THERAPEUTIC EFFECTS OF GENISTEIN®, MINOZAC, AND FOSTEUM® IN A MOUSE MODEL OF MUCOPOLYSACCARIDOSIS TYPE IIIB (SANFILIPPO SYNDROME B)

By

JOSEPH GIBNEY

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Joseph Gibney

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Sanfilippo syndrome, or Mucopolysaccharidosis type IIIB (MPS IIIB), is a genetically inherited lysosomal storage disease caused by the absence of *N*-acetylglucosaminidase (NAGLU). The absence of NAGLU results in accumulation of the glycosaminoglycan (GAG) substrate, heparan sulphate, in the lysosomes causing them to become distended. This leads to the stimulation of a pro-inflammatory state, increasing the activation of immune cells. The immune response may exacerbate damage resulting from abnormal lysosomal function. Sanfilippo syndrome most severely affects the central nervous system with initially delayed behavioral development progressing to neurocognitive regression. Systemic manifestations include organomegaly, diminished vision and hearing, and altered motor function. No curative treatment currently exists for MPS IIIB. Minozac is a CNS-penetrant small molecule previously shown to suppress proinflammatory cytokine upregulation in mouse models of seizure and traumatic brain injury. Genistein® is a tyrosine kinase inhibitor, which acts on both EGF and IGF receptors. Fosteum® is Genistein® with the addition of cholecalciferol and citrated zinc bisglycinate. Evidence suggests that Genistein®

reduces heparan sulfate production causing NAGLU substrate reduction and a decrease in pro-inflammatory cytokine upregulation, while Minozac inhibits the production of proinflammatory cytokines TNFα and IL-1β. We report the effect of treatment with these compounds separately, or in combination, for four weeks in MPS IIIB and wild type mice at ages 10 and 28 weeks. The primary outcome measures include the effects of these compounds on pro-inflammatory cytokine levels and immune cell activation in the brain. Additional measures of effects of these agents on vascular growth factors and lysosomal distention are reported. Genistein® shows a possible role in treatment for Sanfilippo Syndrome, while Minozac and Fosteum® did not show any positive alteration on disease progression. Further studies evaluating the effect of these agents on lifespan and functional aspects of hearing, vision, and motor ability are anticipated.

CHAPTER 1
INTRODUCTION

Mucopolysaccaridoses and Sanfilippo Syndrome

The collective Mucopolysaccaridoses are a group of disorders resulting from the malfunctioning or absence of lysosomal enzymes used to degrade glycosaminoglycans (GAGs). They are part of a larger group of diseases called Lysosomal Storage Diseases (LSDs), all of which result in lysosomal malfunction in one or more essential pathways. All mucopolysaccaridoses result from the absence of one enzyme in the glycosaminoglycan degradation pathway. The absence of this enzyme leads to the cellular buildup of long-chain sugar carbohydrates.

Mucopolysaccaridosis type IIIB (Sanfilippo Syndrome type B) (MPSIIIB), an autosomal recessive disorder, is one of the most common mucopolysaccaridoses, at 1 in 50,000 births (Van de Kamp, 1981). All MPS disorders are all characterized by the accumulation of glycosaminoglycans in the cell lysosome (Heldermon, 2007) (Neufeld and Muenzer, 1995). In addition to MPSIIIB, there are three other versions of Sanfilippo Syndrome. MPSIIIA results from a deficiency of sulfamidase, MPSIIIC results from a deficiency in N-acetylglucosaminide transferase, and MPSIIID results from a lack of glucosamine-6-sulfatase (Nidiffer FD, Kelley TE, 1983). The four disorders are biochemically distinct, stemming from the malfunction of completely different enzymes, yet all of these enzymes act on the HS degradation pathway, and thus result in phenotypic manifestations that are largely clinically indistinguishable (Nidiffer FD, Kelly TE, 1983).

Physical Disease Manifestations

Phenotypically, patients affected with MPSIII often first present with symptoms stemming from deficiencies in the central nervous system. The first characterized phase of the disease is associated with cognitive and linguistic delay. In the early stages the presentation of this disease is readily mistaken for a learning impediment and not as a symptom of a systemic disease. Phase one begins between the ages of one and four. The second phase of the disease, often beginning between the ages of three and four, is hallmarked by severe behavioral disturbances including tantrums, marked hyperactivity, decreased attention span, aggression, heightened night time activity, and disturbed sleep patterns. Physical growth through phase two is normal, with no readily identifiable physical indicators of disease presence (Cleary, 1993).

The third stage of the illness usually begins around age ten and is characterized by readily noticeable developmental characteristics. These may include poor balance and consequently, a reduction in motility. At this time point, a decline in connective tissue quality and joint stiffening is also seen. These factors together usually mark the beginning of the affected individuals becoming wheelchair bound and fully dependent on outside care.

In the fourth and final stage of the disease, swallowing difficulties are common due to a loss of muscular control. Additionally, frequent episodes of aspiration of food and saliva are also seen in the late stages of progression (Cleary, 1993). In the end, death is common in the early-to-mid teens. Death is often due to increased respiratory complications or heart failure. Uncontrolled infection has also been noted as a cause of death (Heldermon, 2007).

Previously Attempted Treatments

Though many therapies have been proposed, no treatment has successfully corrected either the cellular, like the expansion of the lysosome within the cell, or physical manifestations of the MPSIIIB disease, like decreased cognitive ability and motor function loss. Supraphysiologic levels of NAGLU enzyme, administered intravenously, have demonstrated some degree of attenuation of the physical manifestations of the disease, but it has shown little or no significant ability to correct neurologic pathology (Sands et al., 1994).

Further, one group hypothesized that a bone marrow transplant (BMT) into diseased MPSIIIB mice or application of intracranial (IC) AAV 2/5-hNAGLU would attenuate disease symptoms. They found that IC AAV NAGLU increased lifespan and improved motor function the best. They also found that combination of the two treatments decreased the lysosomal inclusion size and improved hearing. Despite the positive effects of BMT+IC AAV NAGLU, no histological improvements were seen (Heldermon, 2010).

NAGLU Mouse Model

A mouse model of MPSIIIB was created by Li et al. in 1999. This was accomplished through the replacement of an 852-bp fragment within exon 6 of the NAGLU gene with a neomycin resistance gene. Also within this vector construct, a cassette with the Herpes Simplex virus thymidine kinase gene was cloned upstream to allow for cellular differentiation (Li, 1999).

Several phenotypic traits such as motor function, Purkinje cell loss, circadian rhythms, auditory response, and retinal function in this model were determined to be

similar to those observed in humans. This mouse was deemed suitable for the study and development of MPSIIIB therapies (Heldermon et al., 2007).

Neuroinflammation

New pathways have been proposed for investigation in an attempt to provide a more comprehensive understanding of treatments that may offer aid to affected individuals.

Lysosomal distention, and thus cellular distention and death, has been hypothesized to play a role in increasing the progression of disease through the activation of the inflammatory pathway (Heldermon, 2010). In normal, non-diseased, brains, activated glia trigger a neuroinflamatory cascade. This cascade is well studied, and its goal is to protect the brain. The neuroinflamatory response is proliferated through the production and secretion of proinflammatory cytokines.

Chronic neuroinflmmation can lead to cellular damage through neuronal and synaptic dysfunction. The ability to alter the body's production and proliferation of proinflammatory cytokines has presented a novel target in a wide array of neurodegenerative disorders like Alzheimers disease or traumatic brain injury (Van Eldik, 2007).

The accumulation of HS has shown the ability to initiate the inflammatory cascade including cytokine and chemokine production, activated glia and microglia, leukocyte recruitment, and the maturation of inflammatory cells (Taylor and Gallo, 1996) (Chrzaszcz et al., 2010). Further, microgliosis and the proliferation of reactive astrocytes have shown a correlation to the glut of undegraded substrate seen in MPSIIIB (Ohmi et al., 2009).

Lending credence to the idea that inflammation plays a major role in MPSIIIB disease progression, an experiment by DiRosario tested if the application of immunosuppressants in an MPSIIIB mouse model would attenuate effects of the disease. They showed that prednisone, increases lifespan and decreases glial fibrillary acidic protein (GFAP) concentration in MPSIIIB mice (DiRosario, 2009).

Administered Drugs

4', 5, 7-trihydroxyisoflavone, or Genistein®, is a soy-derived isoflavone and tyrosine kinase inhibitor that has shown the ability to decrease the production of HS, via receptor inactivation, in an MPSIIIB mouse model (Jakobkiewicz-Banecka et al., 2007) (Ruitjer et al., 2007). Genistein® is also known to act as an inhibitor of different growth factor receptors. For its use in MPSIIIB, Genistein®'s most notable inhibition occurs with the epidermal growth factor receptor (EGFR). This receptor mediates the production of heparan sulfate in the cell.

Additionally, a one year study of human subjects with MPSIIIA or B administered Genistein® at 5 mg/kg/day orally (PO) was performed. They found that Genistein® significantly decreased GAG urine concentration. Additionally, slightly improved cognitive function was observed (Piotrowska, 2008). Genistein® has been shown to be safe in humans in large doses, and it is currently available as a nutraceutical, without a prescription (Ulmann, 2005).

Minozac is a central nervous system (CNS) penetrant chemically manufactured molecule. Minozac has previously demonstrated the ability to decrease neural proinflammatory cytokines such as TNFα and IL-1β (Van Eldik and Wainwright, 2003). Used in mouse models of traumatic brain injury and electroconvulsive shock induced seizures, Minozac showed the ability to lower the impact of the proinflammatory

cascade through its effect on the expression of GFAP, as well as the astrocyte marker S100B(Chrzaszcz et al., 2010).

Fosteum® is a combination of Genistein®, cholecalciferol (vitamin D3) and citrated zinc bisglycinate (Zinc). Zinc supplementation has been shown to decrease levels of TNFα and acute-phase proteins, as well as erythrocyte sedimentation (Prasad, 2009), while vitamin D3 supplementation has been shown to decrease systemic inflammation by reducing C-reactive protein (CRP) levels (Mathias, 2010). Burnett et al. also showed that this FDA-approved dietary supplement aids in the management of osteopenia and osteoporosis (Burnett, 2011).

Cytokines Assessed

The cytokines directly assessed in this study were chosen based on the results of previous literature and preliminary assays performed by our laboratory.

In a preliminary cytokine mapping assay run by Myriad Rules Based Medicine, increased concentrations of FGFb, MIP-1α, VEGF, IL-1α and MCP-1 were found in whole brain homogenates of MPSIIIB mice against aged matched controls; additionally, IL-6 and TNFα are known to be attenuated by Minozac (Chrzaszcz et al., 2010).

FGFb is not strictly a proinflammatory cytokine, however it does have the ability to enhance leukocyte recruitment and CAM expression while in the presence of other proinflammatory cytokines, especially TNF-α (Zitterman et al., 2006). The gene expression of MIP-1α, a cytokine enhancer of macrophage recruitment and macrophages and microglia initiation and creation, has been shown to be increased in the brains of 7-month of MPSIIIB mice (Villani, 2006). VEGF, also not strictly a proinflammatory cytokine, is often seen in the cytokine profile of various causes of inflammation because it stimulates the recruitment of monocytes to the site of

inflammation when in the presence of TNF-α or IFN-γ as well as synergizing with FGFb to boost monocyte and polymorphonuclear neutrophils (PMN) recruitment (Zitterman and Issekutz, 2006). IL-1α, a cytokine commonly associated with apoptosis, has been shown, in combination with TNFα, to increase expression of cyclooxygenase-2 (COX-2) and phospholipase A2 (PLA2). COX-2 promotes inflammation by increasing the production of prostaglandin E2 (PGE2) and PLA2 enhances prostaglandin production (Dinarello, 2000). IL-6 has receptors on neurons and glial cells and is also implicated in the PGE2 production. It is an important inhibitor of TNFα and IL-1 (Fielding et al., 2008). TNF-α has been shown to be significantly upregulated in MPSIIB affected mice (Killedar, 2010).

Hypothesis

We hypothesize that the administration of Genistein®, Minozac, and Fosteum®, separately, or Genistein® and Minozac in combination, will decrease proinflammatory cytokine levels in the brains of MPSIIIB mice. We hypothesize that the combination treatment of Genistein® and Minozac will be the most effective due to Genistein®'s ability to act on heparin sulfate production, and Minozac's ability to decrease the proinflammatory cytokines TNFα and IL-1β.

Aims

This purpose of this experiment is multi-faceted and includes the following aims:

- To further characterize the amount and types of proinflammatory cytokines upregulated in the mouse model of MPSIIIB
- To determine if the administration of Genistein®, with its ability to attenuate heparan sulfate production, will decrease proinflammatory cytokine levels
- To determine whether Minozac application will decrease levels of proinflammatory cytokines, as shown in a model of traumatic brain injury

- To determine whether Fosteum®, a compounding of Genistein® with Zinc and Vitamin D3, will both decrease HS production and lessen immune activation

- To evaluate the potential synergistic effects of Genistein® and Minozac, in combination on neuroinflammation, by inhibiting both GAG accumulation and TNFα and IL-1β upregulation

CHAPTER 2
METHODS

Animals

An established colony of MPSIIIB mice was transferred to the University of Florida from Washington University in St. Louis, Missouri in 2009. This colony was maintained through strict sibling mating; mutant and heterozygous males were crossed with heterozygous females. To genotype pups born, ACS staff at the University of Florida took small tail-tip samples at weaning. These samples were used to genotype the pups via PCR of the NAGLU gene on exon 6 with the neomycin insertion. Results were then verified using a substrate-based fluorescence assay to test for the presence of N-acetylglucosaminidase, the enzyme absent in MPSIIIB -/- mice (Heldermon et al., 2007). All procedures and handling of mice were conducted in compliance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Florida.

Preliminary Cytokine Map

Blood and Tissue Collection and Processing

Twelve mice were used in this study. Two cohorts (mutant and heterozygous) at two time points each (young and old), were randomly selected. This gave each experimental group an n=3. Mice in the “young” group were aged to 6 weeks (+/- 1 day) and mice in the “old” group were aged to 6 months (+/- 15 days).

Before brain tissue collection began, blood was collected from mice via cheek puncture. Using an 18 gauge needle, a small hole was opened at the intersection of the mouse retro-orbital and submandibular veins. Approximately 0.5mL of blood was drained into a 1.5mL heparinized eppindorf tube. Animals were then sacked primarily by

carbon dioxide narcosis and secondarily by cervical dislocation (according to UF IACUC regulations), and blood was again harvested via a cardiac puncture technique. The mouse chest cavity was opened, and an 18 gauge needle was inserted into the left ventricle of the heart. Using a slight vacuum from the attached syringe, blood was collected and then added to the partially filled 1.5mL heparinized eppindorf tube containing the previously collected blood. The harvesting of brains was conducted swiftly after the completion of blood collection. Brains were excised, placed into a 1.5mL cryogenic vial, and immediately flash frozen in liquid nitrogen. Both blood and brain samples were stored at -80° C.

To prepare for analysis, blood samples were thawed and then spun at 13,000xg for two minutes to separate out plasma. Plasma was removed from the original 1.5mL heparinized eppindorf tube, placed in a second 1.5mL heparinized eppindorf tube, and refrozen to -80° C prior to cytokine measurements.

Brain samples were thawed, weighed, and transferred to a 15mL conical filled with 9x volume tissue homogenization buffer, 50mM Tris-HCl with 2mM EDTA in ddH_2O, at pH 7.4. The tissue was then homogenized with an Omni® tissue homogenizer while the tube was submerged in an ice bath. Next, the homogenized solution was transferred from the 15mL conical to 4x1.5 mL eppindorf tubes and centrifuged at 13,000xg for 8 minutes. 500µL of supernatant from one eppindorf tube was aspirated into a clean 1.5mL eppindorf tube and stored at -80° C prior to cytokine measurements. All brain homogenization steps were conducted in a 4° C cold room.

Cytokine Profile Analysis

Prepared samples of blood and brain from each mouse were cold-packaged and sent to a collaborator, Myriad Rules Based Medicine® (RBM), in Austin, Texas. There,

RBM performed a multi-analyte profile combined sandwich immunoassay the samples, testing for the presence and amount of 59 distinct biomarkers and cytokines.

