# TRANS-RESVERATROL AS TREATMENT FOR EQUINE RECURRENT UVEITIS

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## Abstract

Uveitis is the leading cause of vision loss in the equine population with an incidence rate between 10-12%. Uveitis is an auto immune disorder that causes the retinal cells to be attacked and eventually leads to blindness. Current treatment involves the prolonged use of the glucocorticosteroid dexamethasone which, due to the ongoing nature of the disease, leads to detrimental side effects. These include, but are not limited to, drastic change in behavior, liver disease and laminitis.

Resveratrol (trans-3,4',5,-trihydroxystilbene) is a naturally occurring polyphenol molecule found in many plant species including grapes, peanuts, cranberries, Japanese giant knotweed and others. In these plants resveratrol acts as an antioxidant to protect the plant from bacteria, fungi and ultraviolet radiation. When used in human studies, both *in vitro* and *in vivo*, resveratrol was found to protect against oxidative damage and premature aging, aid in vascular support and immune regulation, blocked the carcinogen effects of TNF-α and NF-κB, and down regulated inflammatory cytokines.

In this study human ARPE cell lines were cultured and then treated separately with TNF-α to induce an inflammatory response. From there each cell treatment received resveratrol at 50, 100 and 200μm for 12, 24, and 48 hours. Three controls, non-treated cells, resveratrol treated cells and TNF-α only treated cells, were also included in the experiments. The RNA extracted from these experiments was then reverse transcribed to cDNA and run on a qPCR array that contained eighty-four inflammatory cytokines and receptors. Five genes were selected for validation of their effect on ERU and the remaining data retrieved for further study.

In all five genes selected, Interleukin 33, Interleukin 17F, Fas Ligand, Vascular endothelial growth factor A, and Chemokine ligand 2, resveratrol was shown to have a significant effect on gene expression either in a time, dose or time/dose dependent manner. Pretreatment with resveratrol yielded an attenuation of the expression of several genes after TNF-α treatment. These results show that resveratrol is a viable option for the treatment of equine recurrent uveitis.

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# CHAPTER I

## EQUINE RECURRENT UVEITIS

Equine recurrent uveitis (ERU) is an autoimmune disease of the equine retina that affects 2%-25% of the equine population in the United States and is the leading cause of blindness in the horse (Gerding, 2016). ERU is seen at a higher prevalence in certain areas of the country as well as within certain breeds. It is hypothesized that this is due to environmental factors and genetic predisposition (Deeg, 2008). Although there have been no industry impact studies, it is presumed that the loss of equine performance due to the disease lowers its value, thus impacting the equine industry and owner. Current treatment of ERU is not ideal, due to pharmaceutical side effects, and the fact that it is only palliative.

### Anatomy of the Equine Eye

The equine eye has the largest globe of all land animals and its lateral placement in the skull gives it a 350 degree field of vision; 175 degrees vertically and 190 to 195 degrees horizontally. There is a blind spot a few feet behind the rear and a small blind spot immediately in front of the nose. The horse is considered to have both monocular and binocular vision, with 65% of this estimated to be binocular (Ball, 1999).

The bony socket, or orbit, that the eye sits in the skull is medially surrounded by the sinuses, anteriorly closed by the skull itself, posteriorly open for the orbital blood vessels and

optic nerves, while the ventral plain is made up of soft tissue that creates a barrier between the eye and the oral cavity. Strong optic muscles not only hold the eye within the orbit, but also move the eye allowing for the wide range of vision of the horse. The eyelids of the equid are part of the outermost skin and the orbicularis oculi muscle is responsible for their movement along with the facial nerves; the top lid has eyelashes and cilia while the bottom does not. The eyelid also contains the Meibomian gland that is responsible for the lipid component of tears. The horse has what is known as a 'third eye lid' or nictitans. It moves horizontally across the eye to distribute tears that are produced at its base, as well as to act as a protective layer (Ball, 1999; Brooks, 2008).

The anterior portion of the eye is made up of the precorneal tear film, cornea, iridocorneal angle, iris, lens and ciliary body. The tear film is produced by the lachrymal glands, nictitan glands, and the conjunctival goblet cells. Its purpose is to keep the cornea moist and protected, as well as containing nutritional factors, proteinases, vitamin A, proteinase inhibitors, and cytokines that play a role in corneal health. The cornea itself has four layers and is responsible for the eye's light bending ability. The first layer is made up of three squamous epithelium cell types that act as a barrier to the tear film. The second is a collagen layer, called the stroma, which makes up 90% of the cornea's thickness. This is attached to the third layer which is a basement membrane layer that is rejuvenated throughout life and is only three cells thick. The final layer is only one cell layer thick and contains the ATPase pump responsible for corneal transparency (Ball, 1999; Brooks, 2008).

The iridocorneal angle contains the anterior chamber that separates the cornea and the iris and is filled with aqueous humor. The iris is the tissue responsible for constriction and dilation of the pupil and is made up of two layers of muscle; the sphincter and dilator muscles. The pupil

sits in the middle of the iris and controls, with the iris, the amount of light that reaches the lens of the eye. The ciliary body produces aqueous humor and, like the iris, is responsible for the function of the pupil. Posterior to these structures sits the lens. The lens focuses the light to the posterior chamber of the eye and inverts the image as well; an orientation that is fixed in the brain. The muscles of the lens are also used to focus on objects at varying distances (Ball, 1999; Brooks, 2008).

The posterior portion of the eye is made up of the vitreous chamber, retina, the choroid and optic nerve. The vitreous chamber is filled with a thicker humor than the anterior chamber, providing protection to the retina as well as holding it in place. The retina is also held in place by the sclera and choroid. The choroid provides blood supply to the retina as well as being the grounding tissue for the optic nerve. The posterior orbicularis oculi muscle, at the back of the eye, further holds the eyeball into the orbit of the skull (Ball, 1999; Brooks, 2008).

