Early detection of ovarian cancer requires that some biomarker(s) or protein(s) be present in a higher or lower concentration in the presence of a tumor and the opposite in the absence of a tumor. With the appropriate detection method that relies on the detection of an ovarian cancer protein, oncologists could diagnose ovarian cancer at an earlier and more treatable stage. It has been over three decades since researchers first began their hunt for proteins adhering to appropriate detection guidelines (e.g., levels in affected cells versus unaffected cells), making this a relatively new field of study open to many breakthroughs. In addition to new proteins, researchers are developing mechanisms to mathematically quantify and visually qualify the biomarker levels in hopes of eventually creating increasingly accurate early-detection tests. Our goal is to contribute to the biomarker effort through development of a detection mechanism for B7-H4, a protein only recently discovered to be elevated levels in the presence of a tumor, and thus lending itself quite nicely to the development of sensitive methods for its detection.

At the start of the project, three proteins were identified as potential candidates: Cancer antigen-125 (CA-125), Human epididymis protein 4 (HE4), and Mesothelin. Beginning in the early 1980s, cancer researchers explored the idea of utilizing blood tests that report the levels of tumor-associated proteins to diagnose patients with a given cancer. CA-125 (cancer antigen 125) is one such successful biomarker because elevated CA 125 levels can serve as an indicator for the presence of an ovarian tumor. In the late 20th century, studies were launched to ascertain a rough threshold of when a patient should be considered at-risk for the development of cancer. The results identified "... a CA-125 level of 35 units...to be a useful cutoff point, with 99% of healthy women having values less than 35" [9]. Despite the excitement surrounding this discovery, three primary issues arose with the detection mechanism. First, CA-125 can be absent even when a tumor is present; and the antithesis is also true - that an individual without cancer can be misdiagnosed with the disease because inflammatory conditions of the abdomen also elevate CA-125 levels. Second, although CA-125 is a reliable biomarker when diagnosing women with advanced stages of cancer, it is frequently undetectable in early-stage disease. In fact, the test misses up to 50% of women. Third, values deviate significantly from patient to patient, making it difficult to establish reliable baseline ranges for diagnosis.

In 2008, the U.S. Food and Drug Administration (FDA) approved the HE4 test, which was a slight improvement from the CA-125 test in regards to cancer diagnostic sensitivity. HE4 is secreted by cancerous cells and is detectable in the bloodstream via an enzyme immunoassay with a cut-off level of 150 pmol/L [12]. An experiment conducted by the Mayo Clinic randomly sampled two hundred and thirty-four patients from the population of all women with a pelvic mass. Of those sampled, 67 had epithelial ovarian cancer. Two tests were randomly assigned to each group of one hundred and seventeen subjects: a CA-125 test and a HE4 test. Results proved that HE4 had a higher sensitivity for ovarian cancer detection than CA-125 - 72.9% versus 43.4% at a confidence level of 95%. Subsequent experiments showed HE4 to be "...overexpressed in 93% of serous, 100% of endometrioid, and 50% of clear cell ovarian carcinomas," establishing the protein as a more precise detection mechanism [20]. Drawbacks are still ever present, however, namely that HE4 is elevated in individuals with benign gynecologic conditions, such as ovarian cysts, and that, while undoubtedly promising, it is more useful in monitoring ovarian cancer rather than screening for it.

Many comparison experiments were conducted to relate the effectiveness of CA-125 with HE4. Authored by Rafael Molina, his research article published in *Tumour Biology* cites CA-125 serum levels to be significantly higher in premenopausal women whereas HE4 serum levels are higher in postmenopausal women. Both tumour markers were clearly related to stage with significantly higher concentrations in advanced stages ($p = 0.001$). An assessment of individual performance was also statistically significant, providing convincing evidence that the sensitivity is higher with the combined use of both tumour markers. However, high HE4 serum levels were also recorded for patients with renal failure indistinguishable from ovarian cancer, thus justifying the coupling of two or more biomarkers.

Another promising detection protein is Mesothelin, a 40 kDA secreted protein expressed in normal mesothelial cells and over-expressed in several human tumours. A previous pancreatic cancer detection project enlisted carbon nanotubes to serve as an electric detector. Human mesothelin-specific antibodies were mixed with single walled carbon nanotubes and used to coat strips of filter paper, making the paper conductive. If mesothelin was present in a given blood sample, the antibodies would bind and enlarge, altering the electric properties of the nanotube network. This sensor's detection sensitivity exceeds that of an enzyme-linked immunosorbent assay and it's cost efficiency is over 25,000 times less than current methods [3]. Such promise is novel in the cancer community, but mesothelin was rejected simply because it's presence is dominated in pancreatic cancer as opposed to ovarian cancer.