Statistical Analysis

An F-test was run on plasma and brain homogenate concentration levels to determine significance among samples. If any significance was present, as determined by an F-test $p < 0.05$, comparisons between old heterozygous/old mutant, old heterozygous/new mutant, old heterozygous/new heterozygous, old mutant/new mutant, old mutant/new heterozygous, and new mutant/new heterozygous pairs were achieved by running a Student's 2-sample t-test to determine significance between samples at $p < 0.05$.

One Month Treatment Experiment

Two-hundred-twenty mice, half mutant (-/-) and half heterozygous (+/-), were sex-matched and randomly sorted into five treatment groups: control, Genistein®, Fosteum®, Minozac or Genistein® + Minozac in combination. These treatments were administered at two time points: six weeks ("new") mice or six months ("old"). Twenty two mice were in each treatment cohort, with eleven at each age time point. The treatment time across all mice was 28 days. Table 3-7 describes the setup of each cohort.

Harlan Teklad aided in the production of all specialty research diets. Mice in the control group were fed chow lacking all soy byproducts; this chow was fortified with corn oil to account for the nutrients lost upon removal of soy. Mice in the Genistein® and Fosteum® groups were fed the same chow as the control animals, except this chow was additionally fortified with either Genistein® or Fosteum® at .12%. Genistein® and Fosteum® chow concentrations were based on a mouse average food consumption of 4g/day and an average mouse weight of 30g (Bachmanov, 2006), for a total daily

treatment dose of ~160mg/kg/day. Minozac was dissolved in normal saline solution (0.9 %) at a concentration of 0.15mg/100µL and was SQ injected into mice daily. Each mouse received Minozac at 5mg/kg/day (Chrzaszcz et al., 2010). The amount of Minozac given was weight-adjusted weekly throughout the experiment. The amount of chow eaten each day was measured to ensure normal food consumption.

After twenty-eight days, mice were sacrificed. Eight of eleven in each group were sacrificed primarily by carbon dioxide and secondarily by cervical dislocation (according to UF IACUC regulations). The brains of these mice were excised and flash frozen in liquid nitrogen for later analysis.

Three of eleven in each group were sacrificed via intraperotineal administration of 200µL Beuthanasia-D Special® (390mg pentobarbital sodium + 50 mg phenytoin sodium in a 1:20 dilution in 0.9% NaCl). After death, the chest cavity was opened and a 22 gauge needle connected to a calibrated peristaltic pump was inserted into the left ventricle of the heart and the right atrium was punctured. Ice-cold Tyrode's solution was pumped through the mouse until the fluid exiting the right atria was clear. Immediately following the Tyrode's solution, ice-cold 4% paraformaldehyde was circulated through the mouse until fixation occurred. Brains were then excised and transferred into chilled 4% paraformaldehyde for 24 hours at 4° C with gentle shaking, followed by submersion in a 30% sucrose solution with gentle shaking at 4° C for 24 hours. Brains were stored in this 30% sucrose solution until immunohistochemical analysis was performed.

Homogenization

Brains previously preserved in liquid nitrogen were removed from the freezer and weighed, prior to thawing. The brains were thawed in 500µL of ice-cold tissue homogenization buffer. The buffer consisted of 50mM tris-HCL, 2mM EDTA, and a

cocktail of protease inhibitors all at 1µg/mL (aprotenin, antipain, leupeptin, pepstatin A in 2mM PMSF). Brains were homogenized, submerged in an ice bath, by hand with a pestle, sonicated, while on ice, and centrifuged at 12,000xg for 10 minutes at 4° C. The supernatant was removed with a pipette and stored in a separate 1.5mL eppindorf tube, on ice. More tissue homogenization buffer was added to the tube containing the pellet, raising the volume of pellet + tissue homogenization buffer to 1.5mL. This process was repeated four times. All supernatant was stored, and the brain pellets were frozen pending successful analysis of the supernatant collected.

Bradford Assay Assessing Protein Concentration

A Bradford assay was run on a 250µL microplate. 1X dye reagent was removed from 4° C storage and allowed to warm to ambient temperature. Serial dilutions of 2mg/mL bovine serum albumin (BSA) were performed. 5µL of each standard and unknown sample and 250µL of 1X dye reagent were pipetted into the microplate well; the pipette plunger was depressed repeatedly to mix the solution. The microplate was incubated at room temperature for thirty minutes.

A spectrophotometer was set to 595nm and zeroed with a blank before the absorbance of the standards and samples was determined. Absorbance of the standards and samples was measured, and built-in software was used to plot unknown values against the standard curve. Absorbance values for each sample were then plotted against this standard curve, and protein concentrations for each sample were derived. This data was used to normalize measured cytokine values by the total protein concentration of each sample.

BioRad Custom Array Cytokine Assay

Plate Wash Method

Plates were washed via magnetic separation. The magnetic wash plate carrier on the BioPlex® wash station was installed and primed. The 96-well plate was placed on the magnetic plate carrier and the MagX2 preloaded program was used to wash the wells 3 times.

Plate Layout

The plate layout was then determined. Standards were assigned to columns one and two, and run in duplicate with the highest concentration in row A and the lowest concentration in row H. Wells A3 and A4 served as blanks and were filled with diluent. All remaining wells were available for sample analysis.

Preparation of Standards

BioRad® provided eight standards for the custom array. Standards were reconstituted and diluted in BioPlex® Standard diluent at a 1:4 dilution. The standards were mixed appropriately, a serial dilution was performed, and the dilutions were plated accordingly.

Preparation of Samples and Coupled Beads

Because the assay was analyzing mouse cytokines from tissue lysates, sample dilutions were pipetted at a ratio of 1:2, lysates to sample, per BioRad's® recommendations. This achieved a protein concentration of 200-900µg/mL in each well. The amount of sample and diluent used was adjusted per sample based on the previously conducted Bradford protein concentration assay. Upon completion, each prepared sample totaled 200µL, and this was pipette onto the 96-well plate based on

the previously determined sample layout. Samples were kept on ice throughout this process.

Coupled beads were prepared by adding 72µL of 10X beads for each cytokine type (8 cytokine bead reagents x 72µL each= 576µL) to 175µL of assay buffer. The bead mixture was covered in order to minimize light exposure.

Running the Assay

The diluted coupled beads were vortexed at medium speed for 30 seconds and 50µL of the bead mixture was added to each well, including those of the standards. The wells were then washed twice using magnetic separation. Diluted standards, blanks and samples were gently vortexed for 1-3 seconds. 50µL of each standard, blank and sample were pipetted to previously-assigned wells. The plate was incubated on a shaker at room temperature for 45 minutes.

During incubation, 38µL of detection antibody for each cytokine was pipetted into a tube and diluted with 2700µL of detection antibody diluent. The mixture was vortexed and spun, and 25µL of the mixture was added to each well. The plate was again incubated at room temperature for thirty minutes.

The plate was washed three times via magnetic separation. 50µL of diluted Streptavidin-PE (60µL of streptavidin-PE and 5,940µL of assay buffer) was added to each well and incubated at room temperature for ten minutes. The plate was again washed three times by magnetic separation. 125µL of assay buffer was added to each well before all wells were covered by parafilm and shaken at 1100rpm for 10 minutes.

Reading the Plate

An optimized software protocol for the Luminex 2 machine was prepared via the BioPlex Pro Assay® guideline. The plate was formatted according to the arrangement of

the standards, blanks, and samples. The protocol was run, and initial cytokine concentrations were determined. Concentrations were determined for each sample, and these were then protein-balanced and fit to the standard curve (Table 3-8) to derive the final cytokine concentration values. This assay was run in duplicate to minimize error.

Removal of Outliers

The mean concentration of each treatment group (by age and genotype) for each cytokine was determined from the duplicate runs. If the coefficient of variance for at least one of the two duplicates was greater than 15% from the mean of all values in a treatment group, an algorithm was applied to determine which of the two values (from the duplicate wells) should be retained for final data analysis and which value should be discarded as an outlier.

The algorithm consisted of a formula that considered the two values from the duplicate runs and subtracted these from the treatment group's mean (excluding both outlying data points). The duplicate value with the smallest absolute difference from the adjusted mean was selected, and thus included in subsequent analysis.

Statistical Analysis

Significance, by cytokine, was determined first by using a 1-way ANOVA to analyze only untreated mice across a single age cohort (10 weeks or 28 weeks). Significance was noted with yellow stars. Then a 2-way ANOVA was run to compare all treatments (Genistein®, Fosteum®, Minozac, G+M), ages (10 and 28 week old), and genotypes (Het or Mut) against the whole untreated control group. Significance is reported with white stars. Finally, if any T value in the 2-way ANOVA was near significance, a 1-way ANOVA was run for only that cohort against its matched control.

Significance is reported with red stars (this case happened only once: in the FGFb analysis for the 28 week age group treated with Genistein®).

Secondary Quantification of Cytokines

After acquisition of primary data, the levels of various cytokines for four randomly selected untreated 'old' Mut and Het brains from experiment #2 were measured via a membrane assay from Ray BioTech® [Cat#: AAM-ANG-1] for comparison against the BioRad® bead assay. To do this, 8 detection membranes, see Table 3-9**,** were placed in the provided 8-well tray with 2mL blocking buffer. This was incubated at room temperature for 30 minutes.

Before adding samples to each well, all samples were protein-balanced to 500µg protein/mL (protein values from experiment #2 were used) via dilution in ddH_2O. Next, the blocking buffer was decanted and 1mL of adjusted sample was added to each well overnight at 4° C.

The next day, samples were decanted from each well and the membranes were washed with Wash Buffer One three times at room temperature with shaking for 5 minutes, followed by the same protocol for Wash Buffer Two, twice. Biotin conjugated anti-cytokines were reconstituted with blocking buffer, and 1mL of diluted antibody mixture was added to each well for 2 hrs at room temperature. After incubation, 1mL of diluted antibody mixture was decanted, and 2mL of 1000 fold diluted HRP-conjugated streptavidin mixture (2µL of HRP-conjugated streptavidin + 1998µL of blocking buffer) was added to each well for 2 hours with shaking. After incubation, wash steps were repeated and the membranes were moved to a dark room.

In the dark room, 250µL of detection buffer C and detection buffer D were added, in tandem, to each membrane, after the membrane had been drip dried and placed on a

plastic sheet. Each membrane was incubated for 2 minutes and then was exposed to x-ray film for 4 seconds. Membranes were refrozen at -80° C upon successful x-ray exposure. See Figure 3-14.

The x-ray film was scanned into TIFF format using a 1200 dpi scanner, and the image was prepared using freeware ImageJ® software. With ImageJ®, a negative of the image was taken, and the relative density of each cytokine's expression was measured. Each membrane allowed for duplicates to be run for all cytokines, increasing significance. These values were normalized across positive controls (found on each membrane), separated by genotype, and graphed. A one-way ANOVA was run across all cytokines by genotype, and then a t-test tested between all cytokines.

Secondary Enzyme Assay

After the acquisition of primary data, the level of 4-methylumbelliferyl-β-D-glucuronide, a secondary enzyme in the heparan sulfate pathway, was measured via fluorescence assay. To do this, 50µL of brain homogenate from all samples tested in experiment #2 and 50µL of 4-methylumbelliferyl-β-D-glucuronide were added to an eppindorf tube and incubated for thirty minutes at 37.5° C. After 30 minutes, 250µL of stop solution was added. 200µL of this solution mixture was transferred to a 250µL 96-well black-bottom plate and read by a Tecan® fluorometer (excitation at 364nm and emission 444nm).

Fluorescence values were normalized by the protein concentrations derived from the previous Bradford assay and graphed. Significance was determined via one-way ANOVA, which analyzed the effects of genotype within a treatment group, and two-way ANOVA, which analyzed the effects of age and genotype within a treatment group, with multiple comparison corrections.

Additional Minozac Study

The results obtained for Minozac from the one month treatment experiment led to the conduction of an additional study to assess the efficacies of different modes of administration when this drug is administered acutely. Thirty 6-month-old mice were randomly selected for this study. Six mice, three heterozygous and three mutants, were in each group. Each mouse was weighed and the appropriate dose of Minozac was determined as previously described (5mg/kg). Mice were randomly sorted into five groups, differing by Minozac treatment modality: Control, Subcutaneous (SQ), Intravenous (IV), Intraperitoneal (IP), or Gavage (PO).

Twenty-four hours post-administration, the mice were sacked first by carbon dioxide asphyxiation, and then by cervical dislocation. Brains were excised and flash frozen in liquid nitrogen.

In preparation for the ELISA assay, brains were thawed and moved to a 15mL conical with 4.5mL of ice-cold tissue homogenization buffer, consisting of 50mM tris-HCl and 2mM EDTA. They were then mechanically homogenized using an Omni tissue homogenizer while submerged in an ice bath. The homogenized sample was transferred to eppindorf tubes and spun at 13,000xg for 5 minutes at 4° C. All steps were completed in a 4° C cold room. Samples were then refrozen at -20° C.

FGFb ELISA Procedures

FGFb cytokine quantification was performed on the mouse brain supernatant according to the RayBio® Mouse FGFb ELISA Kit (Cat#: ELM-bFGF-001). A standard curve was created from a 50ng/mL stock provided, via 1:2 fold serial dilutions in tissue homogenization buffer. The layout of the 250 µL 96-well plate was preplanned; samples were run in duplicate. 100µL of each standard and supernatant from sample was added

to each well and incubated at room temperature with gentle shaking for 2.5 hours. The plate was washed four times with 300µL wash solution. 100µL of prepared biotinylated anti-mouse FGFb antibody was added to each well and incubated for one hour at room temperature with gentle shaking followed by the previously described wash step. 100µL of prepared HRP-Streptavadin was added to each well and incubated for 45 minutes at room temperature with gentle shaking followed by a wash step. Then, 100µL of TMB One-Step substrate reagent was added to each well and incubated for half an hour at room temperature in the dark with gentle shaking. 50µL of 0.2M sulfuric acid stop solution was added to each well.

The plate was immediately read at 450nm. A line of best fit was drawn to the standard curve by integrated software, and cytokine concentration was calculated from this line.

MCP-1 ELISA Procedures

MCP-1 cytokine quantification was performed on the mouse brain supernatant according to the RayBio® Mouse MCP-1 ELISA Kit (Cat#: ELM-MCP1-001C). A standard curve was created from a 50ng/mL stock provided, via 1:3 fold serial dilutions in tissue homogenization buffer. The layout of the 250 µL 96-well plate was preplanned; samples were run in duplicate. 100µL of each standard and supernatant from sample was added to each well and incubated at room temperature with gentle shaking for 2.5 hours. The plate was washed four times with 300µL wash solution. 100µL of prepared biotinylated anti-mouse MCP-1 antibody was added to each well and incubated for one hour at room temperature with gentle shaking followed by the previously described wash step. 100µL of prepared HRP-Streptavadin was added to each well and incubated for 45 minutes at room temperature with gentle shaking followed by a wash step. Then,

100µL of TMB One-Step substrate reagent was added to each well and incubated for half an hour at room temperature in the dark with gentle shaking. 50µL of 0.2M sulfuric acid stop solution was added to each well.

The plate was immediately read at 450nm. A line of best fit was drawn to the standard curve by integrated software, and cytokine concentration was calculated from this line.

Data analysis for ELISAs

Data from both FGFb and MCP-1 ELISAs were analyzed using a freeware version of MasterPlex® software. Values were entered into the program and 'control', 'blank', and 'unknown' labels were assigned to each well. The program used internal software to derive a standard curve with a best-fit line. It then determined the adjusted protein concentration values, and averaged all duplicate samples, for all unknowns, based on the standard curve. Protein levels for each treatment type were graphed. T-tests were run to determine difference between Het and Mut controls. Once significance was verified among controls, one-way ANOVAs were run to compare treatment modalities by genotype.