The retina of the horse's eye has ten layers that run seamlessly into the optic nerve. These layers, starting at the vitreous, are (1) the inner limiting membrane that separates the vitreous from the retina, (2) the layer or optic nerve fibers that contain the axons and the ganglion cells, (3) the ganglionic layer that forms the optic nerve, (4) the inner plexiform layer which functions to form the synapsis between the cells of the inner nuclear layer and the ganglion cells, (5) the inner nuclear layer itself that contains the cell bodies of the muller, amacrine, horizontal, and bipolar cells, (6) the outer plexiform layer that forms the synapsis between the axons of the photoreceptors and the dendrites of the bipolar and horizontal cells, (7) the outer nuclear layer containing the rods and cones cellular bodies, (8) the external limiting membrane, (9) the rods and cones photoreceptor layer, and (10) the retinal pigmented epithelium. These combined layers of the retina are responsible for converting light to a chemical reaction

that in turn creates the electrical signal that is sent to the brain which produces the image (Brooks, 2008; Hall, 2011).

## Pathophysiology of Equine Recurrent Uveitis

ERU is categorized as an autoimmune disease of the eye's retina. Autoimmune diseases are defined as the body's T-cells, B-cells, or both, attacking the organism itself, rather than viruses, bacterial infections or injury. This may be caused by a number of factors, including inherited genes that create a failure of self-tolerance, environmental triggers, trauma, and infections that lead to activation of self-reactive lymphocytes. No matter the trigger of the autoimmune disease the outcome tends to be universal; progressive, with reactive periods and relapses, leading to the tissue, organ, or system's eventual failure (Brooks,2008; Deeg, 2007).

There are several proposed etiological modes of ERU, ranging from blunt force trauma, wounds, corneal ulcers, genetic predisposition, and bacterial infection. The first three trigger an inflammatory response that does not become quiescent once the original injury has healed. Instead the immune system starts to identify the self-tissue as foreign, triggering the first autoimmune response that leads to ERU (Witkowski, 2016). Certain breeds of horses, specifically German warmbloods and Appaloosas, have been shown to have a heritable form of ERU, with an occurrence rate of over 20% in the latter (Fritz, 2014). ERU horses that have serologies drawn have a 1-95% rate of bacterial infection stemming from *Leptospira pomona*, *L. bratislava* or *L. autumnalis* that triggers the autoimmune response, depending on the geographic location of the horse (Wollanke, 2001). Along with bacteria, other infectious agents, such as

viruses and parasites, have been shown to trigger the disease. Still, the majority of ERU cases have no diagnosable cause; they just exhibit the pathophysiology of the disease (Gilger, 2015).

In cases where trauma, injury or ulceration of the eye are the cause of ERU, the pathology is believed to be the breaching of the eye's immune privileged state (Whitkowski, 2016). As discussed in Anatomy of the Equine Eye in this thesis, the eye is self-contained within its structure, and in a normal state there is no crossing of immune bodies across the blood-retinal barrier. Once the tissue damage has occurred, causing a breech in the barrier, T-cells start to infiltrate the eye and, not recognizing self, begin to attack the eye's cellular structure, specifically the retinal proteins (Deeg, 2008). Through this modality the immune system starts the autoimmune response triggering ERU.

There are several breeds that show a statistically greater tendency to develop ERU over others. The most documented breeds are the German warmblood and the American Appaloosa. In Germany one study showed that 50% of ERU horses where sired by three stallions and in England ERU became rare after a breeding ban was placed on stallions that showed ERU or cataracts (Alexander, 1990). In the American Appaloosa, ERU is reported as being 3-8 times higher than in other breeds, as well as being more prone to unilateral or bilateral blindness caused by ERU (Deeg, 2008).

Higher clinical statistics of the Appaloosa over other American breeds, and higher incidence rate of ERU among certain eye colors and coat patterns within the breed, have triggered genetic investigations. Human uveitis studies have shown that genes within the major histocompatibility complex (MHC) have a close association with the disease (Levinson, 2007). When a population of 136 Appaloosas was studied, it was found that there was a two to three-

fold increase in ERU incidence rate when certain alleles within the gene were expressed (Fritz, 2014). There have been two specific microsatellites on genes, UM001 and EqMHC1, that have been linked with a higher increase of ERU in the Appaloosa and German warmblood breeds (Brooks, 2008).

In genetic studies of the German warmblood, it was found that genetic components of a single nucleotide polymorphism in leucocyte antigen ELA-9, gave rise to ERU (Deeg, 2004). Moreover, other studies have demonstrated that aberrant genetic regulation of IL-17 and a single nucleotide polymorphism on chromosome 18 cause ERU subjects within this breed to also form cataracts (Kulbrock, 2013). Additional studies are warranted to better understand the difference between ERU that is caused by a genetic predisposition and ERU that is caused by genetic immune regulation.

Bacterial infections have been suspected of triggering ERU since the 1950s when an outbreak of *Leptospirosis* occurred on two horse farms in New York followed by an outbreak of the disease in most horses within 18-24 months (Roberts, 1952). Several strains of leptospirosis have been identified when analysis of the aqueous and vitreous humor is done (Brooks, 2006). It is hypothesized that the bacterium triggers a histocompatibility complex in some individuals which then triggers the auto immune component of ERU (Simeonova, 2016).

When ERU is induced in the laboratory setting there are characteristic cytokine and chemokine profiles that arise, whether *in vitro* or in the intact animal. When uveitis is induced in the horse the cytological and histopathological changes are similar to that of spontaneous ERU. Simeonova and colleagues discovered an increase in lymphocytes, macrophages and lymphoblasts as well as a large number of granular nuclei in the aqueous humor. Along with

these findings a high concentration of the cytokine IL-17 was also observed, linking dysfunctional Th-17 cell expression in equids with ERU. In addition to IL-17, cellular retinaldehyde-binding protein (CRALBP) was also detected inside of the aqueous humor indicating that the immune privileged state had been compromised. In this study ERU was induced by trauma in the left eye and infectious antigens in the right eye, with no significant differences in results between the two (Simeonova, 2016). This strongly suggests that regardless of the trigger, the disease progression does not differ.