Although previous research has been extensive on the abovementioned three proteins, those that have recently been discovered with little headway into their personalized detection mechanism offer more freedom for experimentation. B7-H4, homolog 4 of the B7 family, was discovered approximately three years ago and has since been a candidate of choice for targeted therapy of ovarian cancer. The level of similarity between the mouse and human orthologs suggests that B7-H4 is highly conserved evolutionarily speaking. B7-H4 belongs to the immunoglobulin (Ig) superfamily with single IgV and IgC domains. The Department of Immunology in Rochester, Minnesota sequenced the entirety of the protein, which is encoded by two hundred and eighty-two amino acids with several potential N-glycosylation sites in the extracellular domain. “The amino acid sequence of [human] B7-H4 contains a large hydrophobic transmembrane domain and a very short intracellular domain comprised of only two amino acids,” thereby categorizing B7-H4 as a type I transmembrane protein [18]. All molecules of the B7 family play crucial roles in the fine tuning of antigen-specific immune responses. B7-H4 is a regulatory molecule associated in negative signaling that impacts antitumor responses mediated by T cells. When bound to activate T cell receptors or crosslinked, B7-H4 disable tumor associated antigen (TAA)-specific cytotoxic T cell immunity by delivering a “putative negative signal” [5,18]. Unlike other members of the B7 family, B7-H4 does not bind CTLA-4, a protein receptor that functions as an immune checkpoint to downregulate the immune system. Perhaps most extensive in its inhibition of TAA-specific T cell proliferation and cytokine production is its impact on the cell cycle. A study conducted by Gabriel L. Sica observed B7-H4 intervention in cell cycle progression of T cells to occur during the G0 and G1 phases of mitosis. The final effect of B7-H4 is inhibition of interleukin-2 production, which is a cytokine signaling molecule in the immune system that regulates the activities of white blood cells. Holistically, B7-H4 has a profound effect on the inhibition of T cell responses at a relatively early stage.

The Ovarian Cancer Research Center of the University of Pennsylvania analyzed fresh primary ovarian cancer cells collected from patient ascites and solid tumors, and established cell lines before and after in vivo passaging. Although results confirmed poor cell surface expression of B7-H4 on long-term cultured ovarian cancer cell lines, they found that B7-H4 was readily expressed at the surface of freshly harvested young tumor cells. Likewise, *The Journal of Experimental Medicine* studied ovarian tumor macrophages and discovered that over seventy percent of freshly isolated tumor macrophages and tumor ascites CD14+ expressed cell surface B7-H4 protein while less than five percent of ovarian tumor cell lines expressed surface B7-H4. CD14 is a human gene encoding a protein component of the innate immune system. A tumor ascite is the buildup of fluid in the space surrounding the organs in the abdomen. Based on findings regarding cell surface expression of B7-H4, Dernada Dangaj and his team of data analysts hypothesize that direct ovarian cancer cell eradication can be achieved by targeting B7-H4 .

Weighing 20,743 kDa, MIH43 is the monoclonal antibody to the 30.89 kDa B7-H4 protein. Using immunohistochemical staining coupled with an ELISA test, antibody researchers found high expression of B7-H4 to be correlated with advanced tumor stage and distant metastasis, suggesting that B7-H4 has the combined effects of testing for CA-125 and HE4. MIH43 has been determined to be specific to B7-H4 but it’s tested applications have been limited to flow cytometry, immunofluorescence microscopy, and immunohistochemistry. The latter application refers to the process of detecting antigens in cells of a tissue by exploiting the principle of antibodies binding specifically to antigens in biological tissues. It is a feasible option as a detection mechanism, however the more-sensitive enzyme-linked immunosorbent assay (ELISA) is able to quantitatively determine protein concentration within a given sample, further increasing the specificity with which an ELISA detection mechanism can diagnose any-stage ovarian cancer.

Holistically speaking, an ELISA test involves the binding of a specific antibody to an unknown amount of antigen. This detection antibody is then linked to an enzyme, and when a substrate is added, the enzyme-substrate complex triggers the enzyme to convert to some detectable signal, most commonly a colour change or chemiluminescence. Conventional ELISA tests employ a polystyrene microtiter 96-well plate that is precoated with either an absorptive substance or a capture antibody, as in a sandwich ELISA. After the antigen is immobilized, a detection antibody is added and covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to the enzyme through bioconjugation. A common name for the complex created in ELISA is Ag-Ab, referring to the antigen (Ag) and antibody (Ab).