CHAPTER 3
RESULTS

Preliminary Cytokine Map

Analytes in both brain and blood were measured in four different cohorts: 6 week old “new” heterozygous (unaffected) and mutant (diseased) MPSIIIB mice and 24 week old “old” heterozygous (unaffected) and mutant (diseased) MPSIIIB mice. Protein quantities below the limit of detection (mean + 3 standard deviations of 20 blank readings) were discarded. Statistical significance for each analyte tested was determined by T-test. (*) denotes significance when values were compared within age groups, while (°) denotes significance when values were compared over time. P values were 0.05 or 0.01, and the number of (*) or (°) denote the level of significance achieved.

Brain Homogenate Analysis

Of the 59 analytes surveyed in the combined sandwich immunoassay, 44 were detected at quantifiable levels. Many cytokines involved in inflammatory response, such as MCP1, MIP1β, IL-4, IL-10, etc., were elevated as MPSIIIB mice age. No significant differences in analyte concentrations exist between mutant and heterozygous mice of the 6 week age group, but a large number of biomarkers exhibit statistically significant differences as mouse age approaches 24 weeks (Tables 3-1 to 3-3). Twenty-two of the measured analyte concentrations in 24 week old mutant MPSIIIB brain homogenates are statistically significantly greater than those in normal heterozygous mice (of the same age group), 6 week old mutant mice, or both. The fold-change of 21 statistically significantly different analyte concentrations in 24 week old mutant MPSIIIB mice against matched controls is graphically displayed in Figure 3-1. Oncostatin-M was left off of the graph due to a lack of confidence in the reported values.

Blood Plasma Analysis

As seen in Tables 3-4 to 3-6, the circulating cytokines and biomarkers analyzed are similar to those observed in the brain; however, their concentrations are only slightly elevated in most cases. Notably different though, is the lack of significant differences between the cohorts in terms of concentrations of analytes. MIP-1 α, β, and γ show the highest level of significance between old mutants and controls.

Cytokine Levels Measured by Bead Assay

Brain homogenate levels in MPSIIIB mutant (diseased) and heterozygous (unaffected) mice, across four 1-month treatments (Genistein®, Fosteum®, Minozac, and combination G+M) and two time points, were measured, via a custom sandwich immune assay, for IL-1a, IL-6, MIP-1α, TNF α, FGFb, and VEGF. Figures 3-2 to 3-13 display the results of this assay. Graphs are ordered by analyte and age. A 2-way ANOVA was run on the data from every analyte measured, to determine significance. (*) denotes significance, with * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Basic Fibroblast Growth Factor (FGFb)

Figures 3-10 and 3-11 show that basic fibroblast growth factor (FGF-2 or FGFb) was significantly upregulated in diseased 10 week old and 28 week old mice compared to their unaffected counterparts. Concentrations in 10 week old heterozygous mice averaged 761.2pg/mL and those in 28 week old heterozygous mice averaged 752.9pg/mL. In contrast, the average concentrations found in the 10 week and 28 week old mutant mice were 2030.4pg/mL and 2345.5pg/mL, respectively. These differences were significant, via 1-way ANOVA at $p<0.0001$. Assessed by two-way ANOVA, Fosteum® significantly exacerbated the upregulation of FGF-2 in 10 week old

heterozygous mice ($p<0.01$), 28 week old mutant mice ($p<0.0001$) and 28 week old heterozygous mice ($p<0.0001$).

When assessed by 1-way ANOVA, Genistein® was effective in significantly decreasing the average concentration of FGFb to 1656.2pg/mL in 28 week old diseased mice ($p<0.05$). Overall, the combination treatment was less effective than the administration of Genistein® alone, but more effective than the administration of Minozac alone. Fosteum® significantly increased FGFb concentrations across all mice ages and genotypes.

Macrophage Inflammatory Protein 1α (MIP-1α)

Figures 3-5 and 3-6 show that MIP-1α was significantly upregulated in 28 week old MPSIIIB mice compared to their heterozygous counterparts ($p<0.05$). Though none of the drugs tested significantly decreased the concentration of MIP-1α, certain trends were observable. 10 week old diseased mice expressed an average concentration of 437.8pg/mL and 28 week old diseased mice expressed an average concentration of 503.2pg/mL. Genistein® decreased concentrations of MIP-1α in 10 week and 28 week old diseased mice to 382.90pg/mL and 427.3pg/mL, respectively. The combination treatment of Genistein® and Minozac shows a similar decrease to that described in FGF-2. These attenuations are not statistically significant.

2-way ANOVA demonstrated that Fosteum® increased the concentration of this proinflammatory cytokine significantly in 10 week old heterozygous mice ($p<0.0001$), 10 week old mutant mice ($p<0.01$) and 28 week old heterozygous mice ($p<0.05$).

Other Cytokines Tested

The other four cytokines that were tested, IL-1α, IL-6, TNFα, and VEGF (Figures 3-2, 3-3, 3-4, 3-5, 3-8, 3-9, 3-12, and 3-13) did not yield significant results with respect

to the attenuation of proinflammatory cytokines in the brain homogenates of diseased mice, across treatment groups. In fact, all of these cytokines, showed (in either the 10 week old group or the 28 week old group) a higher cytokine baseline in the unaffected controls over the diseased MPSIIIB mouse.

However, weak trends can be observed. Genistein® consistently decreased cytokine concentrations across most cohorts. Fosteum® intensified the expression of all six of the surveyed cytokines. Minozac did not seem to alter cytokine expression in any discernible pattern. And, the combination treatment of Genistein® and Minozac yielded cytokine levels that were either similar to the expression seen with the Genistein® treatment alone, or worse.

Secondary Quantification of Cytokines

Brain cytokine levels in four randomly selected 28 week old unaffected and four randomly selected 28 week old MPSIIIB diseased mice from the previous experiment were measured via a membrane array (Table 3-9), in an effort to confirm trends seen with the bead assay. The fluorescent membrane array was imaged on x-ray film (Figure 3-14), and the relative amount of each cytokine (grayness) was measured via densitometry software. Values were normalized by positive control for each membrane, and background was removed.

Figure 3-15 shows the graphed values of relative grayness. Significance was determined by T-test. Three of the twenty-four cytokines tested reached significance ($p<0.05$): GCSF, GM-CSF, and IL-12 p70. Several other cytokines nearly achieved significance: FAS1, FGFb, IFNγ, IGF II, IL-12 p40/p70, IL-1α, IL-1β, IL-9, MCSF, Thrombopoeitin, and TIMP-2.

Secondary Enzyme Fluorescence Assay

All brain homogenate samples from the primary experiment were measured for beta-glucuronidase upregulation. beta-glucuronidase is an enzyme that has been previously described as upregulated in MSPIIIB mice. To do this, a fluorescent substrate cleavage assay was performed and a spectrophotometer was used to measure fluorescence.

Figures 3-16 and 3-17 show the average beta-glucuronidase levels in diseased and unaffected MPSIIIB mice at 10 week and 28 week old mice, respectively, across four treatment types. Untreated diseased mice from the 10 week cohort showed a significant difference in beta-glucuronidase level as compared to its matched control ($p<0.05$). Further, untreated diseased mice from the 28 week cohort showed a significant difference in beta-glucuronidase level as compared to its matched control ($p<0.0001$).

Across all treatments in both the 10 week and 28 week old MPSIIIB diseased mice, no statistically significant difference in beta-glucuronidase levels was seen. However, of the four treatments, Minozac seems to show a trend toward the reduction of secondary enzyme upregulation.

Additional Minozac Application Study

Due to the unexpected results from the 1-month primary experiment, an acute study with Minozac was undertaken to determine if the original application mode (SQ injection * 28 days of treatment) was the cause of incongruous results. To do this, unaffected and diseased MPSIIIB mice were treated acutely via several drug-application modalities: gavage (PO), subcutaneous injection (SQ), intravenous injection (IV), or

intraperitoneal (IP). Brain FGFb and MCP-1 levels were measured via ELISA. ELISA values were fit to their respective standard curves (Figures 3-18 and 3-20).

Monocyte Chemotactic Protein 1α (MCP1)

Figure 3-19 shows the adjusted MCP1 values across four treatment modalities. Significance was measured via 1-way ANOVA. Surprisingly, controls of MPSIIIB diseased mice showed significantly ($p<0.01$) less MCP1 than their unaffected counterparts. For the diseased animals, no treatment mode showed a significant difference from another, however, the SQ treatment seems to trend more downward than the control. Additionally, IV treated unaffected mice did reach significance ($p<0.05$) against their matched control.

Basic Fibroblast Growth Factor (FGFb)

Figure 3-21 shows the adjusted FGFb values across four treatment modalities. Significance was measured via 1-way ANOVA. Significance was reached between the MPSIIIB diseased and unaffected controls ($p<0.001$), but it was not reached among any of the Minozac treatment modalities. However, like MCP1, there seems to be a downward trend in the MPSIIIB diseased mice that were treated SQ, against their matched control.

Table 3-1. Myriad Rules Based Medicine® brain cytokine results reported in µg/mL. Average amount of different cytokines found in MPSIIIB mut or het brains in µg/mL. A T-test was used to determine significance. (*) denotes a statistically significant difference within the age group (ie. Mut vs. Het), while (°) denotes a statistically significant difference over time (ie. Old vs. New). * p<0.5, ** p<0.01, ° p<0.5, °° p<0.01

	New Mice		Old Mice	
Analyte	Het (n = 3)	Mut (n = 3)	Het (n = 3)	Mut (n = 3)
Apo A-I	0.0673	0.0661	0.101	0.189
C-Reactive Protein	0.0119	0.0153	0.0205	0.025 °°
Fibrinogen	28.9	38.0	37.7	55.1
Haptoglobin	0.388	0.403	0.399	0.613
IgA	0.196	0.229	0.167	0.423 *°
Serum Amyloid P-Component	0.0516	0.0564	0.0699	0.120 *°
SGOT	39.6	46.3	35.8	41.7

Table 3-2. Myriad Rules Based Medicine® brain cytokine results reported in ng/mL. Average amount of different cytokines found in MPSIIIB mut or het brains in ng/mL. A T-test was used to determine significance. (*) denotes a statistically significant difference within the age group (ie. Mut vs. Het), while (°) denotes a statistically significant difference over time (ie. Old vs. New). * p<0.5, ** p<0.01, ° p<0.5, °° p<0.01

	New Mice		Old Mice	
Analyte	Het (n = 3)	Mut (n = 3)	Het (n = 3)	Mut (n = 3)
Factor VII	2.25	2.51	1.78	4.89 *
FGF-9	2.55	2.96	2.55	3.56 *
FGF-basic	22.5	67.2	5.30	215 **°
GCP-2	0.0593	0.0710	0.0716	0.132
IL-12p70	0.0167	0.0223	0.0249	0.0338
IL-18	2.35	3.29	1.84	3.98
IL-7	0.0100	0.0113	0.0117	0.0196
M-CSF-1	0.216	0.251	0.276	0.414 *°
MIP-1 γ	0.245	0.214	0.179	0.354 **°
MIP-3 β	0.263	0.305	0.366	0.520
MMP-9	2.64	3.01	2.22	6.38
MPO	2.14	2.30	1.85	5.90
Myoglobin	13.7	16.1	27.2	16.5
Oncostatin-M	0.0245	0.0275	0.0231	0.0553 *°
Tissue Factor	0.427	0.770	0.683	1.58
TIMP-1	0.0337	0.0254	0.0196	0.130 *°
VCAM-1	4.03	4.31	3.67	4.56
vWF	1.23	1.55	1.73	1.68

Table 3-3. Myriad Rules Based Medicine® brain cytokine results reported in pg/mL. Average amount of different cytokines found in MPSIIIB mut or het brains in pg/mL. A T-test was used to determine significance. (*) denotes a statistically significant difference within the age group (ie. Mut vs. Het), while (°) denotes a statistically significant difference over time (ie. Old vs. New). * p<0.5, ** p<0.01, ° p<0.5, °° p<0.01

	New Mice		Old Mice	
Analyte	Het (n = 3)	Mut (n = 3)	Het (n = 3)	Mut (n = 3)
CD40	3.97	5.13	5.42	8.12
CD40 Ligand	338	282	361	492
Eotaxin	2.38	3.17	2.83	4.79 **
IFN-γ	2.28	2.51	2.50	3.50
IP-10	8.75	10.7	11.6	34.5 **°°
IL-1 α	49.4	65.0	52.4	120 **°
IL-10	42.5	55.1	40.7	78.7 *
IL-4	7.95	8.52	8.52	13.0
IL-2	3.99	4.23	2.18	6.34
LIF	66.0	63.7	66.0	144
Lymphotactin	6.56	7.83	7.51	15.7 *°
MIP-1 β	13.0	16.9	15.8	90.0 **°°
MIP-2	1.34	1.74	1.67	2.76 **°°
MDC	22.5	27.6	28.0	53.2 *°
MCP-1	3.96	6.44	3.55	20.3 **°°
MCP-3	9.86	13.4	10.0	31.0 **°°
MCP-5	1.90	2.55	2.32	9.58 **°
Stem Cell Factor	293	332	258	334
VEGF-A	156	224	137	392 **

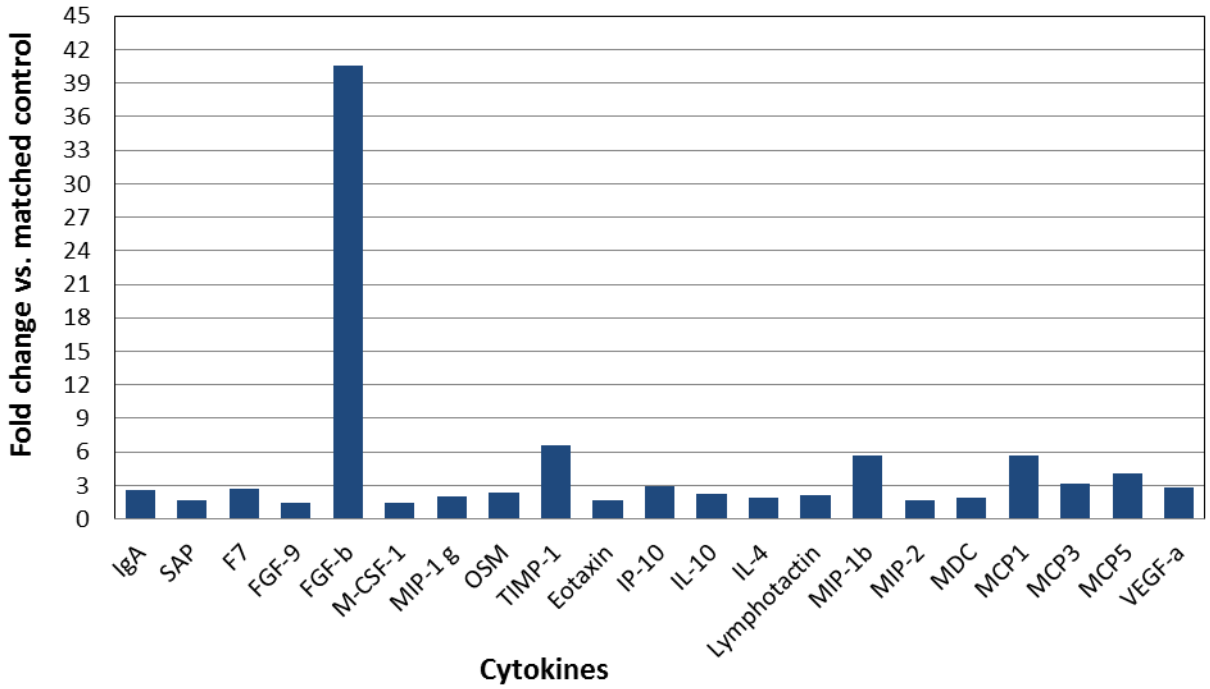

Figure 3-1. Fold increase of 24 week old diseased mice brain homogenate analytes in comparison to unaffected heterozygote brain homogenate analytes. Averages were taken for 24 week old unaffected and diseased mice analytes. The diseased mouse averages were then divided by the unaffected average in order to calculate the fold increase from normal, and plotted on the above graph. All fold increases were significant ($p<0.05$). Oncostatin-M was left off of the graph due to a lack of confidence in the reported values.

