THE EFFECTS OF AN ACUTE BOUT OF EXERCISE ON MELANOCORTIN RECEPTOR EXPRESSION BEFORE AND AFTER 12 WEEKS OF EXERCISE TRAINING IN POST-MENOPAUSAL, OVERWEIGHT WOMEN

By

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ABSTRACT

There is a plethora of evidence to suggest that exercise helps reduce the risk of a variety of inactivity-related diseases, but few studies have examined the mechanisms through which exercise confers its benefits. In recent years, the interest in the melanocortin system has spiked as researchers discovered the wide range of effects this system has on the body. It was only recently that studies began to illustrate the anti-inflammatory effects of melanocortin 1 and 3, especially in relation to exercise. These studies propelled us to examine the effects of an acute exercise bout on the percentage and density of melanocortin 1 and 3 receptors both before and after 12 weeks of exercise training. We will be incorporating both an aerobic and resistance exercise program in overweight to obese, post-menopausal women. We anticipate finding a significant increase in both parameters before training. Post training, however, we anticipate an increase in the percentage of monocytes expressing MC1R, a decrease in those expressing MC3R, as well as a decrease in the density of both receptors on each monocyte. This study is currently ongoing as we finalize the MCR assays and continue recruiting subjects.

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INTRODUCTION

Cardiovascular Disease (CVD), such as heart attack, stroke, and peripheral vascular disease, refers to any class of disease that affects the heart or blood vessels. CVD is considered the number-one cause of death in the United States, accounting for 950,000 deaths each year (1). In comparison, this is twice the number of deaths caused by cancer each year. More specifically, CVD causes more than half of all deaths in American women. (1). Along with CVD, the average age of the United States population is increasing, with now more than 28% of the population over the age of 55 (1). Previous studies associate aging with one of the most common and significant factors that relates to CVD, with as many as 85% of individuals above 65 years old dying from some form of CVD (2). Age therefore perpetuates the already increasing problem of cardiovascular disease-related deaths.

One of the major causative factors of CVD is atherosclerosis. Atherosclerosis was previously considered an accumulation of lipids in vasculature walls, but is now recognized as a chronic inflammatory disease that takes place in blood vessel walls and involves various immune system components (11). Atherosclerosis begins with the inflamed endothelium of blood vessels, which then involves activated white blood cells and platelets that ultimately lead to plaque formation, perpetuation of the atherosclerotic lesions that are formed, and some form of cardiovascular disease (3,4)

Specifically, we chose our population to focus on post-menopausal, overweight/obese women. After going through menopause, a large body of evidence suggests there is an increase in proinflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-alpha) (5). This upregulation of proinflammatory cytokine secretion

suggests a link between post-menopausal women and the development of atherosclerosis. In addition to the change in circulating cytokines, menopause is also commonly associated with increased adiposity, which increases the number of adipocytes that can act as an endocrine organ and secrete adipokines, which in turn have been shown to contribute to the development of atherosclerosis (6).

Atherosclerotic development is a complicated process involving cytokines, leukocytes, platelets, and many inflammatory modulators. The excess secretion of proinflammatory cytokines that results from the increased age and adiposity of our subject population leads to the activation and expression of adhesion molecules on platelets and leukocytes. One of the first steps in platelet-leukocyte aggregation is the expression of P-selectin on platelets binding to the P-selectin glycoprotein ligand on the surface of leukocytes. Secondly is expression of the beta 2-integrin Mac-1 (CD11b/CD18), the adhesive receptor that aids in aggregate survival, and L-selectin, which stabilizes the platelet-leukocyte complex and allows for this survival (7). Through this platelet-leukocyte aggregate formation, the aggregate binds readily to the endothelial lining of blood vessels and promotes plaque formation and atherosclerotic development.

More specifically, platelets are found to bind with a greater affinity to monocytes, large phagocytic cells in the blood, and these monocyte specific aggregates show a higher increase and a longer increase than any neutrophil-platelet aggregates (7). The complexes that platelets form with monocytes contributes to the monocyte ability to infiltrate into the plaque (therefore differentiating into what is known as a macrophage). Platelet-monocyte aggregates are one of the many negative results from lack of exercise. Due to the complexity of this disease and the many modulators involved, the influence of exercise on this disease is not yet fully elucidated.