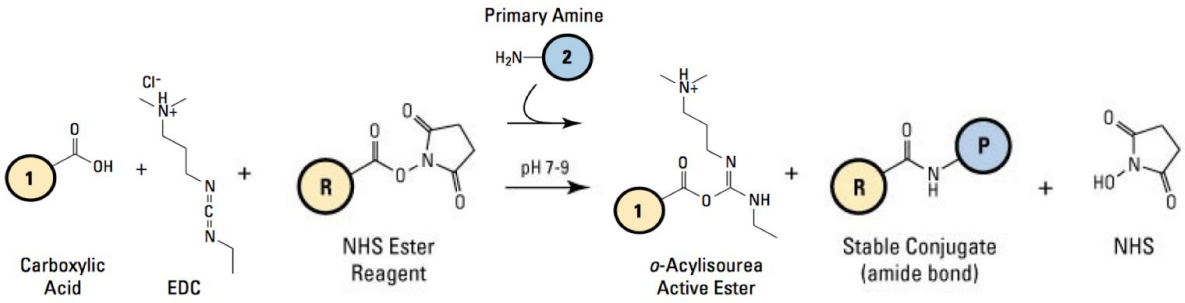
A sandwich ELISA test is favorable when detecting a protein with two or more epitopes.. It is a less common variant of ELISA, but it’s primary advantage “...is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to 2 to 5 times more sensitive than direct or indirect ELISA)” [17]. Sandwich ELISA is a specific subset of this immunostaining technique that utilizes two layers of antibodies - a capture and detection antibody. As a result, the protein contained between the two antibodies must contain at least

two epitope capable of binding to antibodies. It is suggested that either two different monoclonal antibodies be used or one monoclonal antibody and one polyclonal antibody, where the former would serve as the detection antibody. Using the same monoclonal antibody as both the capture and detection antibody could result in an unsuccessful sandwich if the antigen has only one epitope per molecule. The second antibody will never be able to bind because the first is already bound to the unique epitope. Additionally, sandwich ELISA is difficult to optimize, so for the purposes of the project, a tested matched pair of antibodies will be used to ensure that they are detecting different epitopes on the target protein and not interfering with each other's binding.

A crucial aspect of running an ELISA test is washing, which serves to separate bound and unbound reagent components. The liquid used to wash each well is usually buffered in order to maintain isotonicity, since the reaction is successful under such conditions. It is also important to use both a positive and a negative control in order to eliminate lurking variables that could affect the absorbance or cause the color change on its own. Current ELISA tests can be used to detect a myriad of diseases, including HIV, Influenza, and Lyme disease. There does exist an ELISA kit for the human cancer antigen CA125, but it's effectiveness in early-detection is quenched by its limited sensitivity when tested alone. B7-H4, however, has proven to be incredibly overexpressed in early-staged ovarian cancer, so developing a cheaper, efficient ELISA test for B7-H4 is novel for the cancer community.

An Fc receptor is a protein found on the surface of cells, including macrophages, that contribute to functions of the immune system. As previously mentioned, ovarian tumor macrophages were found to readily express B7-H4, thereby indicating the need for a modified ELISA test that exploits the Fc region of an antibody. Our idea, then, is to combine Fc Portion with an ELISA test. Fc Portion achieves the direct antibody conjugation to a gold nanorod via carbohydrate moiety. A heterobifunctional linker with hydrazide and dithiol groups aids in attaching the antibodies to the gold nanorod, and a subsequent sandwich Elisa test converts the enzyme-linked detection antibody into a quantifiable measurement.