Table 3-4. Myriad Rules Based Medicine® blood cytokine results reported in µg/mL. Average amount of different cytokines found in MPSIIIB mut or het blood in µg/mL. A T-test was used to determine significance. (*) denotes a statistically significant difference within the age group (ie. Mut vs. Het), while (°) denotes a statistically significant difference over time (ie. Old vs. New). * p<0.5, ** p<0.01, ° p<0.5, °° p<0.01

	Young Mice		Old Mice	
Analyte	Het (n = 3)	Mut (n = 3)	Het (n = 3)	Mut (n = 3)
Apo A-I	36.8	37.1	37.1	35.5
C-Reactive Protein	5.35	5.95	4.73	6.92 *
Fibrinogen	37400	32700	33400	47000
Haptoglobin	25	27	29	48
IgA	35.6	28.8	45.1	40.3
Serum Amyloid P-Component	19.7	19.7	18.1	27.1
SGOT	161	175	141	163

Table 3-5. Myriad Rules Based Medicine® blood cytokine results reported in ng/mL. The average amount of different cytokines found in MPSIIIB mut or het blood in ng/mL. A T-test was used to determine significance. (*) denotes a statistically significant difference within the age group (ie. Mut vs. Het), while (°) denotes a statistically significant difference over time (ie. Old vs. New). * p<0.5, ** p<0.01, ° p<0.5, °° p<0.01

	Young Mice		Old Mice	
Analyte	Het (n = 3)	Mut (n = 3)	Het (n = 3)	Mut (n = 3)
Factor VII	34.8	33.5	30.7	36.0
GCP-2	10.1	12.5	12.1	17.0
KC/GRO	0.0223	0.0285	0.0623	0.0558
IL-1 β	2.85	2.39	1.31	1.86
IL-18	10.3	11.4	10.9	10.8
M-CSF-1	6.43	6.96	6.19	6.86
MIP-1 α	5.00	5.02	3.61	5.12 *
MIP-1 ɣ	20.9	20.8	14.5	24.7 *
MIP-3 β	2.31	2.59	2.11	2.57
MMP-9	67.6	70.6	70.9	107
MPO	52.5	54.8	56.9	54.6
Myoglobin	627	3300	3760	1890
TPO	33.1	32.2	24.5	32.0
Tissue Factor	7.28	7.28	7.54	8.77
TIMP-1	0.986	0.993	0.874	1.10
VCAM-1	1290	1290	1080	1820 *°
vWF	100	80.4	95.1	113 °

Table 3-6. Myriad Rules Based Medicine® blood cytokine results reported in pg/mL. The average amount of different cytokines found in MPSIIIB mut or het blood in pg/mL. A T-test was used to determine significance. (*) denotes a statistically significant difference within the age group (ie. Mut vs. Het), while (°) denotes a statistically significant difference over time (ie. Old vs. New). * $p<0.5$, ** $p<0.01$, ° $p<0.5$, °° $p<0.01$

	Young Mice		Old Mice	
Analyte	Het (n = 3)	Mut (n = 3)	Het (n = 3)	Mut (n = 3)
CD40	55.9	48.9	36.4	53.4
CD40 Ligand	1390	1400	1990	1670
Endothelin-1	27.8	27.1	33.8	28.0
Eotaxin	463	504	776	658
IP-10	60.4	63.5	55.4	83.2
IL-1 α	276	230	401	367
LIF	943	1140	1050	1110
Lymphotactin	111	96.8	131	166
MIP-1 β	104	140	100	251 *
MIP-2	8.02	7.97	15.5	9.39
MDC	481	549	574	544
MCP-1	61.8	85.7	51.0	86.4
MCP-3	123	196	78.4	127
MCP-5	19.1	23.5	15.5	23.1
Stem Cell Factor	567	637	634	666
T-Cell-Specific Protein RANTES	0.0218	0.0219	0.0244	0.0375
VEGF-A	554	222	7730	291

Table 3-7. Cohort setup. The experimental setup for the brain cytokine analysis across two time points and five treatments in MPSIIIB mice. “New” mice were treated from 6 weeks to 10 weeks of age; “Old” mice were treated from 24 weeks to 28 weeks of age. Each treatment cohort consisted of 22 mice at a given age and Tx type; 11 mice were mutant and 11 mice were heterozygote in each cohort. A total of 220 mice were used.

Tx Code	"Old"	"New"
Diseased (Mut) Untreated (MU)	11	11
Control (Het) Untreated (HU)	11	11
Diseased Genistein® (MG)	11	11
Control Genistein® (HG)	11	11
Diseased Fosteum® (MF)	11	11
Control Fosteum® (HF)	11	11
Diseased Minozac (MM)	11	11
Control Minozac (HM)	11	11
Diseased Combination G+M (MGM)	11	11
Control Combination G+M (HGM)	11	11

Table 3-8. Values used to determine cytokine concentration. Values fit to a standard curve and used to determine cytokine concentration for bead assay (Figures 3-2 through 3-13). Observed concentrations (pg/mL) were matched to Fluorescent intensity. Expected values, given by the assay manufacturer, are noted for comparison.

	Fluorescent Intensity	Exp concentration (pg/mL)	Obs Concentration (pg/mL)
IL-1α	21511	19306	OOR >
	16436.8	4826.5	4611.69
	3211.5	1206.63	1247
	174.8	301.66	283.29
	35	75.41	104
	18	18.85	39.14
	15.5	4.71	2.8
IL-6	15037	11410	11474.98
	6240.5	2852.5	2830.77
	1074	713.13	730.48
	132	178.28	169.08
	43	44.57	53.7
	29.3	11.14	18.43
MIP-1α	17775	39916	40556.12
	15322.8	9979	9970.06
	2689	2494.75	2495.1
	126	623.69	622.94
	75.5	155.92	237.55
TNFα	14329.5	47111	47145.99
	3081.5	11777.75	11754.67
	290.5	2944.44	2954.81
	23	736.11	727.29
	13	184.03	338.11
FGFb	3899	18585.75	18585.31
	245.5	4646.44	4648.12
	35	1161.61	1148.35
	28.5	290.4	641.43
VEGF	5182.5	29861	30070.64
	1703.3	7465.25	7383.6
	275.5	1866.31	1883.62
	33.5	466.58	457.44
	24	116.64	247.03

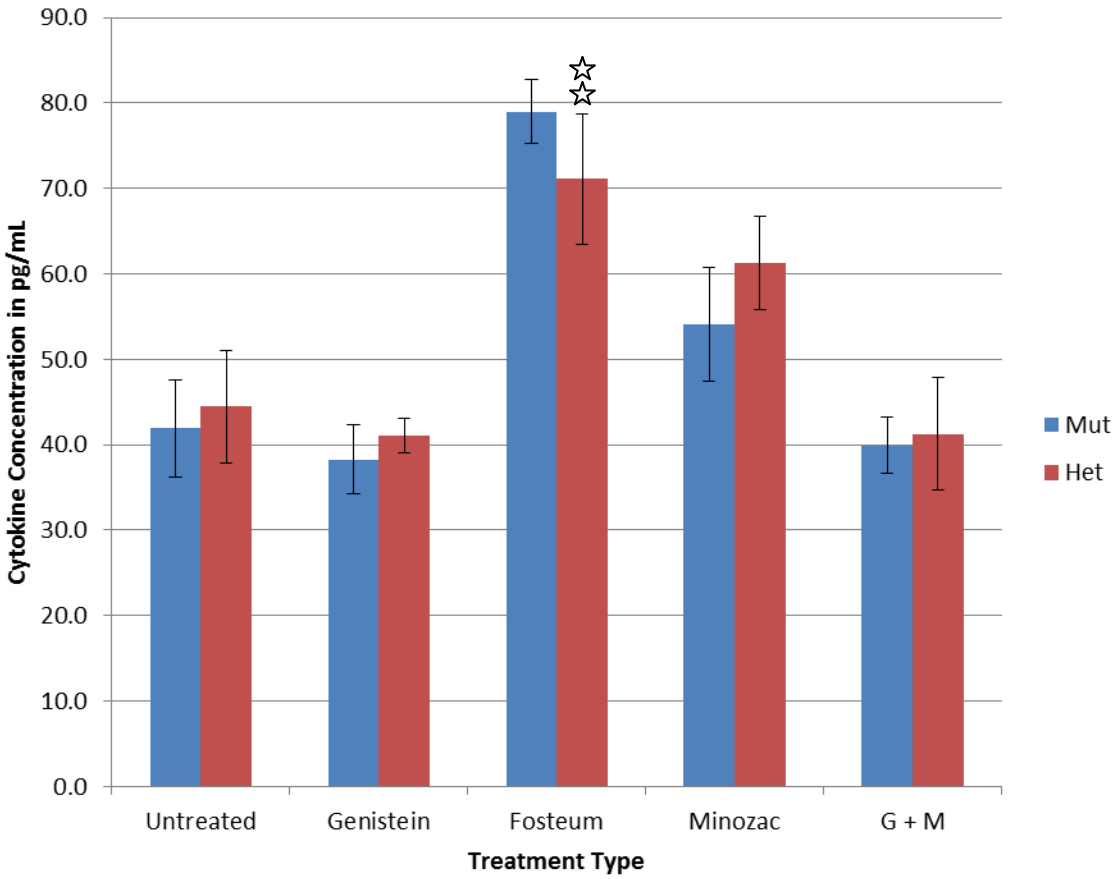

Figure 3-2. Brain IL-1a concentrations across four treatment types in 10 week old MPSIIIB mice. IL-1a concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

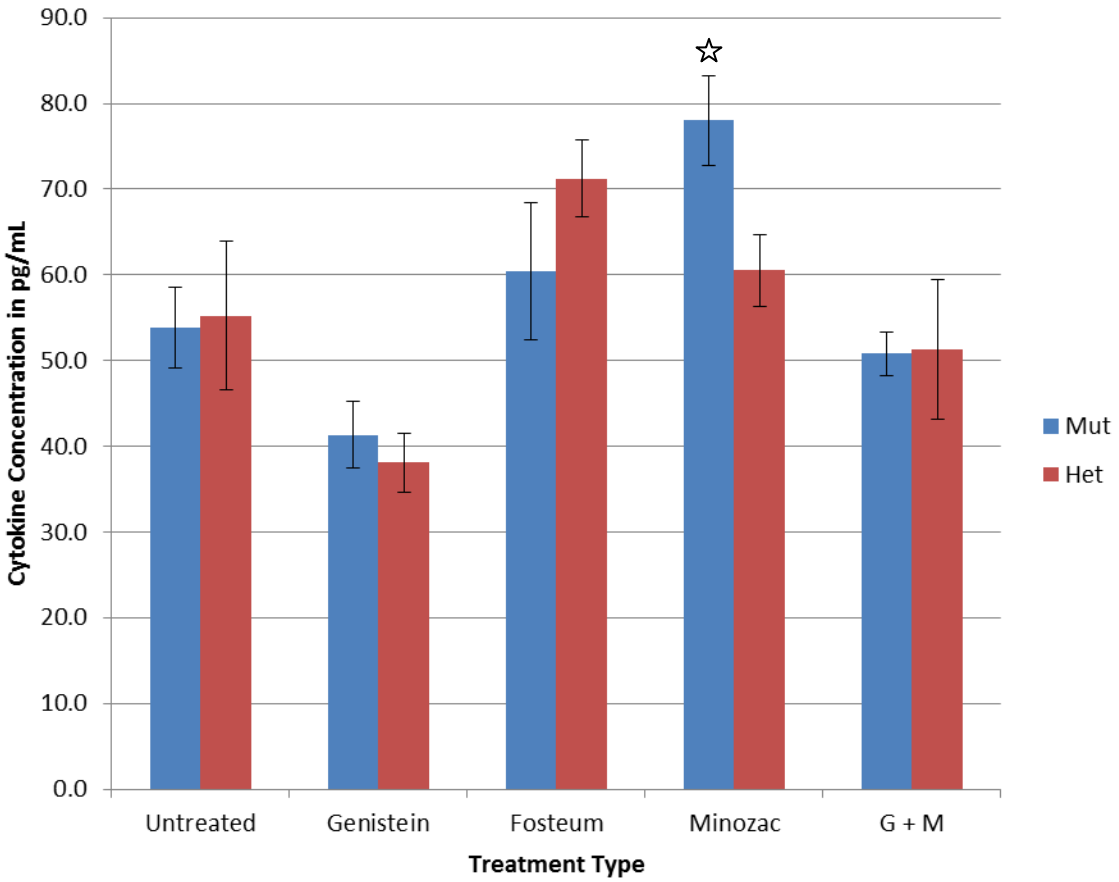

Figure 3-3. Brain IL-1a concentrations across four treatment types in 28 week old MPSIIIB mice. IL-1a concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA.* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

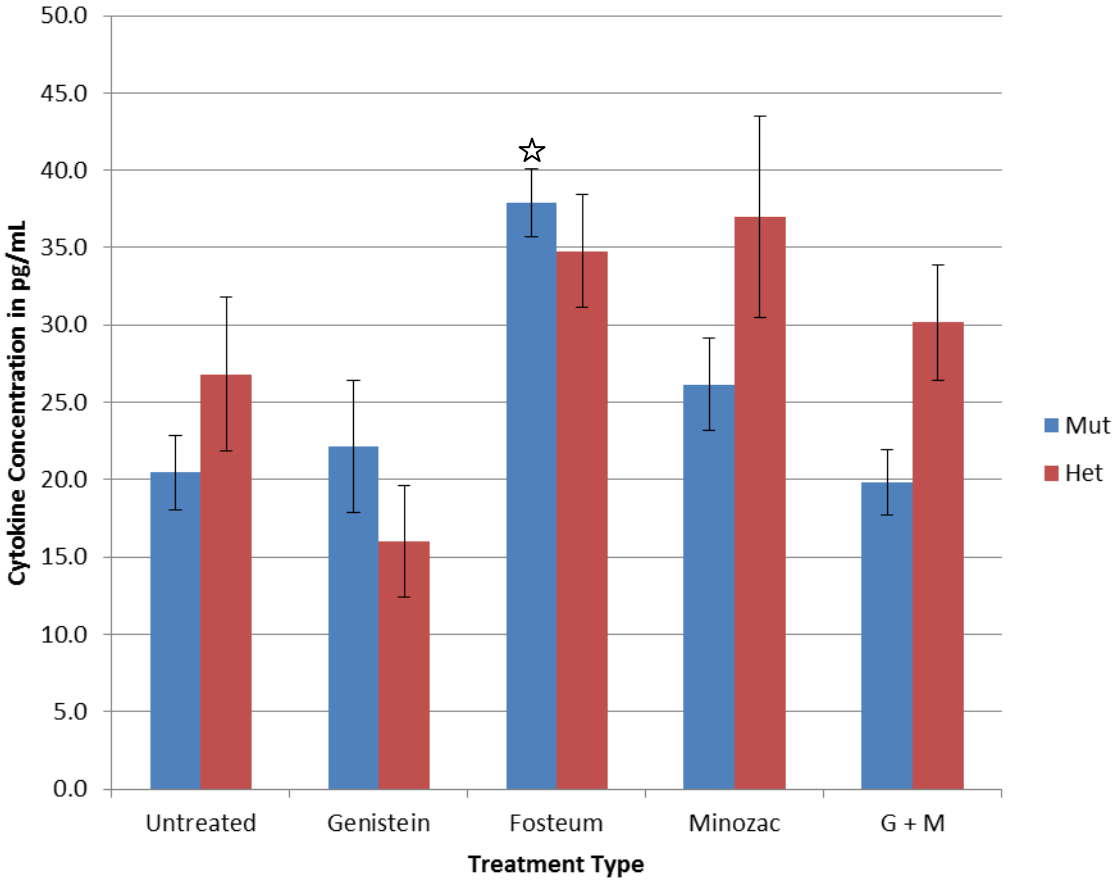

Figure 3-4. Brain IL-6 concentrations across four treatment types in 10 week old MPSIIIB mice. IL-6 concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA.* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