Looking at the aqueous humor of horses that were already clinically diagnosed with ERU, Curto et al. found increased levels of IL-10, IL-1a, IL-4, IL-8, IL-12p70, FGF-2, G-CSF and RANTES (Curto, 2016). When compared with the control group, IL-10, IP-10, and MCP-1 levels were significantly higher in the ERU group. Ten of the thirteen ERU-positive horses in this study also tested positive for the presence of Leptospiral DNA within the eye, suggesting that Leptospirosis does cause the disease.

Regan et al. looked at the enucleated eyes of seven horses that had been clinically diagnosed with ERU at the University of Georgia Veterinary Teaching Hospital. The excised tissue was found to be positive for IL-6, IL-17, and IL-23 by immunohistochemical staining; whereas the controls were not. Staining for *Leptospira* was negative in all of the ERU eyes, however it may be that the disease was advanced enough that the immune system had cleared the initial cause of onset (2012). By creating a cytokine profile of ERU, specifically targeted immunosuppressive drugs could be used as they currently are in human uveitis (Barry, 2015).

Diagnosis and Treatment of ERU

Unless ERU is in an acutely active stage, most equine owners only notice clinical manifestation once the disease has progressed to the point it causes noticeable structural damage to the eye. Clinical appearance is noted by iris color change due to leukocytes attaching to the retinal endothelium, a hazy appearance of the eye, increased tear production during the active phase, corneal edema, cataract formation and a prominent third eye lid do to shrinking of the globe. Exhibition of pain has not been shown in all horses during ERU episodes, and therefore is not used as a clinical diagnostic marker. In advanced stages of ERU clinical observations include endophthalmitis, periorbital cellulitis, complete corneal opacity, and vitreal yellowing (Brooks, 2008; Allbaugh, 2017).

A veterinarian should do a complete ophthalmological exam on any horse suspected of having ERU. Assessment of anatomical correctness should be followed by direct and indirect pupillary light reflexes and stimulant responses. Intraocular pressure (IOP) should be measured by rebound tonometry. A healthy equine IOP ranges between 15 and 30, whereas ERU horses have a mmHG reading of 5 – 12. The eye should also be checked by fluorescein staining for corneal ulcers, as their presence changes treatment options. Dilation of pupils with tropicamide is necessary for examination of the lens, vitreal and fundic areas. Dilation can also reveal aqueous flare, keratic precipitates and cataracts; all associated with ERU. Ocular ultrasound can also be used to diagnose retinal health and possible detachment. A differential diagnosis should also take place to rule out other pathologies including conjunctival foreign bodies, corneal ulcers or corneal stromal abscesses. Blood work and serology should be performed to try to identify the origin of ERU, as well as provide a baseline of the animal's health (Brooks, 2008; Allbaugh, 2017).

Traditional treatment for ERU during its acute stage is topical steroids, prednisolone or dexamethasone, given at least 4 times a day to control inflammation, thereby reducing pain and preserving ocular structures. This is then tapered and discontinued 2 to 4 weeks after a follow up exam has shown the acute phase is over. Unfortunately, this is not a treatment option for horses with cataracts as topical steroids cause continual keratin formation. In these cases topical nonsteroidal anti-inflammatories (NSAIDs) are used. NSAIDs can also be given systemically to aid in the control of intraocular inflammation, discomfort and pain. In severe cases systemic treatment with corticosteroids can be used with caution, as extended systemic corticosteroid use has been shown to induce laminitis (Brooks, 2008; Allbaugh, 2017).

Topical atropine is also traditionally used to minimize synechiase formation, where the iris adheres to the cornea, and relieve spasms of the ciliary body muscles. This can be given up to four times a day, but it should be noted that loss of gut motility and abdominal pain can result from long-term use. Broad spectrum antibiotics are often given during ERU episodes, particularly in cases that tested positive for *Leptospira* infection. However there is concern over whether or not the drug penetrates the BRB, unless it has already been compromised (Brooks, 2008; Allbaugh, 2017).

A surgical procedure called pars plana vitrectomy, is performed to try to debride the eye of fibrin, inflammatory cells and debris in order to improve vision and delay the progression of clinical ocular changes. This surgery has been beneficial to those horses that have confirmed cases of ERU caused by *Leptospira* infections. The postoperative side effects of retinal detachment and cataract formation typically makes most horse owners refrain from this elective surgery (Brooks, 2008).

Treatment options for horse owners have expanded in recent years due to a greater understanding of the disease and pharmaceutical advancements. Implants containing the immunosuppressive drug cyclosporine A are placed within the sclera and in contact with the choroid. Horses that have frequent recurrences and long active periods of ERU are considered to be the best candidates (Brooks, 2008; Allbaugh, 2017). The Appaloosa breed in particular showed a more favorable outcome of the implant over other breeds, with only a 12% vision loss after implantation compared to 50% in other breeds (Gilger, 2010). Acupuncture has also shown promise in the treatment of ERU with all stomach, gall bladder, and eye association points being used. A rise in homeopathic remedies is also being noted, although further studies are recommended (Brooks, 2008).

## ERU and Human Uveitis

The term uveitis in human medicine incorporates a diverse group of chronic diseases that are either localized to the eye or secondary to a systemic disease. Inflammation can be present in either the anterior or posterior portion of the eye and can stem from infectious agents, arise spontaneously, or be autoimmune in nature, but not necessarily arising from the eye (Kumar, 2014). The prevalence in the USA is 17 cases per 100,000 persons and tends to manifest in middle or older aged individuals. Symptoms include pain, photophobia, floaters, blurry vision, blind spots, scarring cataracts and loss of vision. Diagnosis includes a differential workup to determine etiology in order to treat the underlying disease. Treatment is with corticosteroids, either topically, orally or by injection, and based on the etiology. Immunosuppressive

medications, where appropriate, are also given in cases of autoimmunity; while the underlying medical disease, if any, is also being treated (Ferri, 2012).