However, in juxtaposition to the pro-inflammatory properties of these monocyte specific aggregates, we will examine the effects of exercise on the anti-inflammatory melanocortin 1 and 3 receptors that are present on monocytes.

Furthermore, despite extensive efforts to discover the various receptors present on monocytes that are able to downregulate inflammation and slow down progression of this disease, few authors have researched and understand the mechanisms of the anti-inflammatory receptors on monocytes in response to exercise. A primary anti-inflammatory receptor present on monocytes is the melanocortin 3 receptor (MC3R). In 2011, our lab was first to examine the effects of resistance exercise training on the melanocortin 3 receptor mRNA expression in obese, post-menopausal women. We found that 12-week resistance training upregulated melanocortin 3 receptor mRNA in whole blood by 16-fold (8), illustrating a strong relationship between exercise and the density of this receptor present on monocytes.

In recent years, the interest in the melanocortin system has spiked as authors discovered the wide range of effects this system has on the body, such as skin and hair pigmentation, sexual function, energy homeostasis, feeding behavior and fat accumulation (10). It was only recently that studies began to illustrate the anti-inflammatory affects some melanocortin receptors have in response to the binding of certain agonists (stimulating hormones), such as alpha melanocortin stimulating hormone (alpha-MSH) and adrenocorticotropic hormone (ACTH). There are five different melanocortin receptors (MC1R- MC5R), but previous studies have illustrated the anti-inflammatory properties mainly occur through the binding of these agonists to MC1R and MC3R that are present on circulating leukocytes, which is why we chose to specifically look at these two receptors. When ACTH and alpha MSH bind to MC1R and MC3R on circulating leukocytes, the result is a down-regulation of pro-inflammatory modulators such as TNF-alpha

and interleukin-1 beta (IL-1B) (10). Additionally, recent reports illustrate a relationship between these melanocortin agonists and reductions in monocyte adhesion to the vasculature walls and also a decrease in adhesion molecules that aid in platelet-monocyte aggregate formation, such as vascular adhesion molecule 1 (VCAM-1), E-selectin, and monocyte chemoattractant protein-1 (MCP-1) (12). Through these recent studies, alpha MSH binding to MC1R and/or MC3R may function in the decreased secretion of pro-inflammatory cytokines through the inhibition of the NF-kB transcriptional activity, which will therefore decrease the amount of monocytes signaled to the endothelial damage and will, in turn, decrease plaque formation and help slow down the progression of CVD. In response to our recent findings in the 2011 study previously mentioned, we see that melanocortin receptor expression may upregulate in response to exercise. This finding illustrates how the melanocortin pathway is a mechanism by which exercise confers its health benefits.

Our primary goal is to examine the effects of a 12-week exercise training program both the density of melanocortin 1 and melanocortin 3 receptors on each monocyte, and the percentage of monocytes that express these receptors. We will measure these dependent variables at various time points after a single exercise bout (combination resistance and aerobic exercises). We hypothesize that consistent exercise will help those of the post-menopausal, overweight/obese female population to upregulate these anti-inflammatory receptors and their anti-inflammatory properties.

METHODS

Overall Study Design

Forty-four sedentary, overweight to obese, post-menopausal/elderly women between the ages of 55 to 75 will be recruited to undergo preliminary testing. We will explain the procedures verbally and the participants will carefully review and sign the informed consent form, as well as complete a medical history form and a physical activity questionnaire. Those who are cleared for participation will be assigned to one of two groups: the exercise training group (EX) or the education group (ED), which will be the control group.

Once the women have been split into one of these two groups, blood well be taken at two time points: before the 12 weeks of training/education (BT) and after the 12 weeks of training/exercise (AT). Blood will be collected to measure cytokines and chemokines at six different time points: immediately before exercise, immediately after exercise, 30 minutes after exercise, 60 minutes after exercise, 2 hours after exercise, and 24 hours after exercise. We will examine the complete blood count and a 5-part differential of cytokines, chemokines, adhesion molecules, glucose, and insulin. The flow cytometer will measure MC1R and MC3R percentage and cell density and will be assessed at 5 different time points: pre-exercise, post-exercise, 30 minutes post exercise, 60 minutes post exercise, and 24 hours post exercise.