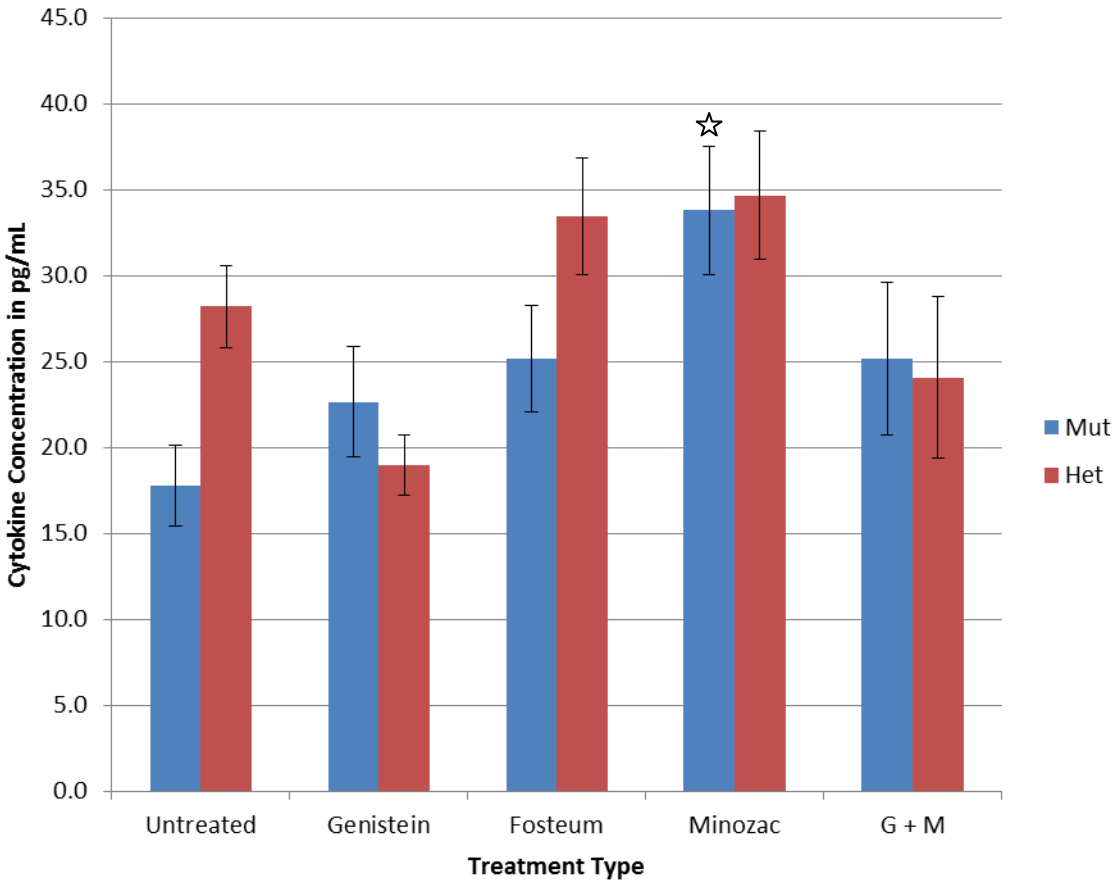

Figure 3-5. Brain IL-6 concentrations across four treatment types in 28 week old MPSIIIB mice. IL-6 concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA.* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

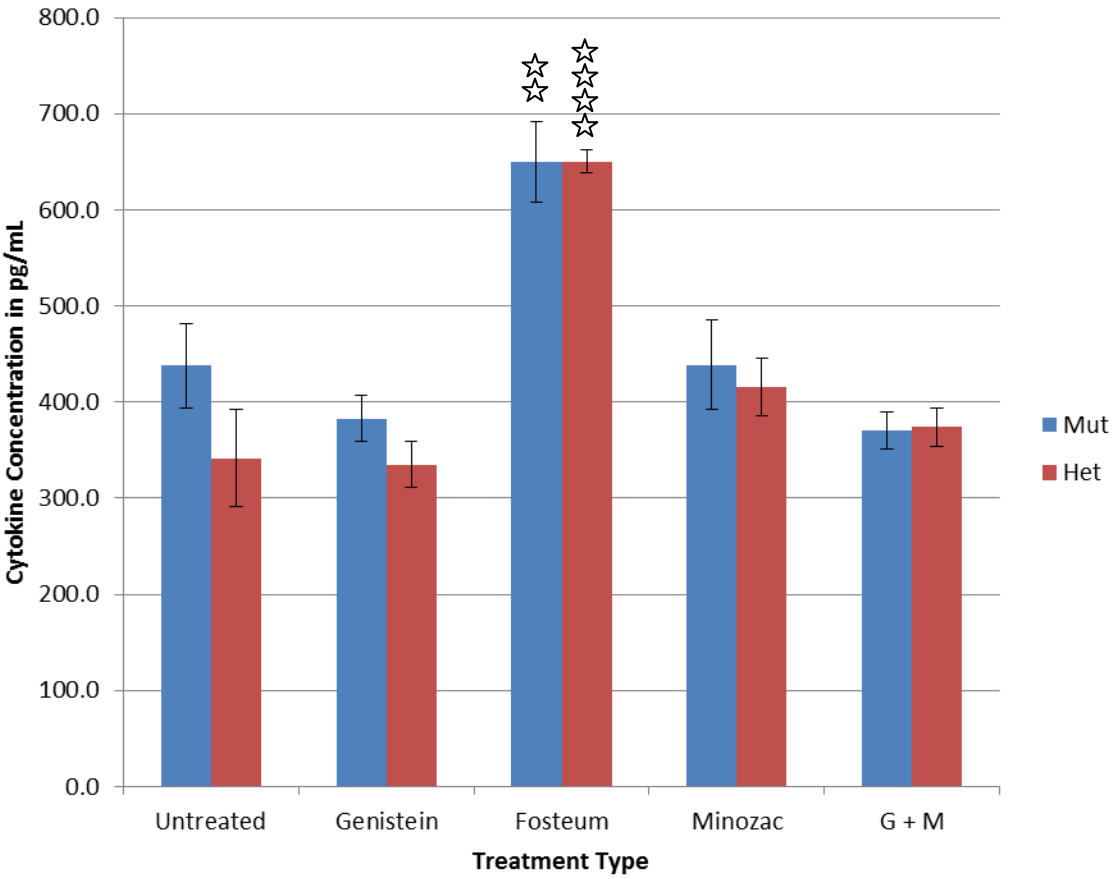

Figure 3-6. Brain MIP-1a concentrations across four treatment types in 10 week old MPSIIIB mice. MIP-1a concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA.* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

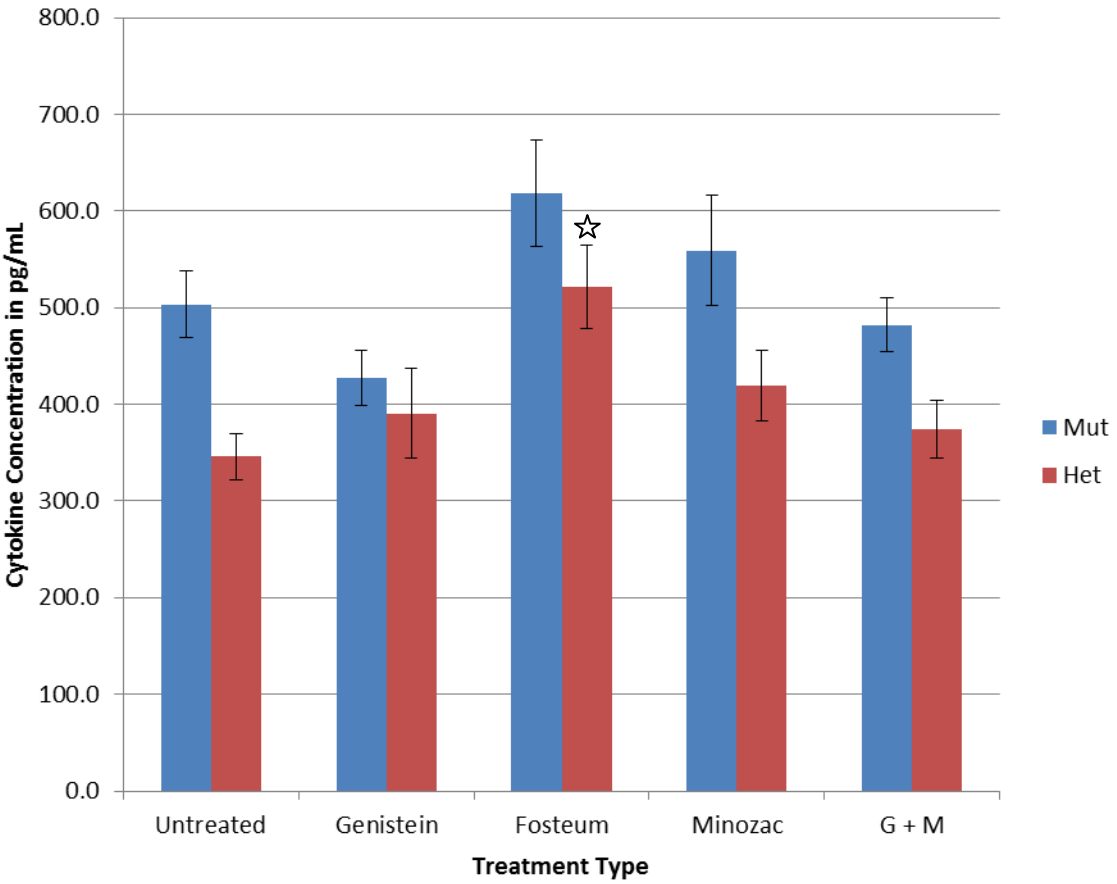

Figure 3-7. Brain MIP-1a concentrations across four treatment types in 28 week old MPSIIIB mice. MIP-1a concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

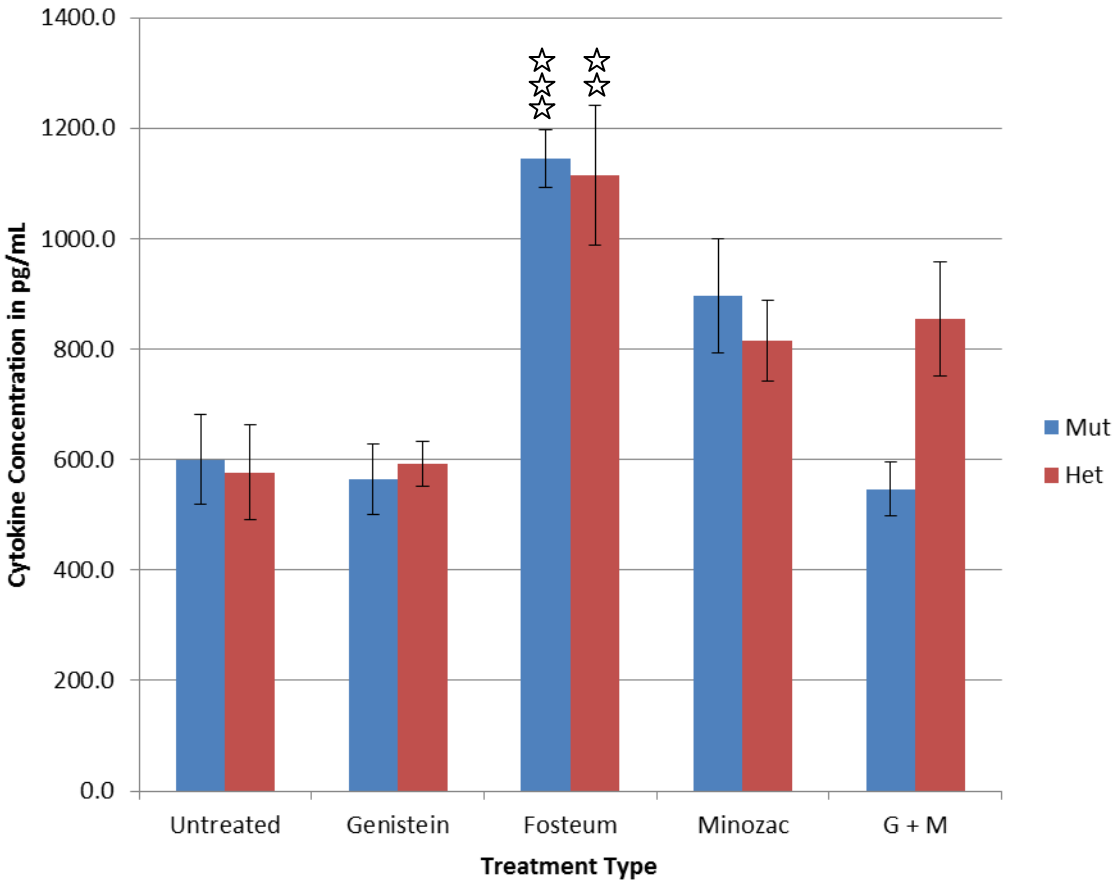

Figure 3-8. Brain TNFα concentrations across four treatment types in 10 week old MPSIIIB mice. TNFα concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

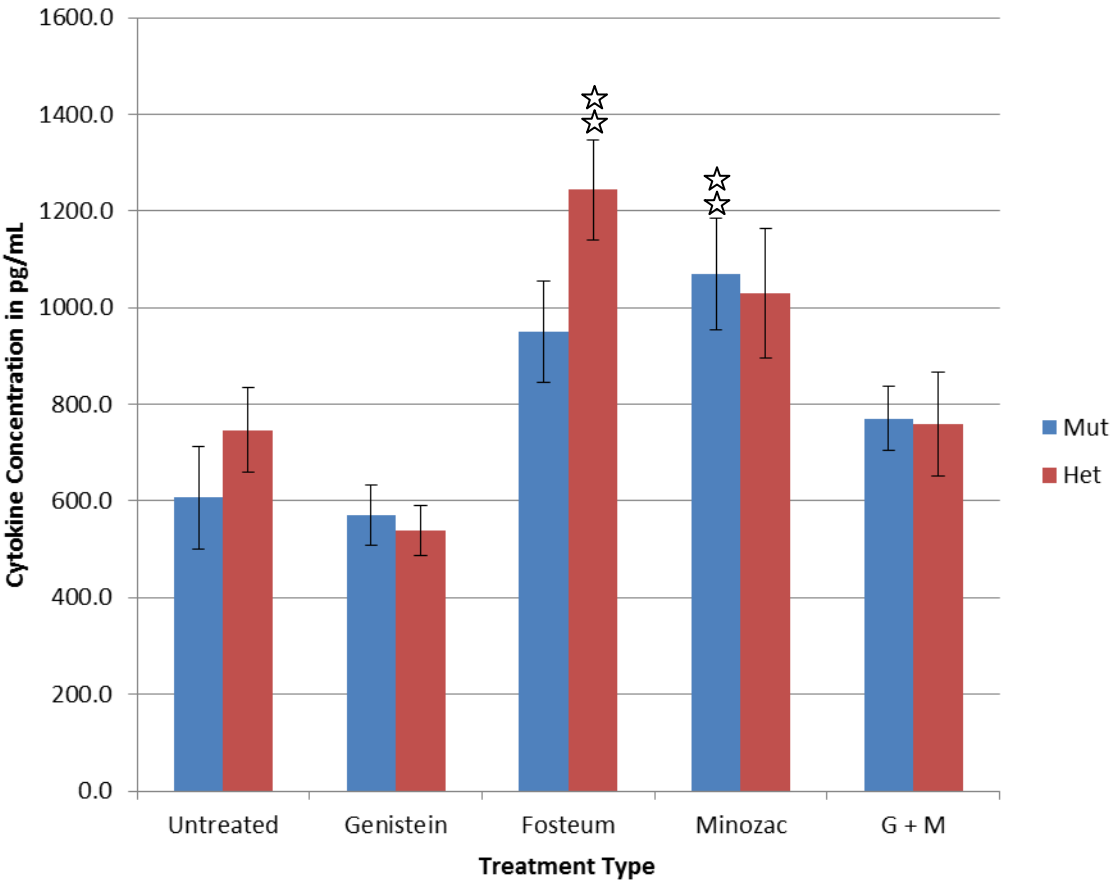

Figure 3-9. Brain TNFα concentrations across four treatment types in 28 week old MPSIIIB mice. TNFα concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA.* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