ERU has become an important model for human recurrent autoimmune uveitis (AU) due to clinical and pathological similarities. The retinal autoantigens CRALBP, IRBP and S-antigen were found in both ERU and AU patients (Deeg, 2006, 2007). Recent studies have found IL-17 expression in human patients that have AU, as well as the autoimmune diseases rheumatoid arthritis, psoriasis, and multiple sclerosis; all of which can give rise to AU secondarily (Curto, 2016). Human patients with AU exhibit an elevated profile for the cytokines and chemokines of TNF-A, IL-2, IL-6, IL-8, IP-10, MCP-1, MIP-1B, and RANTES; several of which also appear in the pathoimmunogical profile for ERU (Lee, 2014; Hauck, 2006; Curto, 2016).

Similarities in treatment of ERU and AU also give rise to similar harmful pharmacological side effects when used over a long period of time. Long term systemic steroidal use to control inflammation has been known to cause behavioral issues and laminitis in horses, and hypertension, diabetes, and psychological disorders in humans. T-cell and immune suppressors are a form of treatment that directly inhibit T-cell activity and cytokine expression. In humans there is the risk for a compromised immune system to become susceptible to an opportunistic infection, while in equids long term treatment may be cost prohibitive to the owner. Antimetabolites are used to block protein synthesis in lymphoid cells thus reducing development of monocyte precursors and natural killer cells in cytotoxic reactions. When used for extended periods of time gastrointestinal function in both humans and equids is observed, with horses exhibiting a loss of gut motility that leads to colic. Steroidal implants into the aqueous humor of the human eye, or the suprachoroidal space in the equine eye, are used to release low dose steroids directly to the affected tissue in order to avoid the systemic long-term effects (Brooks,

2008; Barry, 2014). Due to inflammation flare-ups there is a need to replace the implants in both species, based on a catabolism-dependent time course (Barry, 2014; Gilger, 2010). In the horse this has been estimated to be between 24 and 48 months, which could be cost prohibitive to the owner. Research has also demonstrated that equine implants do not stop all uveitis flare-ups and the need for topical treatment during those episodes (Gilger, 2010).

# CHAPTER II

## RESVERATROL

### Introduction

Resveratrol (3,4',5,-trihydroxystilbene) is a polyphenol compound that is synthesized by plants as an antioxidant form of protection from fungus, UV light, predation, and bacteria. The chemical structure of resveratrol, two phenolic rings bonded together by a double styrene bond, reacts with other molecules via its core phenolic structure, its linkages and the types of glycosides they can form.

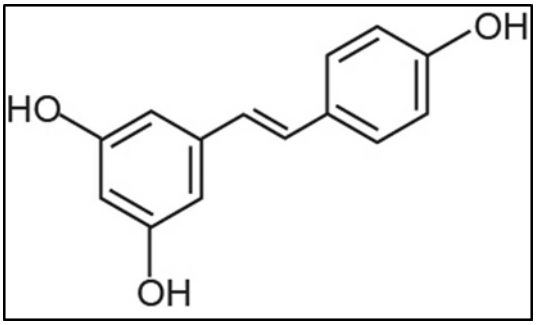


Figure 1 Trans-Resveratrol

These reactions include ionization, oxidation, aromatic transformations, nucleophilic additions, oxidative and hydrolytic bond cleavages, as well as metal complexes (Gambini, 2015). These reactive characteristics enable the compound to act in several positive ways to protect cellular function and longevity. Along with these chemical reactions resveratrol has a binding affinity for proteins, which leads to its forming of soluble and insoluble complexes that directly affects its bio-availability, absorption and metabolism within an organism (Gambini, 2015). The double

bond between the two phenolic rings creates isometric cis- and trans- forms of resveratrol, with the trans- isomer being the more stable of the two.

Resveratrol is found in many plants consumed by humans, such as peanuts, cranberries, Japanese giant knotweed, and grapes thus, leading to the interest in resveratrol's content in red wine. This was sparked by what is known as the "French Paradox", where the French population has a higher consumption rate of red wine as well as a diet higher in fat, yet has a lower incidence of cardiovascular disease than other populations (Marques, 2009). It is hypothesized that the antioxidant effects of resveratrol protect cardiac tissue and the circulatory system from the high fat diet (Rayalam, 2008). This hypothesis has spurred studies looking at resveratrol's other chemical cellular affects in the areas of anti-inflammatory, autoimmunity, cellular immunity, cancer treatment and chemo-preventatives, to name a few.

## Bioavailability, metabolism and pharmacokinetics

Resveratrol's bioavailability is effected by several different factors; its absorption, how it's metabolized and the resulting metabolites, transport with in the body, cellular uptake and possible tissue storage (Walle, 2011). The pharmacokinetics of the compound is thought to be influenced not only by the bioavailability of resveratrol, but also by the bioavailability of its metabolites, the cell type and function, whether the cell is cancerous or malignant, and the dose given, being both dependent or independent of the time course it was given in (Stivala, 2001).

Resveratrol is well absorbed by the body, while its bioavailability is dependent on many factors. In absorption studies, with resveratrol given both orally and intravenously in a single dose of 25mg, there is an average absorption rate of 75%. This was determined by urinary

excretion of total metabolites after radiolabeled doses were given (Walle, 2011). In the intestine resveratrol's lipophilic characteristics allows it to be absorbed by passive diffusion or by forming complexes with membrane transporters.

Once in the blood stream resveratrol's high protein affinity allows it to bind to serum proteins in the blood to be transported throughout the body, and also makes it accessible to cell surfaces. The major plasma protein, albumin, has been shown to interact with resveratrol at two binding sites in both human and bovine serum (Latruffe, 2014). Albumin is made in the liver and acts as a carrier for vitamins, minerals, hormones, and pharmaceuticals. It is water soluble and binds to cell receptors enabling it to deliver what it is carrying into the cell. Human albumin has been shown to stabilize when binding to the trans- isomer of resveratrol, implying that trans-resveratrol has a higher binding affinity for serum proteins, as well as the cis- isomer having different effects from the trans- isomer (Pantusa, 2014).