Preliminary Testing

Height will be measured to the nearest mm, without shoes, using a wall mounted stadiometer. Body weight will then be measured on an empty bladder, in light clothing and without shoes, to the nearest 0.1kg. Body mass index (BMI) will be calculated and waist and hip circumferences will be measured in duplicate to the nearest 0.25 cm and the waist to hip ratio

will be calculated. We will us dual energy x-ray absorptiometry (DEXA) to calculate percent body fat, requiring the subjects to remove all metal objects and lie on the DEXA table for 10-20 minutes. This image will estimate the amount of total body fat, lean tissue, bone mass, and bone mineral density.

After meeting inclusion criteria, the women will be asked to gain approval to participate in the study from their personal physician, and then undergo a medical screening by our study physician (Jay Haynes, M.D.). This medical screening will include a medical history review, a physical examination, and a general screening for dementia. With the study physician or nurse present, all participants will undergo a submaximal treadmill exercise test to estimate maximal oxygen consumption, and an 8-repetition maximum leg press exercise to ensure that the participant can safely handle the exercises during the intervention period. During each test, heart rate and blood pressure will be monitored and acclimation sessions will be allowed prior to each test.

Acclimation Protocol

All forty-four women will follow our week-long acclimation protocol to the following exercises: leg extension, leg flexion, leg press, hip adduction, hip abduction, chest press, seated row, and “lat” pull down. Each participant will perform three acclimation exercise sessions on non-consecutive days. On acclimation day 1, the participants will be taught proper lifting techniques and an 8-repetition maximum will be assessed for each resistance exercise. On acclimation day 2, subjects will perform three sets of each exercise at 50% of their estimated one repetition maximum. During the first two sets, participants will perform eight repetitions and the last set will be to “muscular failure”. Acclimation day 3 will be to reassess all eight repetition

maximum. Those in the education group will go through the same acclimation protocol and strength tests after the intervention period. All acclimation and exercises sessions will begin with 5 to 10-minutes of cycling or walking. All will be performed under supervision of trained exercise technicians.

12 Week Intervention

The exercise group will exercise three times per week on non-consecutive days, with each exercise including a warm-up, a 25-minute aerobic training, two sets of eight resistance training exercises, stretching, and a cool-down. The aerobic will include walking or jogging for 20 minutes, reaching 70-80% of their heart rate reserve. The resistance training will include a set of eight repetitions, then another set that will be until muscular failure. This will be reevaluated biweekly and the exercises will be adjusted accordingly.

The education group will meet twice a week to attend education seminars consisting of health, diet, crafts, safety, and a variety of social interactions. This group will additionally receive a twelve week pass to the TCU recreation center.

For both groups, anthropometric data and exercise results will be acquired after the 12-week intervention period.

Experimental Trials

The experimental trails will be performed both before and after the 12-week intervention period. All participants will arrive at the lab after an overnight fast and will rest in a supine position for 15 minute prior to taking resting blood pressure measurements and a blood sample. The exercise group will then undergo the aforementioned exercise session and will immediately

have a catheter inserted into their arm, post-exercise. Blood will then be taken at the above described time points, still at a fasted state. Total blood volume collected between both experimental trails will be no more than 150 mL. A snack will be provided immediately after the 2-hour post-exercise blood sample.

The following morning, participants will return to the lab for the 24-hour post-exercise blood sample. During this time, the education group will sit quietly in the lab instead of exercising during the experimental trials, but will still have blood taken at the same time points.

Blood Analyses

The blood will be collected into chilled ethylenediaminetetraacetic acid (EDTA) tubes and used to assess CRP, other circulating cytokines, chemokines, and adhesion markers through a milliplex assay. Blood collected into room-temperature EDTA tubes will be used to assess complete blood count and the 5-part differential analysis, as well as for flow cytometry analysis of melanocortin receptors 1 and 3.

Monocyte MC1 and 3 receptor density and the percentage of monocytes expressing MC receptors will be measured using flow cytometery. We will dispense 100 microliters of whole blood into eight tubes (tube one is cells only and will be used as an auto fluorescence control), 2-4 will include isotype controls, 5-7 will have the appropriately designated, single color, fluorochrome antibodies, and tube 8 will have a mix of all fluorochrome-conjugated antibodies. Tubes will be vortexed gently, then incubated in the dark (room temp., 30 min). The following steps were done as quickly and as efficiently as possible: we then dispense 3mL of lysing buffer into each tube, cap the tubes and invert each tube 5 times, making sure to start a timer as soon as the last tube began inversion. We would think quickly and gently vortex all tubes and continue

