Summary
We decided to investigate the claim that the anecdotal health benefits of garlic as a potential anti-cancer agent depend on the method of preparation and storage. We assessed the ability of four preparations of garlic to inhibit cancer cell growth: Frozen, refrigerated, room temperature-stored and boiled.

We also wanted to know whether aqueous extracts of garlic activate cell death mechanisms such as Apoptosis, Autophagy and Necrosis.

By assessing the viability of the HeLa cells after treatment with garlic, we concluded that boiling garlic killed its anti-cancer abilities completely, whilst most other samples inhibited growth by over 90%.

Through protein biochemistry, we discovered that room-temperature-stored garlic activates apoptosis, (through activation of Caspase 9) whilst refrigerated extracts affect both autophagy and necrosis. (Through activation of LC3B and PARP, respectively).

Introduction
Garlic has been used medicinally for thousands of years by the ancient Egyptians and as a protective agent against the sun’s heat by African peasantry and Roman soldiers. Due to its versatility and powerful defence mechanisms, the garlic plant has thrived in all environments across the globe.

Model System for Study:
HeLa cells were used throughout the course of our project. They are a cervical cancer cell line, and the first ever immortal cell line, derived from Henrietta Lacks, after whom they were named.

Research Idea:
The most important objective of our project, is to decide whether preparing garlic in different ways affects its ability to cause cell death in cancer cells. This factor could potentially have a huge effect on the role of garlic in our diets. Is it being prepared in the most beneficial way possible and more importantly, could garlic be used as a therapeutic agent against cancer?

Experimental Methods
Generation of Aqueous Extracts:
We crushed 20g of garlic, before adding 20ml of distilled water, so that the ratio of garlic to water was 1g/1ml. For further purification, extracts were centrifuged at 2,400 x g, for 10 minutes. The extracts were then passed through sterile syringe filters with a pore size of 0.2 microns to remove large contaminants and any microbial contamination.

Cell Viability Assay:
To establish whether the garlic extracts were able to cause HeLa cell death, cells were stained after 24 hours with a dye called crystal violet, which has the ability to bind to cellular DNA. Staining with Crystal Violet is a standard method of predicting cell proliferation.

Spectrophotometric Quantitation of Crystal Violet Assay:
Spectrophotometry was used in order to find the absorbance of cell samples, which directly corresponds to the amount of cells living in that sample. Absorbance was read at 595nm on a spectrophotometer.

SDS-PAGE:
In order to decide whether or not the aqueous extracts were altering expression of cellular proteins, an SDS-PAGE experiment was performed. Once separation of proteins had been achieved, gels were stained with Coomassie dye, de-stained and then scanned using a Bio-Rad Gel Documentation system.

Western Immunoblotting:
Immunoblotting was done to determine the abundance of specific proteins known to be associated with Apoptosis, Autophagy and Necrosis. We blotted for PARP, Caspase 9, SQSTM1 and LC3B. Pre-cast 4-12% gels were used.

Results
Microscopy of Cells:
Higher Magnification: After 1 hour, retraction fibres can be observed (see arrows) in the cell sample treated with garlic extract (RT) and after four hours, membrane blebbing can be seen, both of which are signs of apoptosis/cell death.

Crystal Violet Assay:
The Figure below shows the well plates 24 hours after treatment with garlic extract, having been stained with Crystal Violet. The deeper the colour, the more cells that are present in the sample.

Western Immunoblotting:
The cleavage product of Caspase 9 in the RT extracts, indicates that apoptosis has been activated. The cleavage of PARP in the refrigerated extracts at 55 and 37KDa, shows necrotic type cell death. The lower LC3B band increases in signal strength, meaning that refrigerated extract also affects autophagy (the upper band decreases and the lower band increases in intensity, indicating an activation event).

Statistical Analysis
Unpaired Student t-tests Spectrophotometry Results:
Unpaired t-tests were performed assuming unequal variance between the groups. The absorbance values were expressed as percentages of the control, to normalise data for comparison.

ANOVA:
Having already decided that boiled extracts had no effect on cell proliferation, we wanted to establish whether or not there was a difference in effect between the Frozen, Refrigerated and Ambient extracts. ANOVA (Analysis of Variance) can be used to determine the variance between more than two sets of data. ANOVA also generates a p-value. Similar to a t-test, when the p-value is <0.05, then there is a significant difference between the groups.

P-values of >0.05, mean that Frozen, Refrigerated and Room Temperature-stored extracts had a similar effect on HeLa cell proliferation after 24 hours.

Conclusions
- Refrigerated, frozen and room temperature-stored garlic all reduce HeLa cell growth significantly, and there seems to be no difference between the effects of these three preparations after 24 hours (p-value <0.05). Boiling seems to kill this anti-cancer property in garlic altogether.
- The Western Immunoblot established that room-temperature-stored garlic triggers apoptosis through activation of Caspase 9, whilst refrigerated garlic affects both autophagy and necrosis through activation of LC3B and PARP respectively.
- Overall, I have proved that the anti-cancer effects of garlic are dependant on preparation and storage methods. Garlic seems to have a significant effect on HeLa cell proliferation, with most samples reducing growth by 90% or more. I strongly recommend that further exploration should be done into this particular topic, so that the unknown anti-cancer property in aqueous garlic extracts could be isolated, and hopefully used as a therapeutic agent against cancer in the future.