**How does CagA in *H. pylori* contribute to apoptosis in gastric cancer cells?**

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**I. Introduction**

Gastric cancer, also known as stomach cancer, is cancer that begins in the stomach and can be caused by a decrease in apoptosis or an increase in cell proliferation (American Cancer Society, 2017). In people who do not have cancer, apoptosis will occur which is when cells will trigger a series of events in order to make room for newer cells, eliminate unhealthy cells, or to get rid of unnecessary cells (Apoptosis, 2016). In cancerous cells, apoptosis can be hindered or completely evaded which can lead to uncontrolled cell division (Gutschner & Diederichs, 2012).

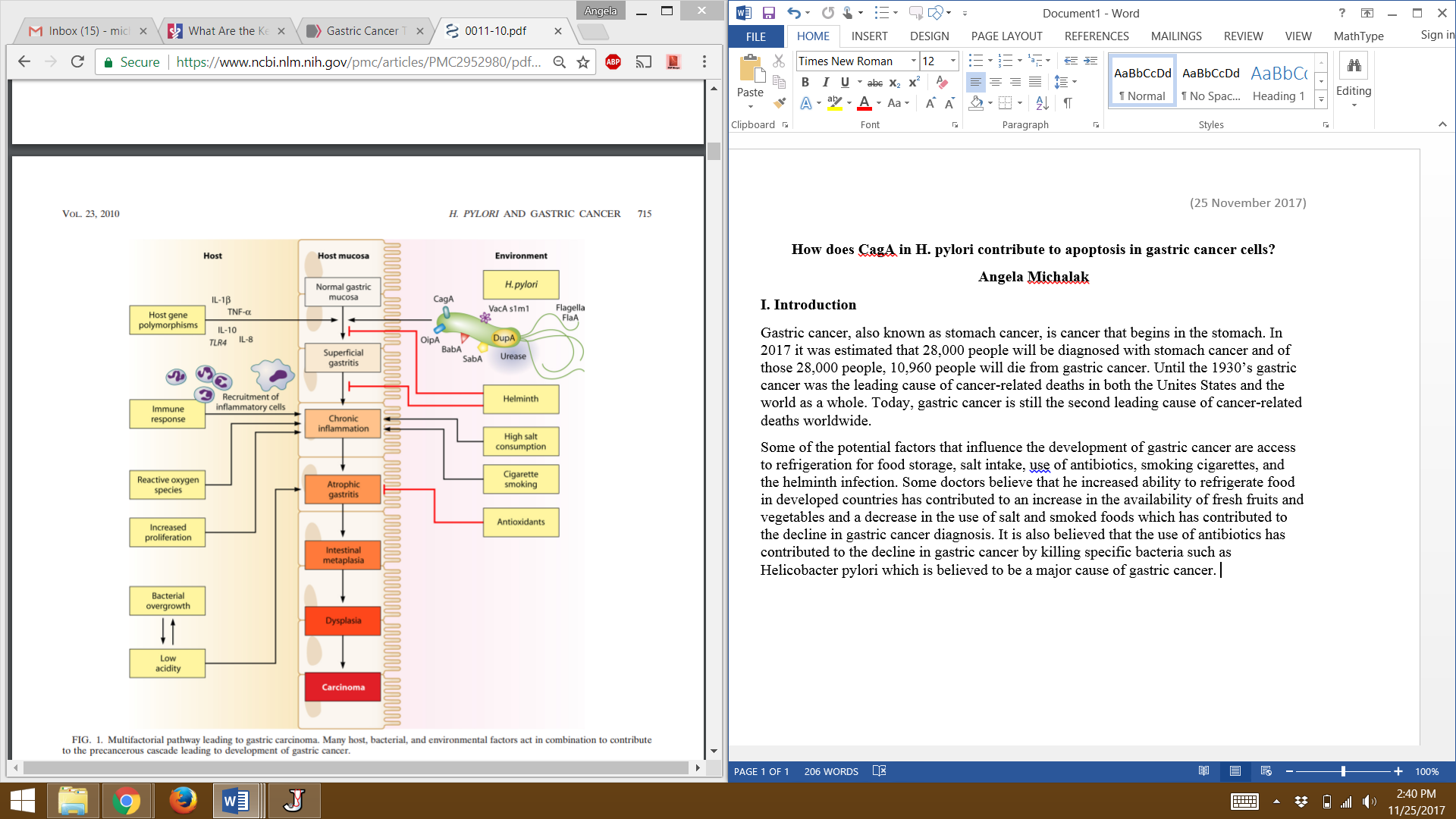
 The American Cancer society (2017) estimated that for 2017, 28,000 people will be diagnosed with stomach cancer and of those 28,000 people, 10,960 people will die. Until the 1930’s gastric cancer was the leading cause of cancer-related deaths in both the Unites States and the world. Today, gastric cancer is still the second leading cause of cancer-related deaths worldwide (American Cancer society, 2017).