inverting as we brought all tubes to the rocker. We would leave the tubes on the rocker for 15 minutes total, as decided by our various lysing experiments. During this time, all tubes are in the dark at room temperature. We then centrifuge our tubes at 500 rcf for 6 minutes at room temperature. The tubes are then carefully removed and, in one motion, we would dispense of the supernatant into a waste beaker, leaving the pellet undisturbed. We would follow this mechanism with a KimWipe to remove excess supernatant, therefore cleaning up the debris that would appear later in our scatterplots. All dispensing is performed in the same order that the tubes received lysing buffer. All tubes would then be "racked" in order to loosen the pellet, and then receive 3 mL of FACS buffer (phosphate buffered saline), following the same order. The cells would be centrifuged and the supernatant decanted in the same mechanism as described above. This would mark the end of the first "wash", and we would then repeat the "wash" to make a total of two washes. After the second wash, we would add 300-400 uL with 1% paraformaldehyde into each tube and mix well to fix the cells. The tubes will be run on the flow cytometer immediately using a BD FACSCaliber cytometer and CellQuest Pro software (BD Biosciences, San Jose, CA). The melanocortin assay will include anti-human CD14 and anti-human MC1R and MC3R.

Data Analysis

Mean and standard deviations will be calculated for all aforementioned variables obtained during preliminary testing. The Shapiro-Wilk test will be used to test for dependent variable normality, which will then be analyzed through a three-way analysis of variance (ANOVA) with repeated measures within the factor. The first factor will be grouped with two levels, exercise training (EX) and education (control; ED). The second factor will be the

intervention time points before training (BT) and after training (AT). The third factor (within subjects) will be the exercise time point and will have varying levels based on the dependent variables. Blood will be taken immediately before exercise, immediately after exercise, 60 minutes after exercise, 2 hours after exercise, and 24 hours after exercise. These samples will be examined using flow cytometry. If data are nonnormal, the log transformation will be utilized. Maulchly's test of sphericity will be employed. A Pearson product-moment correlation will check for correlations among dependent variables, and a multiple linear regression will be conducted to control for potential covariates. Statistical significance will be accepted at $P < 0.05$.

HYPOTHESIS AND SPECIFIC AIMS

The purpose of this study is to examine the effects of an acute bout of exercise before and after 12 weeks of exercise training on cellular modulators of atherosclerotic plaque formation. Of those cellular modulators, the project will specifically examine the percentage of monocytes expressing the anti-inflammatory cell surface proteins melanocortin 1 receptor (MC1R) and melanocortin 3 receptor (MC3R). Additionally, we will also measure the density of each receptor on circulating monocytes.

We hypothesize:

- an acute bout of exercise will upregulate the expression of melanocortin 1 receptors and melanocortin 3 receptors transiently
- an acute bout of exercise will upregulate the percentage of monocytes with MC1R and MC3R transiently
- 12-week exercise training will increase baseline expression of each receptor on monocytes

- 12-week exercise training will increase the percentage of monocytes expressing each receptor at baseline.

The specific aim for this study is to investigate the density of melanocortin receptors 1 and 3 on monocytes and the percentage of monocytes expressing the MC1 and MC3 receptors increases as a result of an acute exercise bout. Additionally, we will examine the effects of 12-week exercise training using a combination of aerobic and resistance exercises on the same variables.

Despite the abundance of literature illustrating the benefits of exercise, it has yet to be elucidated the mechanisms by which exercise confers these benefits. The melanocortin system, through its anti-inflammatory properties, may be one of these mechanisms. There is a paucity of research regarding the melanocortin system with regards to exercise, and this study will further explicate their relationship.

RESULTS AND DISCUSSION

In order to begin our experiment, it was first necessary to optimize the MC3R assay, identifying the correct concentrations of both the primary and secondary antibodies, as well as perfecting the lysing procedure and overall methods. To optimize the assay as a whole, we began by using a rabbit, anti-human IgG (heavy and light chain) primary antibody. IgG is the most abundant immunoglobulin and is evenly distributed in the blood. We use this rabbit, anti-human IgG to bind to the abundant immunoglobulin present on human cells, thus acting as the non-specific primary antibody. We can then obtain a baseline concentration for our secondary antibody, that is donkey, anti-rabbit AlexaFluor 488 conjugated, which will illustrate this antibody's functional ability to bind and fluoresce when excited by the laser. In our first series of

optimization experiments, we used varying concentrations of primary IgG antibody and varying concentration of secondary antibody in order to find the best concentration of secondary antibody without observing significant non-specific binding (NSB).

