective for reduction of depression risk.

“A threshold effect may exist,” Dr. Sanchez-Villegas says. “The noticeable difference occurs when participants start to follow a healthier diet. Even a moderate adherence to these healthy dietary patterns was associated with an important reduction in the risk of developing depression.”

Upon further analysis, the researchers found that absolute adherence to dietary guidelines did not mitigate depression risk as one might initially expect from the early findings. The authors of the study put forth an interesting hypothesis for this observation: psychological elements of neurotic or obsessive behaviors associated with maximal dietary adherence may contribute to the plateau in depression risk beyond moderate dietary adherence.

It is important to note, however, that the present study only provides a correlation, not a causative link, between diet and mental health. Indeed, a variety of other risk factors for poor mental health—genetics, stress, drug abuse, and poverty, among others—have also been identified in other studies.

Nevertheless, many still urge for the relationship between diet and mental health to be further explored and recognized. The International Society for Nutritional Psychiatry Research (ISNPR) is the premiere organization dedicated to research of nutritional therapies for psychiatric illness. Dr. Jerome Sarris, an executive member of the ISNPR, says “It is time for clinicians to consider diet and additional nutrients as part of the treating package to manage the enormous burden of mental illness.”