Figure 1 illustrates some of the potential factors that influence the development of gastric cancer such as access to refrigeration for food storage, salt intake, use of antibiotics, smoking cigarettes, and other infections such as helminth infection (Wroblewski et al., 2010). Some doctors believe that the increased ability to refrigerate food in developed countries has contributed to an increase in the availability of fresh fruits and vegetables and a decrease in the use of salt and smoked foods which has contributed to the decline in gastric cancer diagnosis (American Cancer Society, 2017). It is also believed that the use of antibiotics has contributed to the decline in gastric cancer by killing specific bacteria such as *Helicobacter pylori* (*H.pylori*) which is believed to be a major cause of gastric cancer (Wroblewski et al., 2010).

Figure 1: Multifactorial pathway leading to gastric carcinoma. Many host, bacterial, and environmental factors act in combination to contribute to the precancerous cascade leading to development of gastric cancer. From Wroblewski et al., 2010.

*H.pylori* is a type of spiral shaped bacteria that is grown in the digestive tract and it is estimated that around 50% of the world’s adult population contains *H.pylori* within their digestive tract (Wroblewski et al., 2010). In most cases, *H.pylori* is harmless, however, it can also lead to the development of diseases such as peptic ulcers and gastric cancer. Because of this, *H. pylori* is recognized as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) indicating that they concluded that *H. pylori* has the potential to cause cancer in humans (Hatakeyama, 2017). One of the ways in which *H.pylori* can cause site-specific diseases is by using its spiral shape to penetrate the stomach lining. Once the *H.pylori* penetrates the stomach lining the bacteria is protected by mucus and the body’s immune cells are unable to reach the bacteria preventing it from being destroyed (*H. pylori* infection, 2017). Moss (1998) performed a study in which he examined the effects of *H. pylori* on apoptosis of epithelial cells by staining apoptotic cells. From Moss’s (1998) results he concluded that *H. pylori* induced both proliferation and apoptosis, and that CagA was a likely contributor to the observed proliferation and apoptosis.