Direct antibody conjugation involves two coupling reactions that converts chemical groups on the antibody and nanorod into a new formation that binds the two. Amines are the universal group capable of binding to both a carboxyl group and a thiol group. This also means that in order to convert a carboxyl group to a thiol group, the coupling reaction must go through an intermediate amine. Carbodiimides are used to begin the coupling reaction. Also called zero spacer arm cross-linkers, carbodiimides affect the conjugation of carboxyl groups (glutamate, aspartate, C-termini) to primary amines (lysine, N-termini), resulting in the formation of an amide bond that is shared between the groups. Carbodiimide cross-linkers react and activate the carboxylic acid groups on one molecule to form the active intermediate O-acylisourea. O-acylisourea reacts with the primary amine on the second molecule to form the amide. However, O-acylisourea is unstable, so an *N*-Hydroxysuccinimide (NHS)-ester must be combined in the reaction and the carbodiimide will couple the NHS to the carboxylic acid, creating an NHS-activated molecule that is amine-reactive. O-acylisourea intermediates and NHS-activated molecules will compete for amine targets, but because NHS-esters have a significantly longer half-life than O-acylisourea (several hours/days versus seconds), only NHS-activated molecules will have the ability to attach to thiol groups. Common cross-linkers include N,N'-Dicyclohexylcarbodiimide (DCC) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and common NHS-esters include NHS and Sulfo-NHS. DCC is a water-insoluble carbodiimide-based crosslinker that activates carboxyl groups for spontaneous peptide-bond formation with primary amines. EDC is a water-soluble carbodiimide-based crosslinker that activates carboxyl groups for spontaneous reaction with primary amines, enabling peptide immobilization and hapten-carrier protein conjugation [22].



Following the carboxyl modification reaction, the newly created amide must attach to a thiol (cysteine) group using amine-to-sulfhydryl heterobifunctional protein crosslinking reagents. The difference between the reagents is the type of spacer arms and lengths they create. NHS-maleimide crosslinkers are typically used because they form long, polyethylene glycol (PEG) spacer arms. An alternative to these crosslinkers are isothiocyanates, which are moderately reactive and stable in water and most other solvent buffers. Isothiocyanates form thioureas upon reaction with amines that are reasonably stable for a short period of time. Fluorescein isothiocyanate isomer (FITC) is a widely used amine-reactive fluorophore because it efficiently labels antibodies and other purified proteins at primary amines.

MIH43, the monoclonal antibody to B7-H4, contains amine groups that can be modified by EDC and NHS or Sulfo-NHS to link to carboxylic acid groups found on magnetic nanoparticles. Iron oxide nanoparticles have gained considerable attention due to their superparamagnetic properties and potential in biomedical applications. Recent research reported by Alice Bu suggests that iron oxide nanoparticles that have been converted into water soluble particles can conjugate with other biomolecules, such as proteins. The *Avicenna Journal of Medical Biotechnology* cites the conjugation of monoclonal antibodies to iron oxide nanoparticles for the detection of a protein associated with breast cancer. Researchers led by Fereshteh Shamsipour utilized the EDC method to first bind antibodies to an iron oxide particle and then attach their respective protein (her2/neu) to the capture antibody. The concentration of the conjugated antibodies was measured by Bradford assay, which yielded positive results in showing that the conjugated nanoparticles bound specifically to the her2/neu antigen [16]. B7-H4 and MIH43 have similar properties to the above experiment, except a bicinchoninic acid assay (BCA assay) must be applied because it works without regard to the size of the magnetic nanoparticle. The BCA assay is a widely used assessment for the total protein concentration found in a sample compared to a protein standard. “It combines the well-known reduction of Cu2+ to Cu1+ by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu1+) by bicinchoninic acid” [21]. The first step is the biuret reaction, which is the chelation of copper with protein in an alkaline environment to form a light blue complex. The second step introduces the bicinchoninic acid to react with the reduced cation (Cu1+) that was formed in step one. This intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. Cysteine, cystine, tyrosine, and tryptophan are the four amino acid residues that lead to the BCA color formation, and B7-H4 has intrachain disulfide bonds formed by cysteines, making it compatible with the assay.

Following the BCA assay, which provides less sensitive results, a sandwich ELISA test will yield a specific quantification of B7-H4 concentration. Rather than utilizing an additional monoclonal or polyclonal antibody, modern ELISA tests make use of a biotinylated antibody. A biotin-conjugated detection antibody binds to the captured antigen and an avidin-horseradish peroxidase (HRP) conjugate is added. The HRP enzyme is triggered by a 3,3',5,5'-Tetramethylbenzidine (TMB) substrate, resulting in color development. As in the case of the BCA assay, the antigen concentration is calculated with respect to values of the standard curve. For the purposes of a biotinylated antibody, the luminescence would be tested and a positive result would only occur in contrast to a negative result. In other words, a high luminescence value is significant only in comparison to a lower luminescence by the negative control.

Such as extensive literature reviews guided the project from it's flowering stages, providing adequate background knowledge to assist us during protocols and assessment of results. Given that the literature was read and written prior to the beginning of the project, some of the information included may be irrelevant to our project but are no less important in knowing. Likewise, there are parts that were unintentionally omitted from our research, consequently providing learning opportunities and instances for modification.