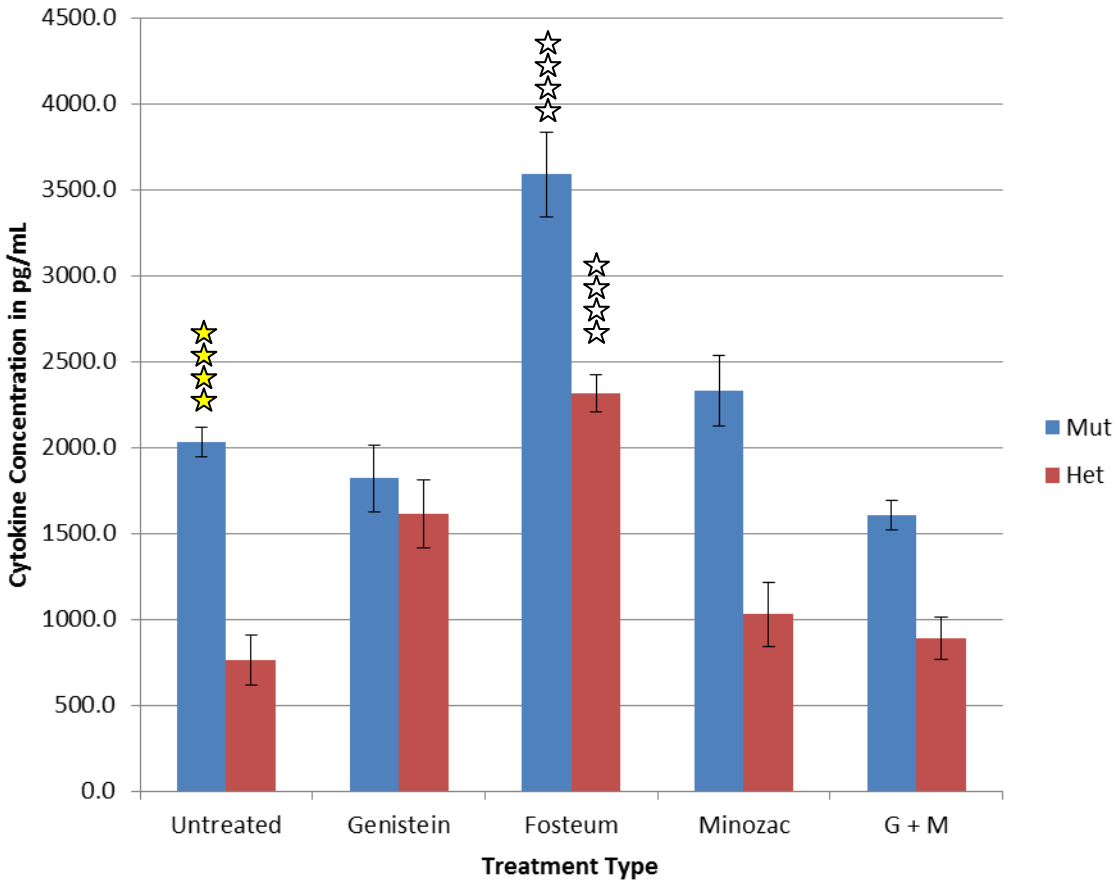

Figure 3-10. Brain FGFb concentrations across four treatment types in 10 week old MPSIIIB mice. FGFb concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA. Yellow * indicates significance, as determined by 1-way ANOVA, in Mut v Het controls for the 10 week old time point only. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

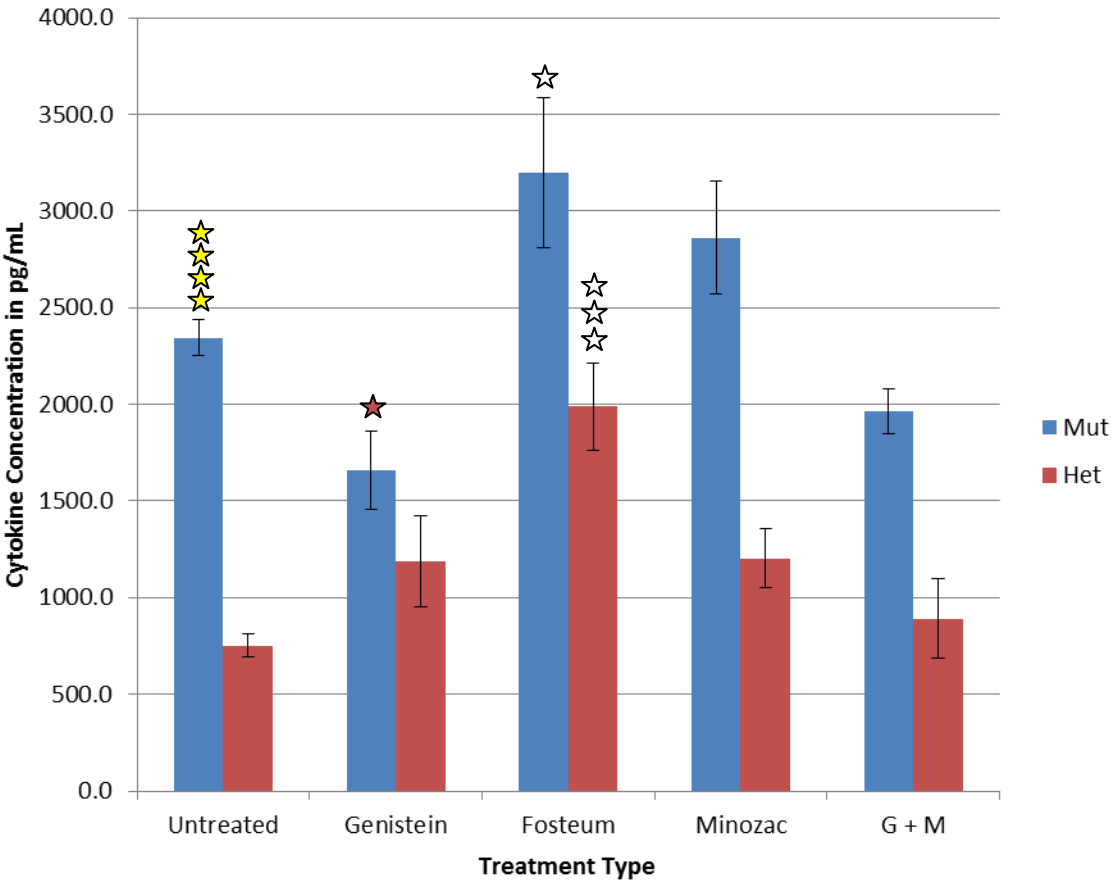

Figure 3-11. Brain FGFb concentrations across four treatment types in 28 week old MPSIIIB mice. FGFb concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls, All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA. Yellow * indicates significance, as determined by 1-way ANOVA, in Mut v Het controls for the 28 week old time point only. Red * indicates significance as determined by 1-way ANOVA for the Genistein® treatment only against matched controls, for only the 28 week old time point. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

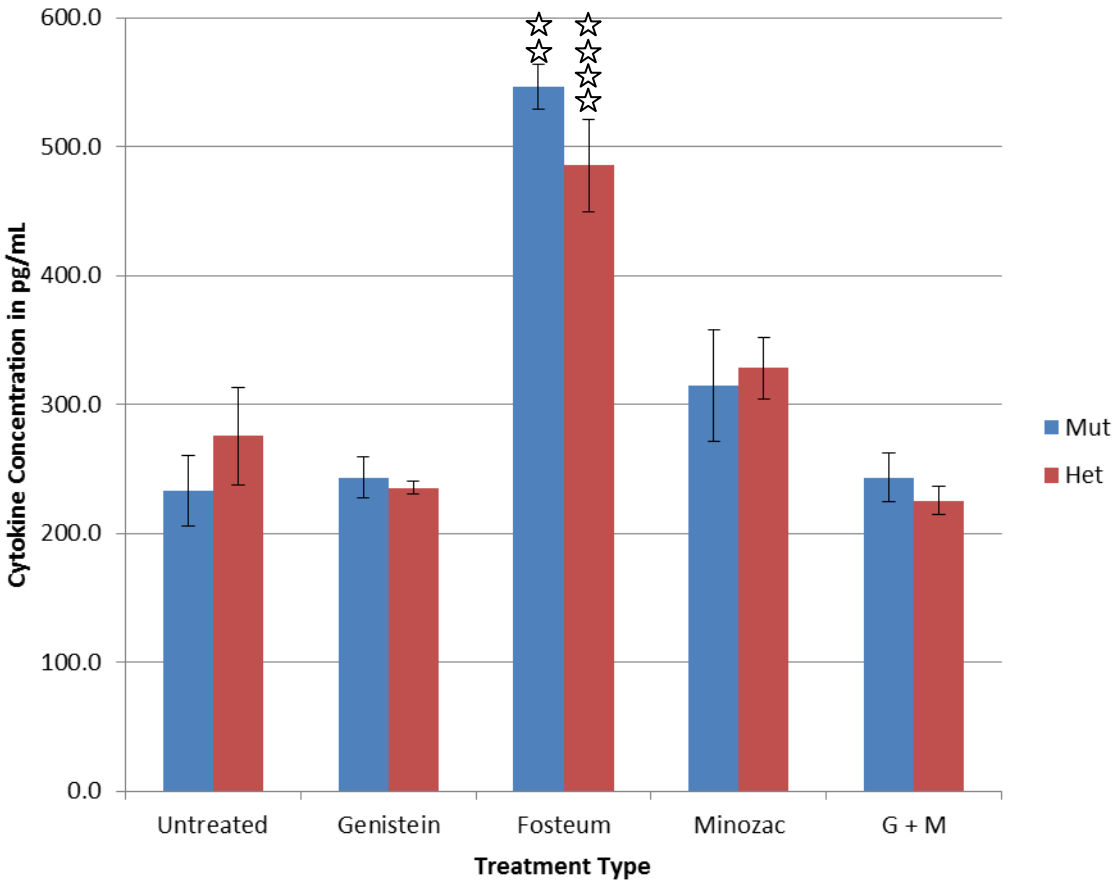

Figure 3-12. Brain VEGF concentrations across four treatment types in 10 week old MPSIIIB mice. VEGF concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA.* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

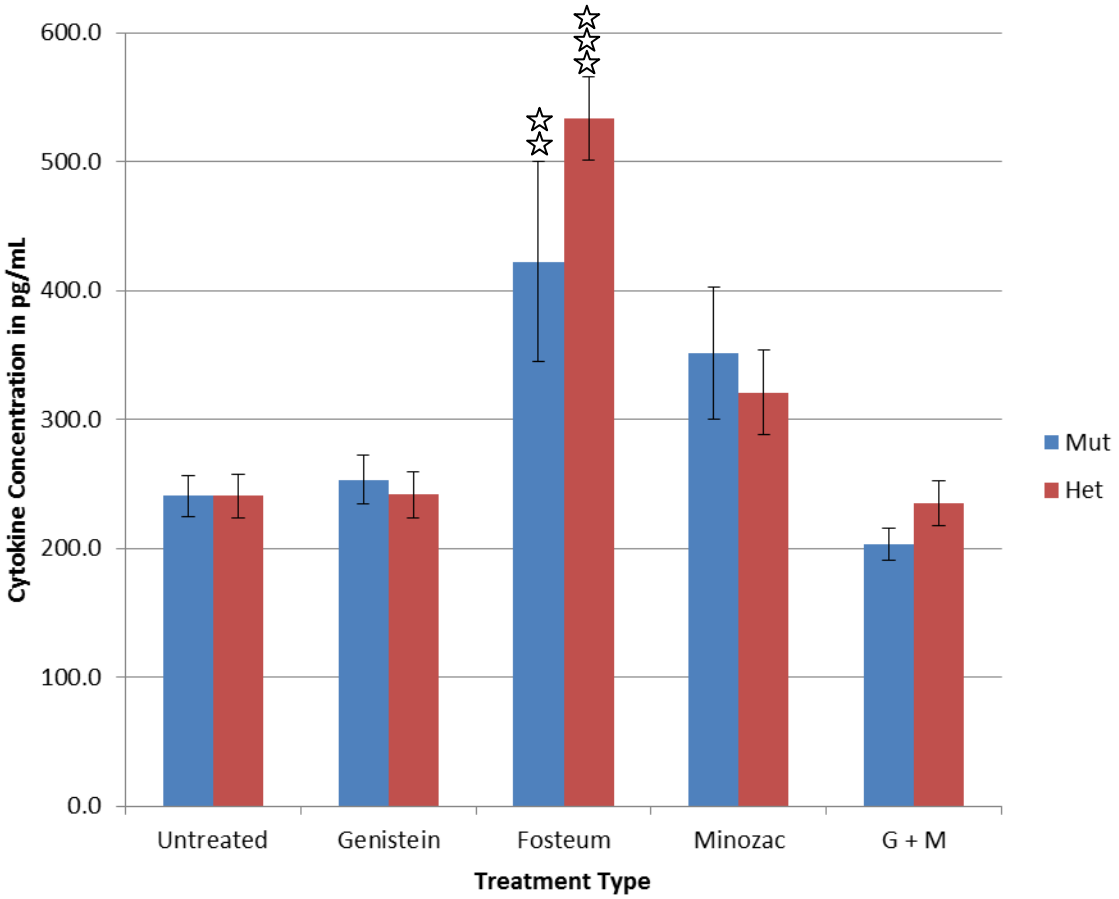

Figure 3-13. Brain VEGF concentrations across four treatment types in 28 week old MPSIIIB mice. VEGF concentrations for het and mut MPSIIIB mice in four different treatment groups: Genistein®, Fosteum®, Minozac, and combination Genistein®+Minozac. (*) indicate significance as determined by 2-way ANOVA against matched controls. All cohorts (10 week old mice and 28 week old mice) were considered in the 2-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Table 3-9. Membrane array map. Provided by Ray Biotech® as a part of the membrane assay kit. The position of each antibody blot on the membrane array is displayed. All cytokine blots were run in duplicate.