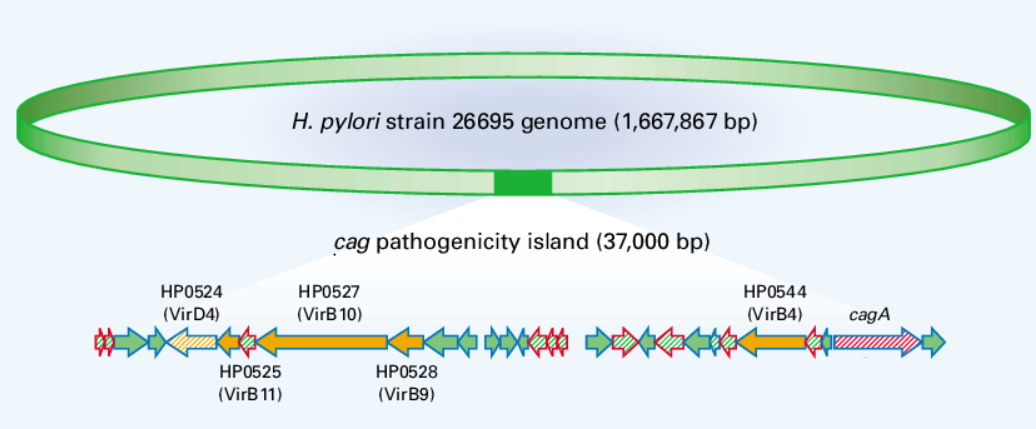
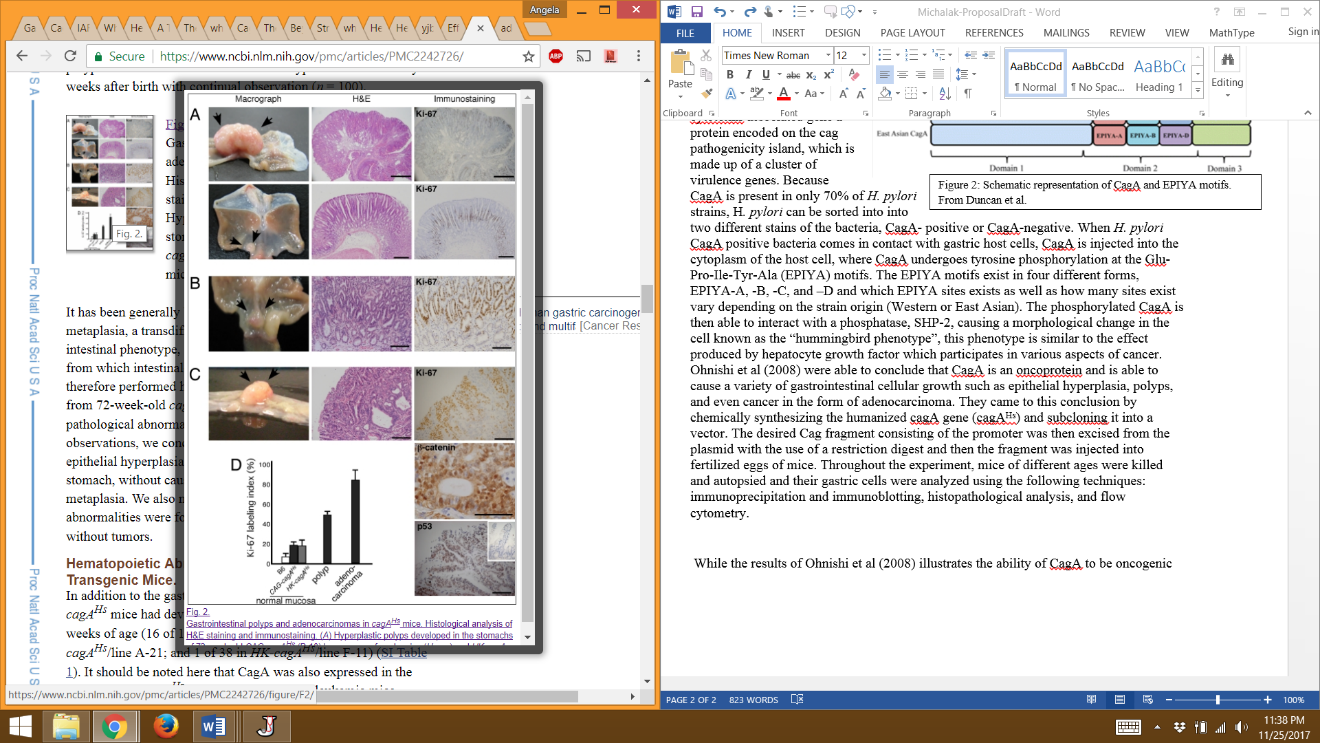
One identified virulence factor of *H.pylori* is CagA or cytotoxin-associated gene A, a protein encoded on the cag pathogenicity island, which is made up of a cluster of virulence genes (Jones, et al., 2010). Figure 2 illustrates the presence of the CagA gene within the pathogenicity island (Suerbaum &n Michetti, 2002). Because CagA is present in only 70% of *H. pylori* strains, *H. pylori* can be sorted into two different strains of the bacteria, CagA- positive or CagA-negative (Jones, et al., 2010) CagA-negative strains, however, not only lack the CagA gene but also lack the pathogenicity island (Terry, et al., 2005).When *H. pylori* CagA positive bacteriacomes incontact with gastric host cells, CagA is injected into the cytoplasm of the host cell, where CagA undergoes tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs (Jones, et al.). The phosphorylated CagA is then able to interact with a phosphatase, SHP-2, causing a morphological change in the cell known as the “hummingbird phenotype”, this phenotype is similar to the effect produced by hepatocyte growth factor which participates in various aspects of cancer (Churin et al., 2003).