Hemoglobin, a plasma protein that is responsible for transporting oxygen and carbon dioxide in the blood, has also been reported to bind with resveratrol and transport it throughout the body (Latruffe, 2014). Hemoglobin's affinity to bind with resveratrol is less than that of albumin due to the different bonds that are created. Albumin and resveratrol create a hydrophobic bond, while hemoglobin and RV interact through a hydrogen bond. Hemoglobin and albumin both have been shown to accumulate resveratrol when concentrations are high enough in the body, creating a reservoir for the compound (Delmas, 2011).

Another carrier molecule of resveratrol in the blood stream is the lipoprotein LDL (low-density lipoprotein). LDL binds to resveratrol in a noncovalent manner and due to its different cell receptors, gives resveratrol yet another pathway to enter the cellular cytoplasm (Dalmas,

2011). Resveratrol's binding to LDL also gives it the ability to work outside of a cellular matrix and within the arterial system were oxidation of LDL occurs, resulting in damage to the circulatory system (Delmas, 2011).

Metabolism of resveratrol is hypothesized to be its rate limiting step. When ingested orally this is completed in three passes; the first is absorption in the digestive tract, the second as it passes through the liver, and finally there is evidence that a third pass, that of bacterial digestion of the metabolites of resveratrol might contribute to chemoprotective effects (Stivala, 2001). Once free resveratrol enters the liver it is broken down into metabolites. These metabolites have their own carrier affinities as well as cellular interactions that are tissue specific (Stivala, 2001).

There are several noted metabolites of resveratrol; resveratrol monosulfate, two isomeric forms of resveratrol monoglucuronide, dihydroresveratrol monosulfate and dihydroresveratrol monoglucuronide. Due to their individual chemical structures it is hypothesized that each metabolite is able to affect cellular biochemistry in a different way. Glucuronides of resveratrol have been shown to be a reservoir of the parent compound and it is the liver that does the reassembling. Resveratrol sulfates bind to cyclooxygenase sites on certain enzymes and inhibit their activity, as well having an antiproliferative effect on the cell cycle of human colon cancer cells (Delmas, 2011). Further studies on resveratrol's metabolites are needed to discern more of their unique cellular pathways.

Cellular uptake of resveratrol is mediated by cell receptors that recognize the specific carrier protein or lipoprotein, as well as by passive diffusion across the cell membrane. As a result, resveratrol's mechanism of action may be by activating signaling pathways upon binding

to membrane receptors, activating intracellular pathways, or developing its effects inside the nucleus of the cell. Fluorescence microscopy has shown that resveratrol exists in the cytoplasm and nucleolar region of treated cells in vitro (Lancon, 2004). This would explain its effect related to regulation of cell-cycle genes, activating transcription factors, inhibiting protein kinases, suppressing expression of antiapoptotic genes, upregulation of antioxidant enzymes, and inflammatory regulation.

Studies also show that cellular uptake is dependent on the form of resveratrol, the cell type, and possibly whether or not the cell is malignant or benign (Walle, 2011). There have been studies demonstrating that sulfate metabolites of resveratrol are taken up specifically by human breast cancer cells (Murias, 2008) as well as having the ability to reform the parent compound in the liver (Wenzel, 2005). Hepatic cells have been shown to take up resveratrol and metabolize it into its mono- and disulfate derivatives, but not glucuronide metabolites (Lancon, 2007). Studies in rats have shown not only cellular uptake, but also storage of resveratrol by the kidney and liver as well as the intestinal tissue in mice (Sale, 2004; Bertelli, 1996).

Cellular uptake is greatly influenced by the dose of resveratrol given, and the frequency and duration of treatments. Multiple studies have sought to determine a saturation point as it relates to dosage in order to increase the availability of resveratrol. Concentration of resveratrol in plasma reached only about 500ng/mL even after the highest dose of 5,000mg was used; this was thought to be attributable to solubility (Boocock, 2007). A study that administered varying doses over twenty-nine days showed that plasma concentration increased with dose (Meng, 2004). Human studies of resveratrol's long term cellular effects have focused primarily on blood serum levels rather than on the duration of dosing. The trouble lies in tracking and following up with the subjects while the trial is ongoing. The current focus of research is to

determine greatest efficacy rather than highest concentration in identifying resveratrol's effect on the cellular pathway of interest (Chow, 2010).

There have been reported cases of side effects when resveratrol was administered orally in higher concentrations; most within an hour of caplet ingestion at 2.5 or 5.0 g (Brown, 2010). In another four-week study, resveratrol was shown to be tolerated and have the greatest effect when administered at 1 g per day (Chow, 2010). The most common side effects of overdose were diarrhea, nausea and abdominal pain.

The creation of synthetic, or encapsulated, forms of resveratrol is currently being explored in order to increase bioavailability. The aliphatic acid of resveratrol has been synthesized and shown to have a 40-fold higher affinity for the plasma protein albumin, as well as having a greater solubility in water (Chen, 2009). Other synthetic derivatives have been shown to have a 10-fold greater efficacy when studied in cancer cells; the hypothesis behind this is that the derivatives have a longer half-life and therefore a higher bioavailability (Mulakayala, 2013). Encapsulating resveratrol with lecithin-based nanoemulsions resulted in a doubling time of cell permeability, protection against chemical degradation, and sustained release when administered by dialysis (Sessa, 2014). Currently both methods of increasing resveratrol's bioavailability are undergoing studies to ensure that the compound maintains the same effects once it reaches its target tissue.

## RV and Cellular Biological Activity

Resveratrol's affinity for transport proteins and lipoproteins, its ability to passively diffuse across the cellular membrane, and its binding capabilities with cellular receptors, give it a

wide range of influence on cell signaling, replication and regulation. Its affects have been shown to differ between cell types as well (Gambini, 2015). These combined properties of resveratrol influence the control of inflammation, antioxidant activity, regulation of cell immunity and longevity, autoimmune response, and chemopreventive potential. Further investigation of each of these properties is warranted to attain a better understanding of how resveratrol might be used as an intervention.