Each experiment contained two “cells only” tubes that did not receive any primary or secondary antibody. This controls for the natural auto-fluoresce of a cell. Additionally, each experiment also contained a series of tubes that did not receive primary antibody, but did receive varying concentrations of secondary antibody, similar to those of the previous series that did receive primary antibody in this same experiment. This controls for nonspecific binding.

In our first experiment, we saw that our first three concentrations (1:800, 1:400, 1:200 dilution) successfully avoided significant nonspecific binding, but the last and highest concentration of secondary antibody (1:100 dilution) led to a significant amount of nonspecific binding. We then examined the tubes that did receive primary antibody and observed that they each exhibited the same results: once the secondary antibody increased to the highest concentration, there was an increase in the amount of positive events, likely attributed to the nonspecific binding we saw in the NSB control series.

From these results we can decide an ideal range (around 1:200 dilution) for the secondary antibody concentration that we will use when binding to the actual anti-MC1R primary antibody in future experiments.

Tube	Primary Concentration	Secondary Concentration	# Events	% Parent	Mean	Median
5a	None	1:800	36	0.5%	465	407
5b	None	1:400	36	0.5%	602	441
5c	None	1:200	36	0.7%	474	373
5d	None	1:100	627	9%	454	325

Table 1. Comparison of the NSB control tubes that received no primary antibody, but did receive varying concentrations of secondary, anti-MC3R antibody.

Once we began to finalize the concentration for the primary antibody and the secondary antibody together, we began to see a significant amount of non-specific binding. We ran an optimization experiment using primary antibody concentrations that varied between 1:25, 1:33, and 1:50 combined with secondary antibody concentrations that varied between 1:133, 1:100, and 1:75, including, as usual, a cells only tube and 3 tubes with no primary antibody added.

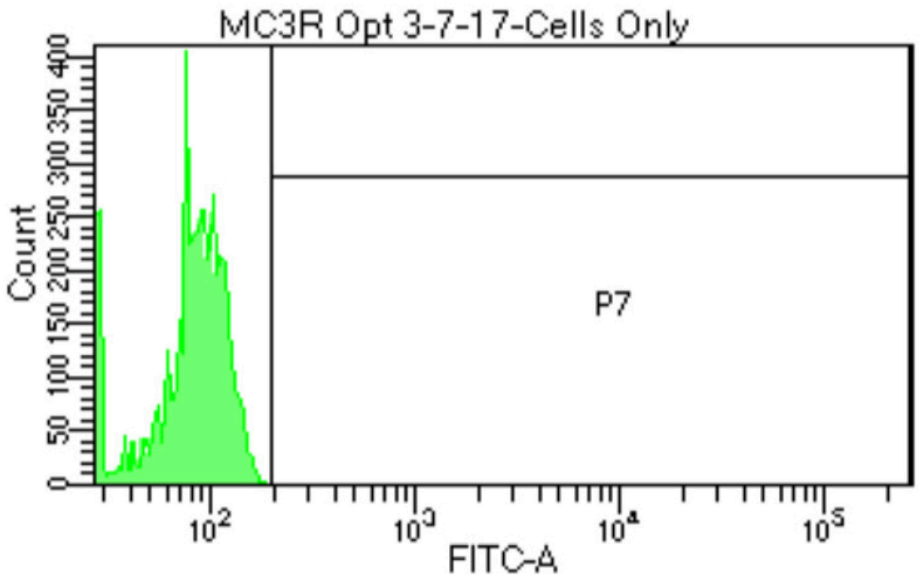

Figure 1. This histogram illustrates “cells only” control tube for auto fluorescence.