Figure 2: The cag Pathogenicity Island. Most H. pylori strains that cause disease (so-called type I strains) contain the cag pathogenicity island, a chromosomal region with about 37,000 bp and 29 genes, whose location is indicated by the arrows. Modified from Suerbaum & Michetti, 2002.

Ohnishi et al (2008) were able to conclude that CagA is an oncoprotein and is able to cause a variety of gastrointestinal cellular growth such as epithelial hyperplasia, polyps, and even cancer in the form of adenocarcinoma. They came to this conclusion by chemically synthesizing the cagA gene and subcloning it into a vector. The desired Cag fragment consisting of the promoter was then excised from the plasmid with the use of a restriction digest and then the fragment was injected into fertilized eggs of mice (Ohnishi et al., 2008). Throughout the experiment, mice of different ages were killed and autopsied and their gastric cells were analyzed using the following techniques: immunoprecipitation and immunoblotting, histopathological analysis, and flow cytometry. Ohnishi et al. (2008) observed a number of growths in 72 week old mice containing the CagA gene, as illustrated in figure 3 and determined that at this point two mice had developed adenocarcinoma in the stomach and four mice had developed adenocarcinoma in the small intestine.

The findings of Ohnishi et al (2008) as well as Moss (1998) illustrates the ability of CagA to be oncogenic and influence apoptosis. However, it is still not completely understood how apoptosis is affected by CagA. I will design an experiment that aims to isolate the effects that CagA in *H.pylori* has on apoptosis in gastric cancer cells. By determining the effects that CagA has on apoptosis in gastric cancer cells we may become one step closer to understanding how to more effectively treat gastric cancer, decreasing the large amount of deaths from the disease each year.

Figure 3: Gastrointestinal polyps and adenocarcinomas in cagAHs mice. Histological analysis of H&E staining and immunostaining. (A) Hyperplastic polyps developed in the stomachs of 72-week-old CAG-cagAHs (B-10) homozygous female mice (Upper) and HK-cagAHs (A-21) heterozygous male (Lower) mice. Scale bars, 300 μm. (B) Adenocarcinoma developed in the stomach of a 72-week-old CAG-cagAHshomozygous male mouse (B-10). Scale bars, 100 μm. (C) Adenocarcinoma developed in the small intestine of a 72-week-old CAG-cagAHsheterozygous male mouse (B-10). In the p53-immunostaining panel, matched control is shown in Inset. (Scale bars, 100 μm.) (D) Ki-67 labeling indexes of gastric lesions in cagAHs mice. Error bars indicate mean ± SD. Modified from Ohnishi et al.