*Antioxidant Properties*

As cells carry out metabolic processes waste products are created, including free radicals, which have a single unpaired electron in their outer orbit, and oxygen derived free radicals known as reactive oxygen species (ROS) from mitochondrial respiration. Under normal conditions the body removes these from the cell by enzymes, mineral and transport protein interaction, or antioxidants. If left in the cell, free radicals and ROS cause a condition called oxidative stress which leads to lipid peroxidation of membranes, oxidative changes of proteins, lesions and cross linkages in DNA, and cellular apoptosis. This has been linked to cell injury, cancer, aging, degenerative diseases of the circulatory and neurological systems, as well as initiating inflammatory reactions (Stivala, 2001).

Cancer can be defined as unregulated cell proliferation or differentiation, as a result of cellular or genetic damage. This change usually arises from a single cell's genetic change within its DNA, followed by self-replication, metastasis, and ultimately invasion of secondary tissues of the host organism. There are genes within the nucleus of cancerous cells that lead to an expression of its characteristics, whether it be transcribed for growth factors, cellular signaling or

receptors, nuclear regulatory proteins, or cell cycle regulators. Resveratrol has been shown to act as an inhibitor of these genes and their function.

There are cellular proteins and receptors that affect a tumor's sustainability and growth which resveratrol has been shown to influence the expression of, in an anti-neoplastic way. NF-kB is a protein complex that controls the transcription of DNA for a range of cellular functions such as cell proliferation and survival, apoptosis, immune response, and inflammation, which is hypothesized to lead to tumor metastasis. In a prostate cancer study resveratrol was shown to block NF-kB activation (Benitez, 2009). In another study resveratrol induced apoptosis in MCF-7 cells, a breast cancer cell line, by creating a pro-oxidative effect via the NF-kB pathway (Filomeni, 2007). Resveratrol can also bind to estrogen receptors, activating genes in their downstream pathway, some of which are shown to have antioxidant properties (Borra, 2005). The binding of resveratrol to estrogen receptor rich MCF-7 cells was shown to antagonize the effects of estradiol, thus decreasing metastasis (Ashby, 1999).

Apoptosis of cancerous cells is another mode of action for resveratrol, induced by cell cycle arrest via tumor suppressor protein p53. Hepatocarcinoma (HepG2) and HCT116 cell lines were shown to arrest in the G1/G2 phase of the cell cycle causing apoptosis, after treatment with resveratrol (Amiri, 2013; Yu, 2009). Resveratrol has also been shown to induce apoptosis by the extrinsic pathway which involves transmembrane receptor-mediated binding leading to a formation of the death-inducing signaling complex (DISC) in H411E (rat hepatoma cell line) and HT-29 (colon cancer cell line) cells (Michels, 2006; Miki, 2012).

Resveratrol's antioxidant properties have been linked to positive effects in cardiac tissue and the circulatory system. Nitric oxide (NO) is a signaling molecule that relaxes vascular

smooth muscle and increases blood flow by preventing platelet aggregation and leukocytes from adhering to blood vessels. Resveratrol has been shown to increase NO production through an extracellular signaling kinase triggered by resveratrol's binding to estrogen receptors on vascular cells (Klinge, 2008). In a study where cytotoxicity was first induced, treatment with 50μM resveratrol increased NO production to levels that reversed this effect (Takahashi, 2012).

High levels of c-reactive protein (CRP) in the blood are an indication of vascular compromise and atherosclerosis caused by inflammation. Following induced inflammation with IL-6 and IL-1B, Hep 3B cells showed an increase in CRP and were subsequently treated with resveratrol. The result showed that resveratrol inhibited CRP expression and was therefore hypothesized to promote vascular health (Kaur, 2007). Platelet aggregation in blood vessels leads to clot formations which in turn can lead to a stroke or heart attack. Studies done in vivo and in vitro proposed that resveratrol's binding to calcium channels reduces thrombin by as much as 50%, thus leading to a decrease in blood clot formation (Zhu, 2002). At 100uM resveratrol was shown to help in regulation of triglycerides as well as lipolysis, suggesting a potential treatment for obesity (Lasa, 2012).

Resveratrol's effects on triglycerides were further investigated in a study where mice were fed a high fat diet with and without resveratrol supplementation. The group receiving resveratrol supplementation showed reduced fat deposits on abdominal organs compared to the control group (Baur, 2006). When resveratrol was given to rats that had induced diabetes Insulin sensitivity was shown to improve by evidence of lower blood glucose and triglyceride levels, as well as glucose uptake into tissues (Su, 2006). Fatty liver disease is associated with obesity and can lead to steatohepatitis and cirrhosis. Resveratrol supplementation in rats who were fed a high fat diet showed an inhibitory effect on developing fatty liver disease (Bujanda, 2008).

Anti-aging effects of resveratrol are seen not only in its anti-neoplastic and cardiovascular implications, but also through enzyme and kinase pathways. The SIR1 and SIR2 enzymes are known to mimic the effects of caloric restriction which has been shown to increase longevity (Guarente, 2005). In a study in which mice were fed a high calorie or standard diet, with or without resveratrol, the high calorie diet group that was fed resveratrol showed increased insulin sensitivity, less fat deposition in the liver and an increased number of mitochondria per cell (Baur, 2006). It is hypothesized that activated protein kinase (AMPK) is effected by resveratrol, as AMPK influences fat metabolism in the liver, and down regulates the rate limiting step in cholesterol synthesis. When resveratrol was given to mice on a high fat diet their metabolic rate, physical stamina, and the number of mitochondria were all higher than that of the group not supplemented with resveratrol; although mice that were genetically bred to lack AMPK showed no metabolic effects (Gonzales, 2008).