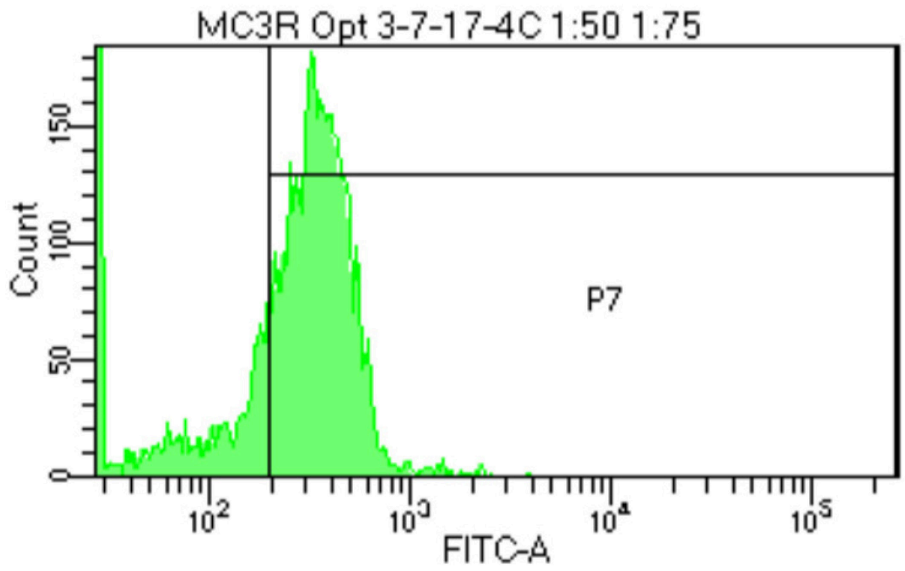

Figure 2. This histogram illustrates the combination of the most diluted amount of primary antibody and the most concentrated amount of secondary antibody (using the same concentration as above), 1:50 and 1:75 respectively.

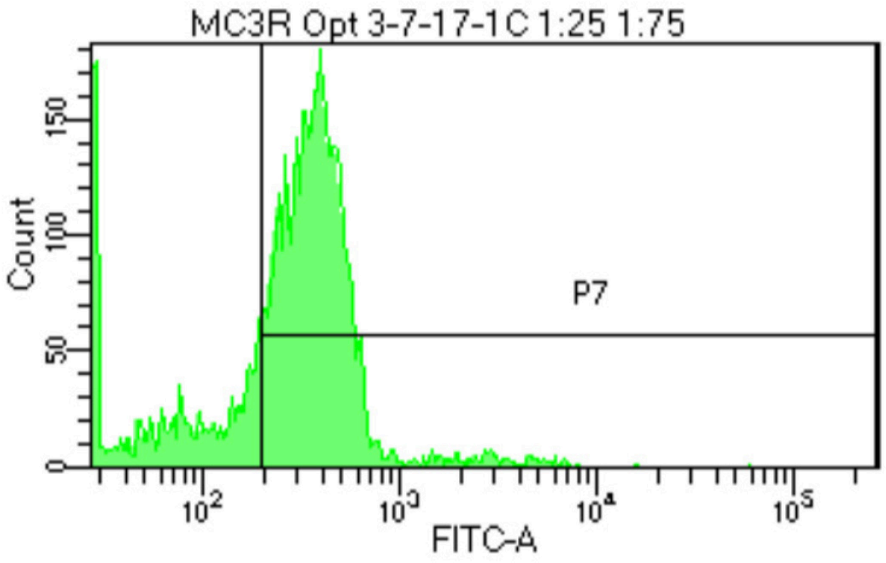

Figure 3. This histogram illustrates the combination of the most concentrated amounts primary and secondary antibodies, 1:25 and 1:75 respectively.

Tube	Primary Concentration	Secondary Concentration	# Events	% Parent	Mean	Median
5a	None	1:133	3564	63.1	310	287
5b	None	1:100	3383	60.7	313	289
5c	None	1:75	2995	56.3	307	278

Table 2. Comparison of the NSB control tubes that did not receive any primary antibody, but did receive varying concentrations of secondary antibody. Illustrates an increased amount of positive events and NSB present

From Figure 1 that contains cells only with no added antibodies, we are able to set a baseline for what is considered a "positive event", represented by section P7, accounting for the natural auto-fluorescence of a cell. In Table 2, the tubes present are the NSB control tubes that did not receive any primary antibody, which should therefore illustrate a very low percentage of positive events, but we instead see a very significant amount of binding from our secondary, fluorescently tagged antibody. This would be a significant problem during the actual experiment as we would obtain false positive results, thus nullifying our experiment.