**II. Experiment**

The goal of this experiment is to synthesize a CagA-negative strain through the use of PCR performed on a CagA-positive strain in order to generate CagA-positive and CagA-negative colonies that can be analyzed by flow cytometry in order to determine how CagA affects apoptosis of gastric cancer cells. The 26695 strain is commonly used in order to study the CagA Pathogenicity Island due to the presence of the CagA gene (Terry et al., 2005). In this study the only difference between the H. pylori 26695 (CagA-positive) strain that will be used and the CagA-negative strain that will be generated is the difference in the presence of the CagA gene.

This experiment will generate two gastric cancer cell lines one containing CagA-positive H pylori and one containing CagA-negative *H. pylori*. Because the *H. pylori* had identical genomes before the removal of the CagA gene, any difference in apoptosis, measured through the use of flow cytometry, can be attributed to the presence of the CagA gene.

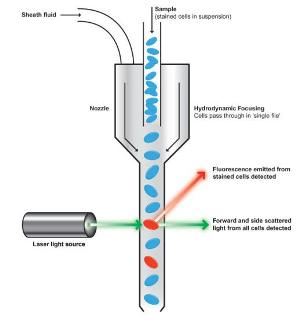
In order to perform this experiment gastric cancer cell lines will need to be obtained from ATCC (2016), an organization that collects and cultures a variety of cell lines for uses in clinical studies and scientific research (ATCC, 2016). In order to maintain the cell cultures the cells will be maintained in a humidified incubator. This technique was also used by Mia et al (2017). The gastric cancer cell lines will be split onto two different plates containing a transport media that will allow for the proliferation of gastric cancer cells and help to inhibit the growth of unwanted bacteria.

The next step in this experiment is to grow and replicate the 26695 strain of H. pylori on a plate in vitro, this plate will be referred to as PlateI in order to separate the numerous plates used throughout the experiment. By replicating the bacteria in vitro, it helps ensure that the genomes of *H. pylori* are as identical as possible, to control for gene variation. In order to grow the *H.pylori*, the bacteria will be cultured on a nutrient-rich agar. Because an environment with reduced oxygen is ideal for growth of *H. pylori* a closed system that can produce this environment is required. In order to generate the closed system an anaerobic jar which can simulate microaerophilic, low oxygen concentrations, conditions (Blanchard, et al. 2006).

After the 26695 strain culture on PlateI has been grown, the existing culture must be split into two separate cultures, one culture on PlateI and one culture on PlateII. At this point both PlateI and PlateII will contain the exact same 26695 strain. In order to generate a CagA-negative strain, the CagA gene from the 26695 strain on PlateII will need to be removed and plated on a separate plate, PlateIII. This process ensures that any effect on apoptosis is due to the CagA gene and the expression of its produced protein and not a difference in the H. pylori genome between cultures.

Once the gastric cancer cells and *H. pylori* bacteria are cultured and maintained, the regions upstream and downstream of the CagA gene in the 26695 strain on PlateII are amplified using PCR. The primers used to amplify the entire H. pylori plasmid, including the pathogenicity island but excluding the CagA gene are: Forward- 5’-CCCAGTTTGTCGCACTGATAATCAAATACACCAACGCCT-3’ and Reverse- 5’ ATCCACTTTTCAATCTATATCTATGACTAAGCCACTGCCGT-3’. These specific primers were used in Zeng at al. (2005) because this sequence flanks the CagA genome. These primers were determined from the 26695 strain of the *H.pylori* genome that had been sequenced prior to the study. By using these primers the CagA-positive genome will be amplified excluding the CagA gene, and therefore will create a CagA-negative strain. The PCR product will be plated on PlateIII and after growth will be compared to the CagA-positive strain on PlateI in order to determine whether there is a change in apoptosis.

The CagA-positive and CagA-negative cultures created, PlateI and PlateIII, will then be used to infect the gastric cancer cell cultures through injection. At this point one gastric cancer cell culture contains *H. pylori* CagA- negative strain and the other gastric cancer cell culture contains *H. pylori* CagA-positive strain. These cells will then be left to incubate for 1 month checking on the plates every 2 days, as *H. pylori* colonies begin to visualize as early as 2-4 days after plating (Blanchard et al, 2006).