*Inflammation and Inflammatory Conditions*

Inflammation is a reaction produced in an effort to return the body to homeostasis and is caused by chemical signals produced by cells, or plasma proteins, in response to injury, foreign bodies, immune reaction or infection. Inflammation causes a change in vascular flow and cellular permeability in order to bring plasma proteins, leukocytes, mast cells and macrophages to the area to promote healing. These changes are brought about by chemical signaling from cytokines, chemokines, and hormones that in turn cause a cascade of enzymes which further direct the process. Inflammation can be acute, where the initial cause is gradually resolved, or chronic, where the initial cause is not resolved and becomes altered in nature.

Cyclooxygenase is an enzyme produced by injured cells to mediate cellular response by the formation of prostaglandins which are responsible for the inflammatory symptoms of pain and fever. The enzyme has two forms, COX-1 and COX-2, the first being responsible for both inflammation due to injury, and homeostatic regulation in the digestive tract and electrolyte balance. COX-2 is the principle form of the enzyme that deals solely with the inflammation response. The hydroxylated form of resveratrol showed an affinity to the binding sites of both forms of COX while the methoxylated forms did not (Murias, 2004). Resveratrol has also been shown to inhibit COX-2 without having an effect on COX-1's positive homeostatic role (Martinez, 2000). When examining the broader biological process of inflammation, resveratrol has also been shown to inhibit COX-2 activation by interfering with NF-KB's signaling pathway (Surh, 2001).

NF- κβ's role in inflammation is to increase the transcription of genes that lead to cytokine expression. Adipocytes that were treated with TNF-α to induce inflammation showed a down regulation of NF-κβ activated cytokines IL-2 and COX-2 after treatment with resveratrol (Gonzales, 2008). When looking at synovial cells in arthritic patients, NF- κβ was shown to be activated and therefore responsible for transcription of the inflammatory TNF-α and IL-1B genes. After treatment with resveratrol the cartilage matrix and synovial fluid of the experimentally induced inflammation group showed less change over the control group (Elmali, 2007). IL-1B was also examined in a study were human chondrocytes were treated with IL-1B to induce inflammation and degradation of the mitochondria, leading to apoptosis of the cells. Treatment with resveratrol in a time- and dose -dependent manner showed suppression of not only IL-1B, but of ROS and tumor suppressor p53 production as well (Csaki, 2008). In a two-week study of surgically induced chondrocyte osteoarthritis in rabbits, three different

concentrations of resveratrol were used to observe the effects of the compound. Resveratrol treatment decreased the rate of chondrocyte apoptosis, and reduced the production of NO in a dose-dependent manner (Wang, 2010).

Studies of other physiological systems that are susceptible to developing inflammation have also used resveratrol to determine whether its anti-inflammatory mechanisms can be observed as well. Using resveratrol to treat inflammation in the GI tract has shown promise in chronic inflammatory bowel disease. A study using mice that were fed 3mg/kg body weight of resveratrol, showed differences in in the expression of several cytokines compared to the control group's diet without resveratrol. The resveratrol group showed a decrease in the pro-inflammatory cytokines TNF- α and IL-1B, and a significant increase in the anti-inflammatory cytokine IL-10 (Sanchez-Fidalgo, 2010). Intestinal ischemia-reperfusion was induced in rats and followed by treatment with resveratrol during the surgery. The resveratrol group had a lower level of liver enzymes and the histological damage of the intestinal tissue was reduced by 60% over the control group (Yildiz, 2009).

Asthma is an inflammatory disease in the lungs that blocks the exchange of air into and out of the body. It is estimated that 358 million people globally suffer from the disease with the conventional pharmaceutical treatment being steroids (Wenzel, 2016). When treating A549 lung epithelial cell lines with 100 uM resveratrol it was observed that iNOS activity and COX-2 gene expression was inhibited, along with the inflammatory cytokine IL-8. The study further showed that resveratrol did not act on estrogen or glucocorticoid receptors, suggesting that resveratrol may be therapeutic where glucocorticosteroids have been ineffective, as in the case of COPD (Donnelly, 2004).

*Immune Cell Function and Autoimmune Disease*

The body's immune system is responsible for protection against any foreign object that enters the host organism and any resulting infection. The cells that are responsible for this are B and T lymphocytes, dendritic cells, lymphocytes and macrophages. These cells then cause immune responses in cell signaling via cytokines and interleukins in order to enlist the necessary biological functions to return to homeostasis. When the body no longer recognizes self, or the above cellular signaling does not work correctly, a condition known as autoimmunity arises. Autoimmunity can also arise from inflammation that does not resolve itself and becomes chronic.

Macrophages are phagocytic cells of the immune system that are responsible for not only destroying foreign cells in the body, but also producing antigens for T cell recognition, thus aiding in T cell activation. The body's unchecked production and chemotaxis of macrophages leads to a variety of diseases including heart disease, HIV infection, cancer, and unchecked granulomas in other autoimmune diseases. Macrophages that have been treated with resveratrol show a decrease in the secretion of TNF-α and an inhibition of NF-κβ leading to a decrease of macrophage chemotaxis (Bi, 2005). Macrophages also have a pro-inflammatory cytokine affect that has been shown to be attenuated when they are pretreated with resveratrol (Martinez, 2000).

Dendritic cells (DCs) are the sentinels in the immune system and are the chief antigen-presenting cells. They also have the capability to be inflammatory or tolerogenic depending on activation from their immature state. Inflammatory DCs can produce IL-1b, IL-12, and TNF-α; while tolerogenic DCs produce the immunosuppressive cytokines IL-10 and TGF-B. When resveratrol is present during cellular maturation the tendency is to become immunosuppressive in

nature as well as increasing immunoglobulin transcripts known to promote immunosuppressive cytokines such as IL-10 (Svajger, 2010; Manavalan, 2003). Over activity of inflammatory DCs have been linked to several autoimmune diseases such as lupus, Crohn's disease and ulcerative colitis.