	A	B	C	D	E	F	G	H
1	Pos	Pos	Neg	Neg	Blank	Eotaxin	Fas Ligand	bFGF
2	Pos	Pos	Neg	Neg	Blank	Eotaxin	Fas Ligand	bFGF
3	G-CSF	GM-CSF	IFNg	IGF-II	IL-1a	IL-1b	IL-12 p40/p70	IL-12 p70
4	G-CSF	GM-CSF	IFNg	IGF-II	IL-1a	IL-1b	IL-12 p40/p70	IL-12 p70
5	IL-13	IL-6	IL-9	Leptin	MCP1	M-CSF	MIG	PF-4
6	IL-13	IL-6	IL-9	Leptin	MCP1	M-CSF	MIG	PF-4
7	TIMP-1	TIMP-2	TNFα	Thrombo	VEGF	Blank	Blank	Pos
8	TIMP-1	TIMP-2	TNFα	Thrombo	VEGF	Blank	Blank	Pos

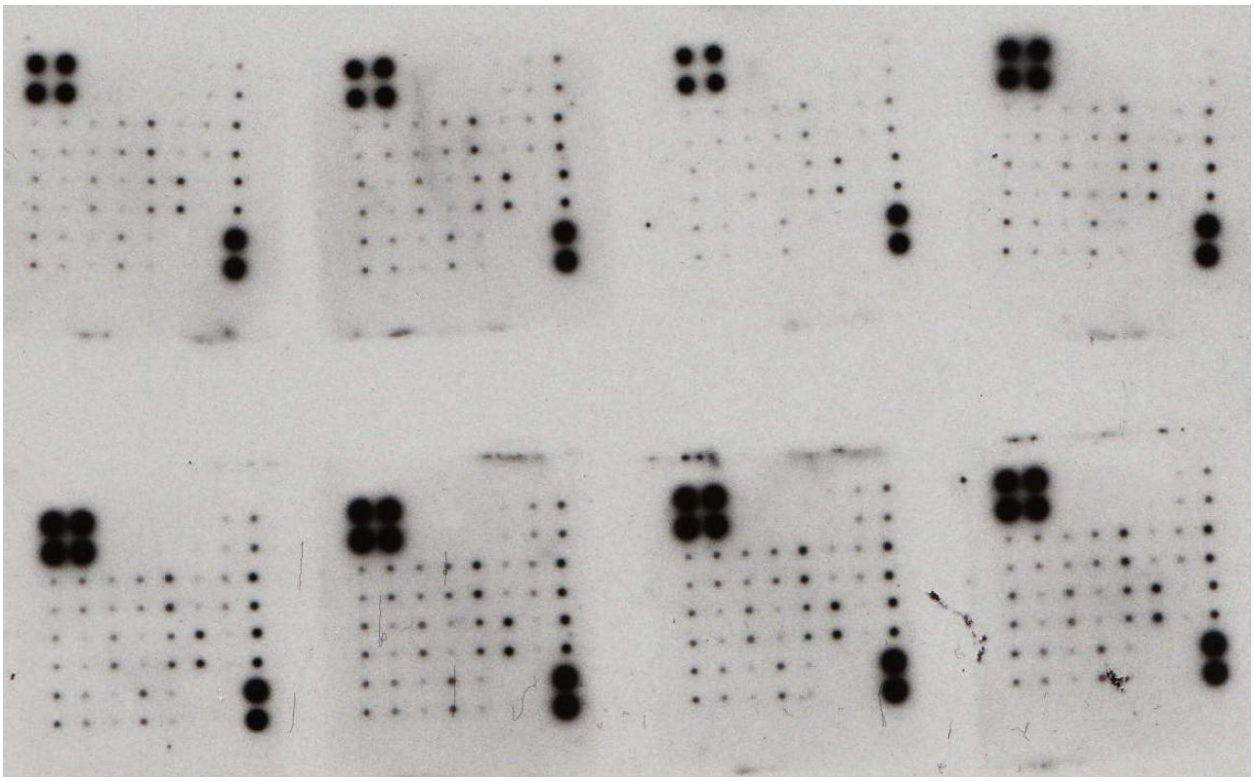

Figure 3-14. X-rayed image of membrane assay. 8 total cytokine detection membrane assays were completed. The top row represents four, randomly selected, 28 week old control untreated brain homogenates; the bottom row represents four, randomly selected, diseased untreated brain homogenates. These homogenate samples are from Table 2.

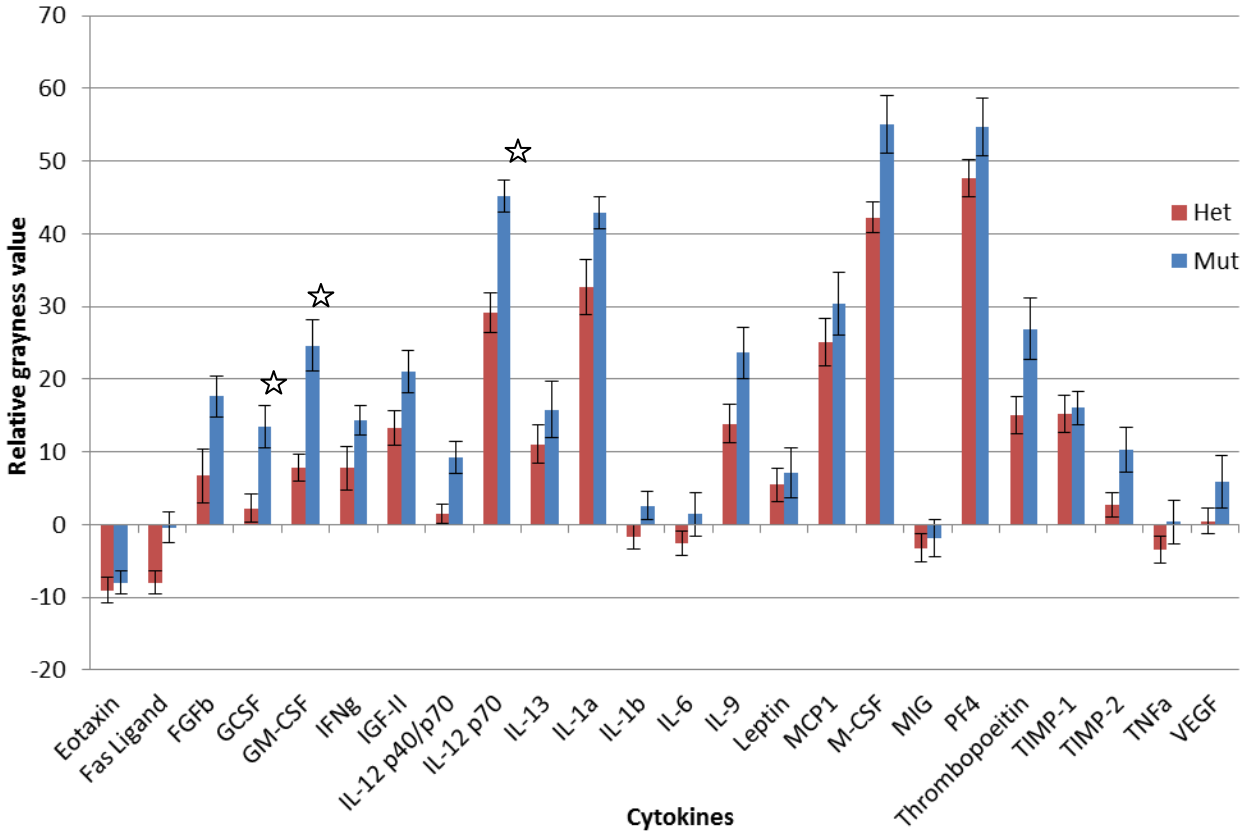

Figure 3-15. Paired differences in cytokine expression. Individual blots from the membranes in Fig. 3a were measured for density. These duplicated measurements were normalized by positive control relative density. Negative blots were subtracted as background; values below background are negative as shown for Eotaxin, Fas Ligand, etc. The average cytokine expression across the all four membranes in each group (het or mut) was determined. These averages were plotted, and T-tests were run on each group (Het v Mut) to determine significance. (*) denotes significance ($p<0.05$)

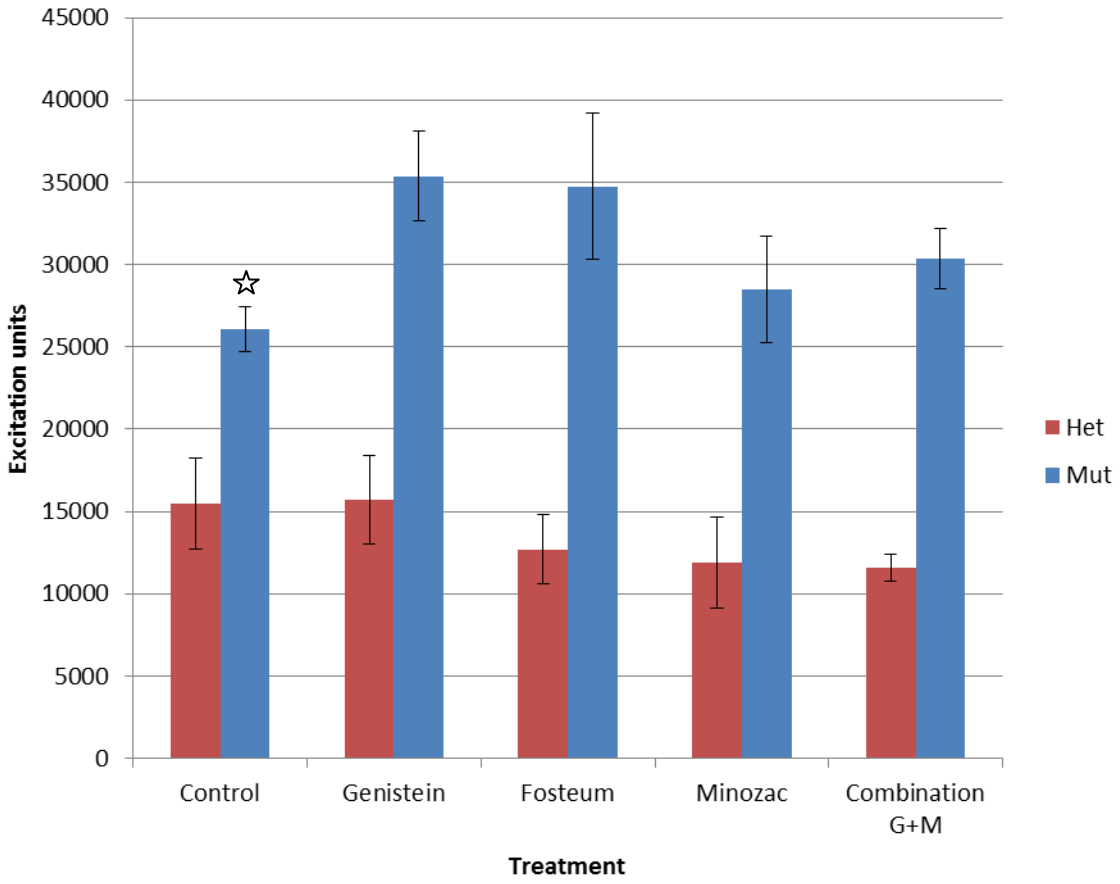

Figure 3-16. Secondary enzyme fluorescent assay of 10 week old MPSIIIB mice. Levels of β-glucuronidase, as determined by a substrate based fluorescence assay, in MPSIIIB 10 week old control and diseased mice across four treatments. Significance was reached using a 1-way ANOVA for controls. No significance was found using a 2-way ANOVA across groups vs. untreated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

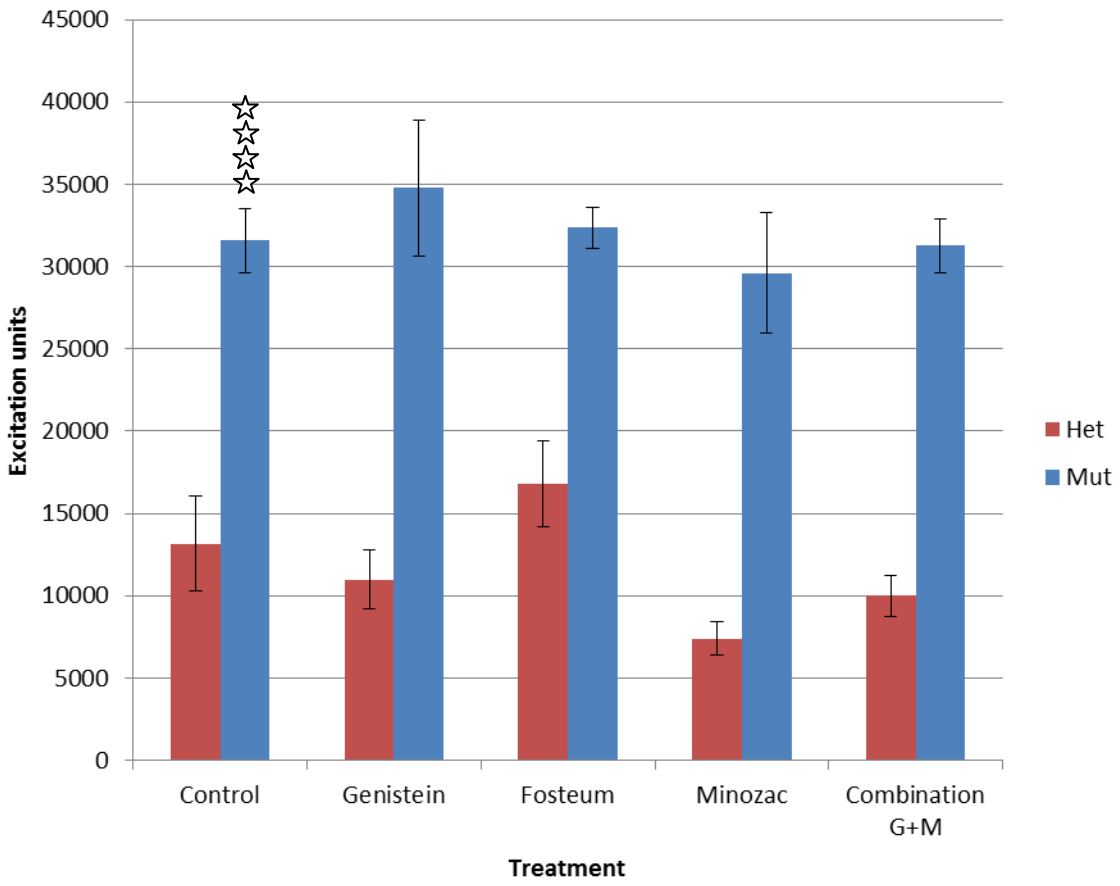

Figure 3-17. Secondary enzyme fluorescent assay of 28 week old MPSIIIB mice. Levels of β-glucuronidase, as determined by a substrate based fluorescence assay, in MPSIIIB 28 week old control and diseased mice across four treatments. Significance was reached using a 1-way ANOVA for controls. No significance was found using a 2-way ANOVA across groups vs. untreated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

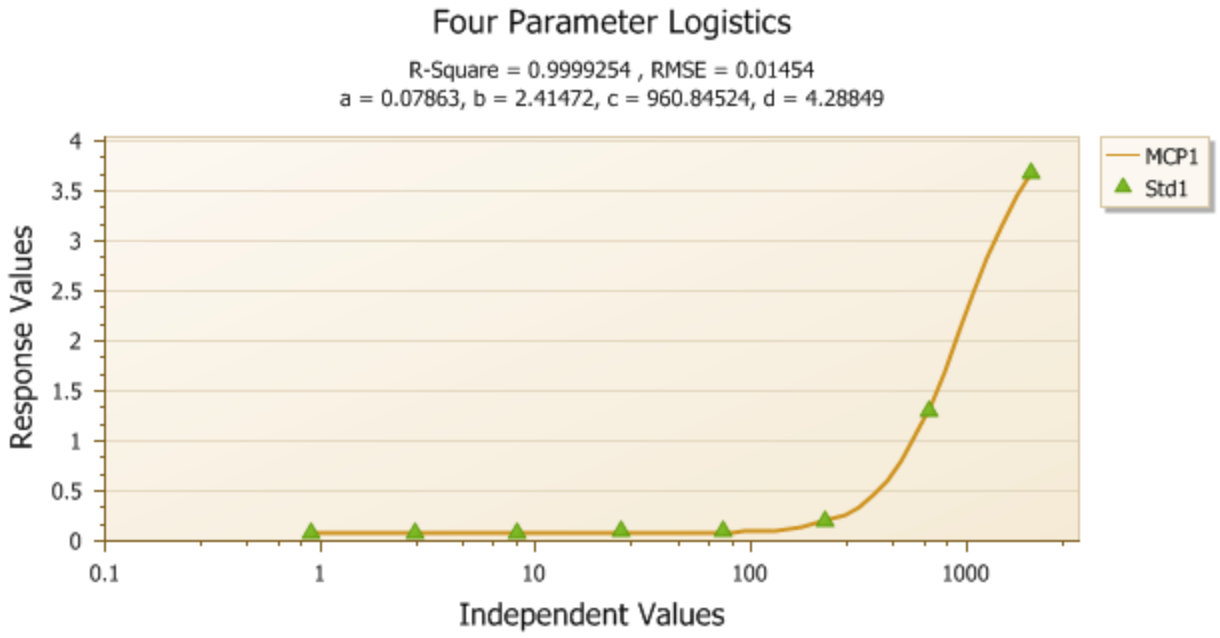

Figure 3-18. Graph of standard curve used to derive the MCP1 cytokine levels in Figure 3-19. The best fit line to the standard dilution that came with the MCP1 ELISA kit was determined using MasterPlex® 2010 freeware software. Best fit curve was defined using four parameter logistics. The range of detection for MCP1 was 0-2000pg/mL, r^2 = 0.9999254.

