INTRODUCTION
Macrophage populations with distinct phenotypes have recently received attention as predictors of disease pathogenesis in processes such as cancer, atherosclerosis, periodontitis, and fibrosis, among many others. Specifically, macrophages have also been described as having phenotypes that range along a spectrum from pro-inflammatory (M1) to anti-inflammatory/regulatory (M2). Endometriosis is a disease characterized by endometrial glands and stroma being present outside of the uterus; inflammation, adhesions, pain and infertility are also associated with the disease. The disease represents the third leading cause of hospitalization for gynecologic indications and affects over 5 million individuals in the United States.

Despite the prevalence of the disease, the etiology is unknown. Endometriosis is assumed to result from retrograde menstruation or from a dysregulated inflammatory response. To date, the inflammation has largely been assessed in the peritoneal fluid, rather than on a tissue level. A small number of studies that have investigated endometriosis on the tissue level have shown macrophage polarization may play a role in helping the endometrial tissues survive outside of the uterus. Perplexingly, while the peritoneal fluid shows an increased level of inflammatory factor, tissue level examinations show that the anti-inflammatory response is prevalent within endometriotic lesions. The discovery suggests while endometriotic lesions are considered to be benign, a number of parallels can be drawn between the inflammatory process in cancer and endometriosis, implying the cells are able to evade the immune system through manipulation of a locally favorable environment; in fact, there are several patients on the study who have come back after undergoing surgery to excise lesions only to find that they have developed ovarian cancer as a direct result of their endometriosis.

OBJECTIVE
The aim of this project was to develop methods for quantitative assessment of macrophage phenotype at the tissue level in endometriotic lesions from the peritoneal cavities of human patients, in the hopes of predicting the downstream possibility of ovarian cancer, which would be represented by a predominance in the M2 phenotype.

HYPOTHESIS/SUCCESS CRITERIA
The success criteria of our experiment is twofold. First, we would like to develop a staining protocol that could be applied to all of the patients that are currently a part of will become a part of the study. Using the staining protocol, we will apply all methods to the second part of our criteria, that being the development of a method to label and identify the stained lesions. We developed the following method for identifying the lesions: if a lesion were to present with more positively stained inflammatory cells, we would label the lesion as the M1 phenotype, whereas if we were to see a higher amount of positively stained anti-inflammatory cells, we would label the lesion as M2, and consider further labeling the lesion as possibly cancerous.

METHODS

Tissue Collection

Tissues were collected from sixty-nine human patients who were diagnosed with endometriosis. The patients underwent laparoscopic surgery and biopsies were taken from several sites of endometriosis (including peritoneum, uterosacral ligament, and ovarian fossa) and a control section (no lesions).

Immunofluorescent Labeling

Half of the biopsies were embedded in optimal cutting temperature compound and flash-frozen in liquid nitrogen at -80°C. Tissue sections were taken at 6 microns and incubated in PBS + 2% bovine serum albumin for 1 hour at room temperature and then overnight at 4°C with mouse CD68 mAb (clone number KP-1, Abcam), rabbit CD86 mAb (clone number EP1158Y, Abcam), and goat polyclonal CD206 (Santa Cruz), staining cell markers for macrophages, M1 macrophages and M2 macrophages, respectively, over a range of dilutions from 1:50 to 1:150 after a multitude of preceding steps in the protocol to ensure proper staining; endothelial cells were identified by staining with rabbit polyclonal CD31 (Abcam). Primary antibodies were identified by using Alexa Fluor 594 donkey anti-mouse, 568 donkey anti-rabbit, 488 donkey anti-goat, and 488 donkey anti-rabbit (Invitrogen) using a range of dilutions from 1:100 to 1:250. Slides were counterstained with DAPI and examined using a FLoid Cell Imaging Station. Parallel slides in which primary antibody had been omitted were used as negative controls.

Immunoperoxidase Labeling

The other half of the biopsy punches were embedded and processed in paraffin wax and sectioned at 7 microns by histologists at Magee-Women’s Hospital, then sent to me for staining. The slides were subjected to PBS + 1.5% horse serum for 1 hour at room temperature and then overnight at 4°C with mouse CD68 mAb (clone number KP-1, Abcam), rabbit CD86 mAb (clone number EP1158Y, Abcam), and rabbit polyclonal CD163 (Abcam), staining cell markers for macrophages, M1
macrophages and M2 macrophages, respectively, over a range of dilutions from 1:50 to 1:100 after a multitude of preceding steps in the protocol to ensure proper staining; secondary application of antibodies/development of slides was done through the use of a VectaShield Elite ABC Kit, using a peroxidase enzyme to catalyze a chemical reaction at positively marked cells to turn them brown; slides were then counterstained with hematoxylin, turning the background blue.

RESULTS
Paraffin-embedded slides exhibited a better preserved tissue morphology, as well as providing brighter and clearer stains than their frozen tissue counterparts. Furthermore, the immunofluorescently tagged slides showed a penchant for generating too much autofluorescence, increasing the amount of false positives. Comparatively, the immunoperoxidase slides, when treated properly, showed little to no background staining and easy identification of the positively labeled cells.

Figure 1 below displays a comparison between the frozen (see left side) and paraffin sections (see right side) and how the different methods of labeling appeared for a treated frozen slide & treated paraffin slide.

Figure 1: Pictures of a frozen slide having undergone immunofluorescent treatment (left picture) and a paraffin slide having undergone immunoperoxidase treatment (right picture). Both sections were stained for CD163 and were stained with the same dilution factors.

Figure 2 below shows an evaluation of the three different antibodies used for immunoperoxidase labeling (CD68, CD163 and CD86) and how we hope to compare these lesions in the future.

Figure 2: Pictures of immunoperoxidase labeled sections for CD68 (top row), CD163 (middle row) and CD86 (bottom row). Positively labeled sections are marked with red circles and all images were taken in approximately the same area across three samples; the only difference is the antibody with which they were treated.

As we can see in the above figure, we can see a large area of macrophage infiltration in the CD68 designated images. Looking to the middle row of images, we see that those areas of high infiltration can be designated as M2, due to the higher amount of positively stained cells seen, compared to the lack of positive cells seen for the row of images corresponding to the CD86 stain. Overall, these kind of results were what was expected of the lesions, however, the results don’t necessarily bode well for the patients themselves.

DISCUSSION
The difference between frozen and paraffin sections is easily seen; the paraffin sections show a much brighter stain and did not require the reduction of autofluorescence, unlike the frozen sections. Furthermore, we can see the frozen sections show slight disturbance in their overall morphologies, whereas the paraffin sections remain largely undisturbed. Going forward in this project, we propose to stain primarily paraffin sections due to their more favorable outcomes in the process of immunofluorescent labeling and keeping of the overall structural integrity of the sample. We would also like to begin the quantification of the ratios of M1 and M2 macrophages seen in the lesions, as well as begin correlating the macrophage differences seen between lesions and their locations, as well as begin correlating the polarization of macrophages and the recurrence of endometriosis.

ACKNOWLEDGMENTS
The authors would like to acknowledge the assistance of Deanna Rhoads and Esther Elishaev in tissue procurement, preparation, and staining.

REFERENCES