In order to visualize the differences in apoptosis between the two different gastric cancer cell cultures, flow cytometry will be used. In order to perform flow cytometry the cells that were grown on PlateI and PlateIII need to be heavily diluted and passed through the column of the flow cytometer. The column of the flow cytometer is narrow enough and therefore, allows only a few cells to pass through the column at a time. Figure 4 demonstrates that as the cells travel down the column, each cell is stuck by a laser which causes light scatter. The direction in which the light scatters generates a graph that can be used to determine whether apoptosis is occurring in the cells or not as shown in figure 5.

When cells undergo apoptosis the morphological changes in the cell are able to be measured through flow cytometry by understanding the light scatter that occurs with the interaction of the particle (Vermes, et al, 2000). When performing flow cytometry the interaction of the particle and laser result in light scattering in both the forward (FSC) and lateral (SSC) direction. During apoptosis there is an initial increase in SSC coupled with a decrease in FSC followed by a decrease in both FSC and SSC during later stages of apoptosis. It is hypothesized that the initial increase in FSC and SSC that is observed during apoptosis is due to chromatin condensation and the shrinking of the cell (Vermes, et al., 2000). Chromatin condensation is not an unusual occurrence for apoptotic cells as nuclear condensation is one of the criteria in identifying apoptotic cells (Toné et al., 2007). The decrease in FSC and SSC is attributed to the emergence of necrosis, cell death (Nicoletti et al.). As mentioned previously, by diluting the cells obtained from PlateI and PlateIII and passing them through the flow cytometer the difference in the FSC and SSC observed will allow us to distinguish between cells that are apoptotic and those that are not

Figure 4: Visualization of flow cytometry. From Faculty of Medicine and Dentistry at University of Alberta, Canada.