We can then compare the cells only histogram in Figure 1, to Figure 2 which has the lowest concentration (or most diluted) amount of primary antibody with the most concentrated secondary antibody. From this histogram, we observe a small positive population that is farther along the x axis, illustrating that there are two separate populations. When we observe the NSB control tubes in Table 2, we can hypothesize that the large peak on the x axis is likely primarily attributed to the nonspecific binding observed in the control tubes. Furthermore, when looking at Figure 3, we observe the two most concentrated amount of antibodies. This gives us an even more prominent peak to separate the two populations. We believe that the population on both

histograms (Figures 2 and 3) that is farther along the x axis is the true population, while the larger peak in both cases is a mix of both true positives and nonspecific binding.

From these results, we decided to proceed by using an Fc blocking reagent. This reagent uses a high concentration of immunoglobulin that floods the Fc-receptors, binding, and therefore blocking, a significant amount of the non-specific binding sites present on our monocyte population. This should help minimize the amount of NSB observed in our results. We then conducted an experiment to compare the results of tubes, both with and without the Fc block present, using the following concentrations. We obtained the following results:

Tube	**Primary Concentration**	**Secondary Concentration**	**Fc Block Presence**	**# Events**	**% Parent**	**Mean**	**Median**
1a	1:25	1:133	No	4556	87	1714	632
1a with FC	1:25	1:133	Yes	2638	49.1	3041	383
4a	1:50	1:133	No	4474	80.6	896	494
4a with Fc	1:50	1:133	Yes	3380	60.4	1155	408
5	None	1:133	No	1055	18.6	368	338
5 with Fc	None	1:133	Yes	1052	18.9	370	338
Cells only	None	1:133	No	2	0	388	388

Table 3. Comparison of all tubes in the FC blocking experiment, including the varying combination of primary and secondary antibodies.

We observed a decrease in the amount of binding, as depicted above in Table 3. Although in most cases we observed this significant decrease, the amount of binding is still higher than desired in the NSB control tubes (labeled 5 and 5 with FC), thus illustrating that, although the FC blocker helped, it did not eliminate the NSB enough to continue using the same blocker in the future.

Further work needs to be done, and we most likely will proceed by using a different FC blocker and running further experiments to trouble shoot the likely causes of the spike in NSB. Once we are able to control this NSB, we can then proceed to finalize the optimization of both antibodies combined, thus finalizing our assay as a whole for MC3R. It is once this is finalized that we can begin the experimental trial portion of our study.

REFERENCES

1. Cardiovascular Disease. Retrieved January 05, 2017, from http://cvri.ucsf.edu/disease/

2. Custom Research Papers on Cardiovascular Disease. Retrieved January 05, 2017, from https://www.papermasters.com/cardiovascular-disease.html

3. Ross, R. Atherosclerosis is an inflammatory disease. *American Heart Journal.* 138: S419 S420, 1999.

4. Packard, RR, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clin Chem*. 54:24 38, 2008.

5. Pfeilschifter J, Ko¨ditz R, Pfohl M, Schatz H. Changes in proinflammatory cytokine activity after menopause. *Endocr Rev.* 23(1):90–119, 2002.

6. Lyon, C.J., Law, R.E., and Hsueh, W. A. Minireview: adiposity, inflammation, and atherogenesis. Endocrinology. 144:2195 2200, 2003.

7. Patko, Z, A Csaszar, G Acsady, I Ory, E Takacs, J Furesz. Elevation of monocyte platelet aggregates is an early marker of type 2 diabetes. *Interventional Med Appl Sci*. 4(4): 181 185, 2012.

8. Henagan, TM, MD Phillips, DJ Cheek, KM Kirk, JJ Barbee and LK Stewart. Melanocortin 3 Receptor: A Novel Mediator of Exercise Induced Inflammation. *Journal of Aging Research*, Article ID 512593, 2011.

9. Catania, A, S Gatti, G Colombo, JM Lipton. Targeting melanocortin receptors as a novel strategy to control inflammation. *Pharm Rev.* 56: 1 29, 2004.

10. Maaser, C, K Kannengiesser and T Kucharzik. Role of the melanocortin System in Inflammation. *Ann N.Y. Acad. Sci.* 1072: 123 134, 2006.

11. Wilund, K. R. (2007). Is the anti-inflammatory effect of regular exercise responsible for reduced cardiovascular disease? *Clinical Science, 112*(11), 543-555. doi:10.1042/cs20060368

12. Yang, Y, W Zhang, L Meng, H Yu, N Lu, G Fu, Y Zheng. Alpha melanocyte stimulating hormone inhibits monocytes adhesion to vascular endothelium. *Exp. Biol Med*. 0: 1 6, 2015.