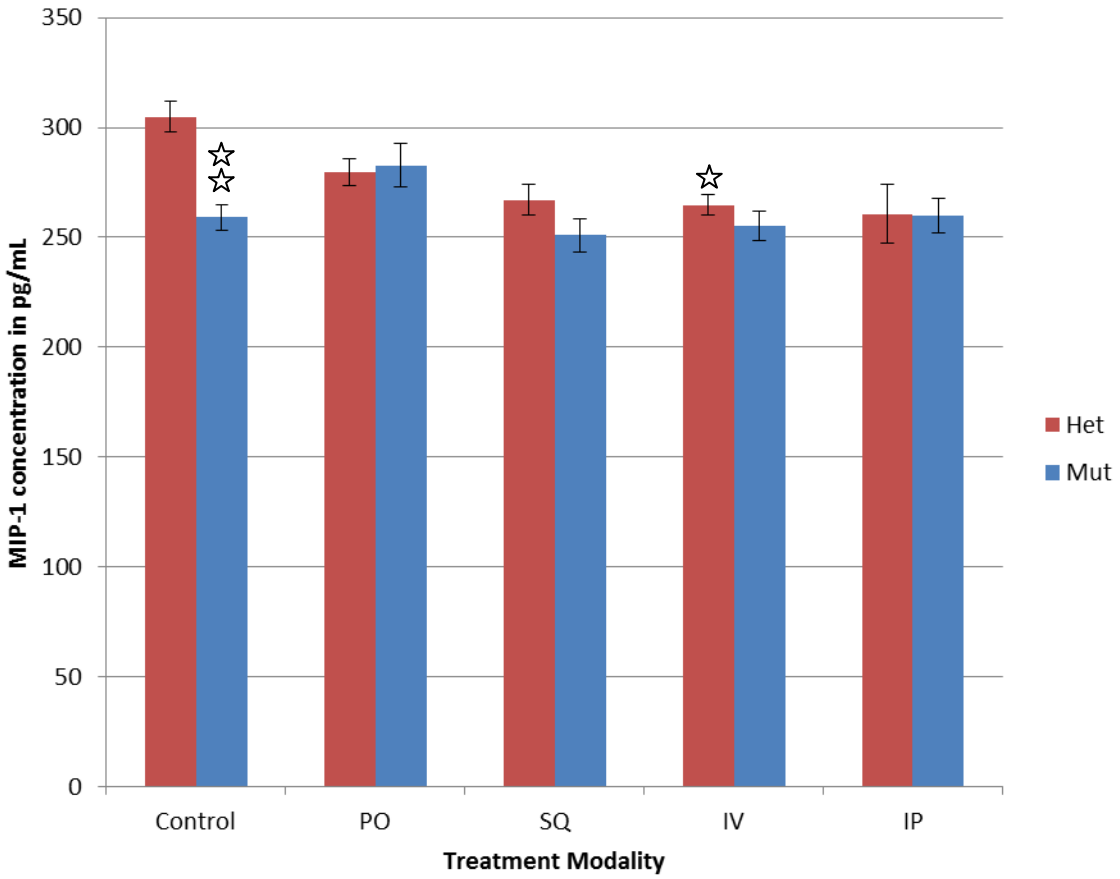

Figure 3-19. MCP1 ELISA. This graph represents the amount of the cytokine MCP1 in brains from the Minozac modality study. There were four treatment modalities: gavage (PO), subcutaneous injection (SQ), intravenous injection (IV), and intraperitoneal injection (IP). Significance was determined via 1-way ANOVA vs. matched controls. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

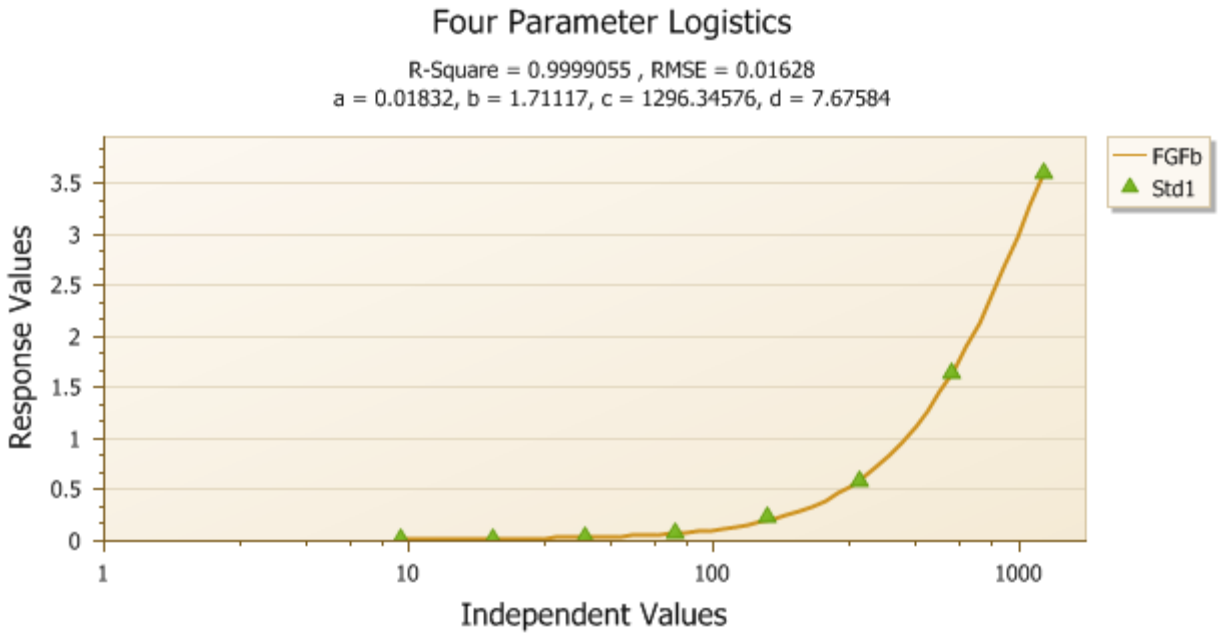

Figure 3-20. Graph of standard curve used to derive the FGFb cytokine levels in Figure 3-21. The best fit line to the standard dilution that came with the FGFb ELISA kit was determined using MasterPlex® 2010 freeware software. Best fit curve was defined using four parameter logistics. The range of detection for FGFb was 0-1200pg/mL, $r^2 = 0.9999055$.

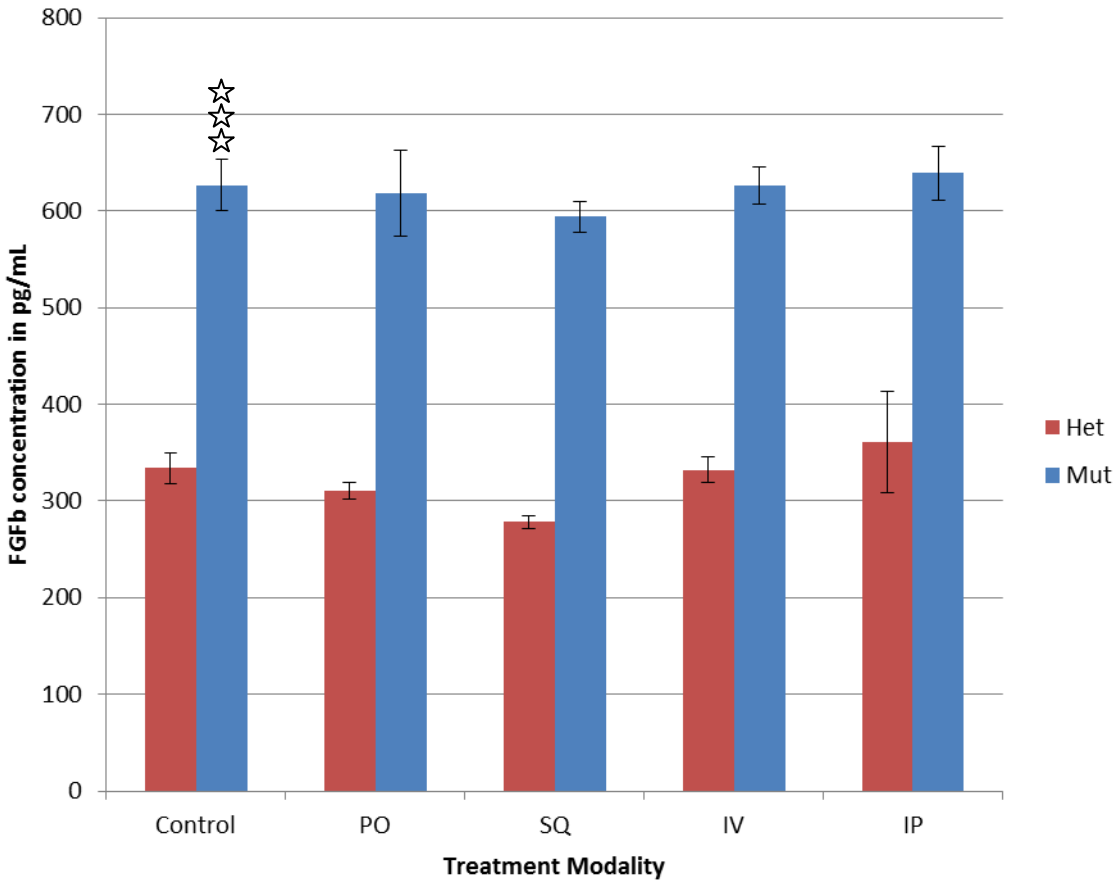

Figure 3-21. FGFb ELISA. This graph represents the amount of the cytokine FGFb in brains from the Minozac modality study. There were four treatment modalities: gavage (PO), subcutaneous injection (SQ), intravenous injection (IV), and intraperitoneal injection (IP). Significance was determined via 1-way ANOVA vs. matched controls. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

CHAPTER 4
DISCUSSION

These experiments offer an important view into the neuroinflammatory processes that occur in lysosomal storage diseases, specifically in MPSIIIB. In the primary brain and blood exploratory map we found that circulating proinflammatory cytokine levels may not accurately represent the degree of proinflammatory cytokine proliferation found in the brain. We also note that many cytokines, some of which are proinflammatory, are indeed upregulated in diseased MPSIIIB brains at a 24 week time point.

We confirmed our original assessment that both MCP-1 and FGFb were upregulated in MPSIIIB diseased mice in our 28 day treatment experiment. Untreated diseased MPSIIIB mice showed a significant upregulation of MIP-1α and FGFb over their matched unaffected controls.

FGFb is an important cytokine that aids in cell growth and, in the presence of other proinflammatory cytokines (TNF-α, etc.), synergistically acts to increase the recruitment of polymorphonuclear (PMN) leukocytes, monocytes, and T-cells, thereby exacerbating inflammation. Thus, because FGFb participates in the signal transduction of various inflammatory pathways, we hypothesize that high levels of FGFb may be caused by this inflammatory pathway activation (Quarto, 1994).Further, FGFb forms a complex with its receptor (FGFR-1) that is known to be stabilized by heparan sulfate (Quarto, 1994). We hypothesize that MPSIIIB mouse accumulation of HS brain cells enhances the expression of FGFb, via positive feedback or retention/stabilization.

MIP-1α is involved in the induction of leukocyte chemotaxis and has been shown to induce the release of histamine from basophils. Antiserum MIP-1α studies have demonstrated the significant role of this proinflammatory cytokine in models of acute

lung injury (Cook, 1996). We believe that FGFb and MIP-1α provide likely targets by which future therapies for the treatment of MPSIIIB may be based.

Additionally, Genistein®'s ability to reduce GAG production and accumulation in MPSIIIB mice resulted in our using it as a possible reducer of proinflammatory cytokines or proinflammatory-related cytokines. Our 28 day treatment experiment showed that Genistein® significantly prevented FGFb and nearly significantly prevented MIP-1α upregulation in 28 week old MPSIIIB diseased mice. In all additional cytokines, Genistein® seemed to instigate a downward trend in proinflammatory cytokine production that does not quite reach significance. However, neither Genistein®, nor any other drug assessed, was able to decrease secondary enzyme upregulation. Throughout the literature, the effectiveness of Genistein® is unclear. A newly published, double- blind, study demonstrated a small, yet statistically significant reduction in GAG concentration in urine and blood plasma following oral administration of Genistein® to patients with MPSIIIA or B at 5mg/kg/day for one year. However, drug application had no effect on behavior or histology, as measured by hair morphology (de Ruitjer, 2012). The idea of substrate reduction is an interesting secondary pathway that, when targeted, may offer therapeutic benefit to MPSIIIB patients. However, Genistein® may be best used as supplement to other more aggressive treatments (gene therapy, BMT, etc.) by providing the added effect of some substrate reduction, thus positively impacting treatment outcome.

Our Minozac results were in direct contrast to the effects described by Chrzaszcz et al. in a mouse model of traumatic brain injury and electroconvulsive-induced seizures. We found that Minozac did not mitigate levels of neural cytokines, including

the expected TNFα and IL-1α. However, in our 28 day treatment groups these cytokines were not elevated. Chrzaszcz et al. administered Minozac intraperitoneally and acutely (Chrzaszcz et al., 2010). Our trial administered the drug subcutaneously and chronically.

Due to the unexpected results from Minozac application, a second 24-hour trial was run, comparing the acute application of Minozac to diseased MPSIIIB mice intraperioneally (IP), subcutaneously (SQ), intravenously (IV) or orally (PO). Both the FGFb and MCP-1 ESLISAs run on the brain homogenates of these treated mice did not show any significant differences in cytokine concentration among the various modes of administration, although subcutaneous injection had the highest downward trend in both cytokines measured. From this data, we conclude that Minozac does not seem to be a viable drug for decreasing the chronic neuroinflammation seen in MPSIIIB mice.

Fosteum®, greatly aggravated neuroinflammation as evidenced by the heightened concentrations of neural cytokines measured. We speculate that the cholecalciferol supplement in the Fosteum® caused this increase. Research has shown that house mice and several species of rats have a cholecalciferol LD50 of ~44 mg/kg (Marshall, 1984). In fact, previous research has suggested the addition of cholecalciferol as a secondary lethal agent to rodenticides (Marshall, 1984). Humans have a much higher LD50 to cholecalciferol than mice. We believe that sub-toxic levels of cholecalciferol exacerbated the stress and inflammation seen in the brains of MPSIIIB mice, thus rendering Fosteum® an ill-advised treatment for mouse MPSIIIB. Interestingly, because cholecalciferol increases calcium absorption, which can cause hypercalcemia and calcification throughout organ systems, Fosteum® may have elucidated a heretofore

unknown interaction between calcium absorption and inflammation in the brains of MPS patients that aggravates inflammation and escalates disease progression.

CHAPTER 5
FUTURE AIMS

The results of this study are limited by several factors. The definitive role of neuroinflammation and the roles of specific upregulated cytokines, such as FGF-2 and MIP-1α, in the disease pathology of MPSIIIB remain to be definitively determined. Even if neuroinflammation is proven to play a key role in MPSIIIB, future studies must evaluate whether cytokines are a viable biomarker for predicting lifespan or symptomatic improvement of Sanfilippo Syndrome.

Genistein® and Minozac also should be tested to determine their effects on lifespan and potential synergistic effects with other treatment modalities such as gene therapy via intracranial AAV, BMT (Heldermon, 2010), or ERT (Sands, 1994). The potentially detrimental effects of excess zinc bisglycate and cholecalciferol in the diets of patients with Sanfilippo syndrome, elucidated by the Fosteum® treatment findings, should also be evaluated.

CHAPTER 6
ONGOING EXPERIMENTS

Immunohistochemical analysis of brains from the primary 28 day treatment experiment is ongoing. Brains from all cohorts are being graded for the presence of: glial fibrillary acidic protein (GFAP), an astrocyte marker; NeuN, a neuronal marker; heparin sulfate (HS); lysosomal associated membrane protein 1 (LAMP-1); IBA-1, a microglia marker; FGFb, and MCP1.

Further analysis is ongoing for the 24-hour Minozac experiment. Brain homogenates have yet to be immunohistochemically analyzed (described above) or analyzed for GFAP and MIP-1α concentration.

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BIOGRAPHICAL SKETCH

Joseph Gibney graduated from the University of Florida College of Agriculture and Life Sciences in May 2010 with a bachelor's in biology and two minors in environmental science and agricultural and natural resource ethics and policy. He followed this degree with master's in Medical Science from the University of Florida College of Medicine in August 2012. He plans to attend medical school in the coming years.