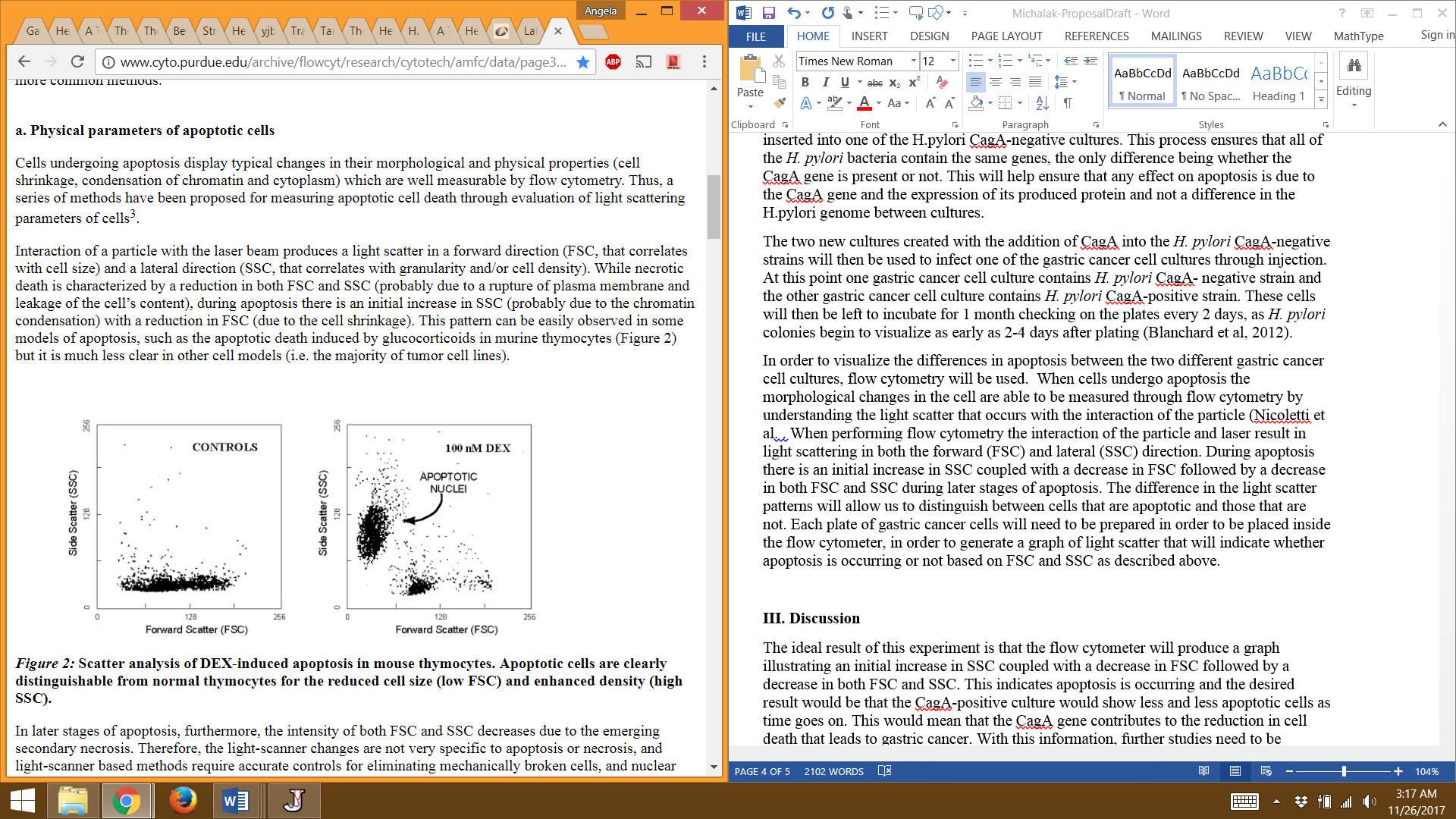


Figure 5: Scatter analysis of DEX-induced apoptosis in mouse thymocytes. Apoptotic cells are clearly distinguishable from normal thymocytes for the reduced cell size (low FSC) and enhanced density (high SSC). Modified from Nicoletti et al.

**III. Discussion**

The ideal result of this experiment is that the flow cytometer will produce a graph illustrating an initial increase in SSC coupled with a decrease in FSC followed by a decrease in both FSC and SSC. This indicates apoptosis is occurring and the desired result would be that the CagA-positive culture would show less and less apoptotic cells as time goes on. This would mean that the CagA gene contributes to the reduction in cell death that leads to gastric cancer. With this information, further studies need to be performed in order to better understand the CagA gene and it’s interactions with other genes that may contribute to gastric cancer as well as a way to inhibit the injection of CagA into gastric host cells by *H. pylori* due to its ability to be an oncoprotein.

If the graphs created from the use of flow cytometry do not exhibit a difference between the gastric cancer cells containing CagA-positive and CagA-negative *H. pylori* strains, then we can conclude that either CagA is not affecting apoptosis or that a mistake was made throughout the procedure preventing a difference from occurring or being visualized. In this case further studies would need to be performed in order to determine whether CagA has an impact on apoptosis. If a repeated trial of this experiment or a similar experiment to test the effects of CagA on apoptosis still does not yield the desired results then different genes of the *H. pylori* as well as the interactions of genes need to be further examined to determine what genes in *H. pylori* may affect apoptosis in gastric cancer cells.

Some limitations of this experiment is that mutations during replication of H. pylori are not accounted for. This experiment assumes that the *H.pylori* used in both gastric cancer cell cultures will have identical genomes other than whether the H. pylori is CagA-negative or CagA-positive. This assumption of correct replication is also upheld for the gastric cancer cell lines as well as the replication of the CagA gene through PCR.

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