CD4+ and CD8+ are T cells that play a role in the immune system's defense. CD4+ cells secrete cytokines which direct macrophages and B lymphocytes in combating infection, while CD8+ cells function as cytotoxic killer cells. Studies with resveratrol treatment, at varying concentrations and time exposures, have shown a change in the behavior of these cells in vitro. When parent T cells were treated with a lower concentration of resveratrol CD4+ and CD8+ formation was increased; while higher concentrations blocked their development (Falchetti, 2001). The same study showed that CD8+ cells had a significantly higher sensitivity to the effect of resveratrol than that of the CD4+ cells. Resveratrol was also shown to inhibit IL-2 mRNA expression at high doses, with IL-2 being the major secreted cytokine of CD4+. This suggests that resveratrol can be used in variable concentrations for different immunological needs (Gao, 2001). High concentrations of resveratrol have been shown to suppress CD8+ and lymphocyte function. As a result, graft acceptance and immunosuppressive effects with transplants have been examined with positive outcomes (Gao, 2001; Wu, 2006).

CD4+ cells were once believed to secrete the pro-inflammatory cytokine IL-17, which has been linked to autoimmune and chronic inflammatory diseases. In 1995 it was discovered that IL-17 was actually secreted from its own T helper lymphocyte, appropriately named Th17 (Korn, 2007). Th17 is derived from CD4+ cells when stimulated by IL-1B and IL-23 and in turn have their own cytokines IL-17A, IL-17F, IL-21 and IL-22. Th17 T cells have been documented to play a role in rheumatoid arthritis, type 1 diabetes, experimental autoimmune uveitis, asthma

and Crohn's disease (Murdaca, 2011). Resveratrol's effects on autoimmune disease have been studied from several different angles, ranging from arrest of Th17 cell differentiation to the suppression of its IL-17 expression. Resveratrol was shown to prevent Th17 cells from reaching maturity by blocking IL-10 signaling and reducing the secretion of IL-17 in mononuclear cells (Lanzilli, 2012; Petro,2011). The immune diseases of type 1 diabetes, asthma, rheumatoid arthritis, Crohn's and inflammatory bowel disease, uveitis, and multiple sclerosis all exhibit over expression of Th17 T cells or IL-17. Further discussion of the interactions between resveratrol and IL-17, and the role in autoimmune diseases, will be discussed in a later section.

## Resveratrol and the Eye

There are several studies reporting that resveratrol is capable of passing through the blood-retinal barrier (BRB) which helps maintain the eye's immune privileged state. Resveratrol's highly lipophilic property allows it to passively diffuse through the cells of the BRB (Wentzel, 2005). This is highly important as most topical treatments are only able to treat the anterior rather than the posterior section of the eye, which requires that treatment be administered systemically for cellular uptake (Toda, 2011). As resveratrol diffuses through the BRB it also lends its anti-inflammatory properties to the cells of the BRB. Degeneration of the BRB by inflammatory cytokines is just as detrimental to the eye as that of the retinal cells (Kim, 2012).

Treatment with resveratrol has been studied in several ocular diseases including glaucoma, cataracts, retinal glioblastoma, diabetes, uveal melanomas, age-related macular degeneration, multiple sclerosis, and uveitis. The pathological modes for diseases of the eye

vary, as well as resveratrol's molecular mechanism of action in each pathology. Glaucoma, cataracts and age-related macular degeneration (ADM) are all ocular diseases that are caused by sustained oxidative stress. In glaucoma, production of ROS in the cells leads to blocked circulation of aqueous humor via tubular meshwork, causing ocular hypertension (Lancon, 2016). Luna et al. showed that resveratrol inhibited the production of ROS, not only preventing oxidative stress, but also prohibiting the cytokine release that follows, causing inflammation (Luna, 2009). In a mouse model of glaucoma resveratrol was also shown to have a protective role if given before glaucoma was induced (Li, 2012). When resveratrol was given prior to oxidative agents, it prevented oxidative stress in a dose-dependent manner in human retinal cells (Pintea, 2011). Cataracts are thought to form due to oxidation of the lens' proteins, thus causing clouding of the lens and visual impairment. The action of resveratrol has been shown to prevent oxidation of the lens and increase the levels of glutathione in the lens, preventing further ROS activity and preserving lens function (Doganay, 2006).

Age-related macular degeneration (AMD) is caused by oxidation of the retinal cells and leads to loss of vision in those affected. Resveratrol's anti-oxidative abilities were demonstrated in retinal cells at low doses, thus preventing AMD (Sheu, 2010). Another characteristic of AMD is the abnormal repair of cells leading to angiogenesis of the retinal tissue. Similar to AMD in pathology is macular telangiectasia (MT), in which the photoreceptors associated with the retina deteriorate due to abnormal neovascular proliferation (Yannuzzi, 2006). Abnormal cellular growth is attributed to an over expression of VEGF in the cells. Resveratrol is known to be an inhibitor of VEGF expression, and when used in mouse studies, has been shown to suppress VEGF in retinal cells (Richer, 2013; Hua, 2011).

Directly linked to resveratrol's anti-angiogenesis activity in the eye is its anti-tumorigenic effects. Inhibiting VEGF suppresses tumor growth, but resveratrol has also been shown to induce apoptosis in malignant cells. Uveal melanoma is a malignancy of the uveal tract that initiates positive apoptosis when treated with resveratrol. Local injection of resveratrol to the area showed that normal cells did not undergo apoptosis similar to their malignant neighbors (van Ginkel, 2008). When resveratrol was used to treat retinoblastoma cell lines, it was shown that growth of the cell line was not only inhibited, but apoptosis was induced in a time- and dose-dependent manner (Sareen, 2006).

Apoptosis of retinal cells is observed as retinal diseases advance, thus leading to loss of cellular function and vision impairment. Most of the apoptotic events can be tied to an inflammatory response that continues unchecked. In the case of diabetic retinopathy, high levels of blood glucose cause inflammation of retinal cells leading to deterioration of the BRB and vision loss. When resveratrol was given in a dose-dependent manner it blocked activity of the cytokines responsible for the hyperglycemic inflammation of the retina, and increased the integrity of the BRB (Losso, 2010). In a rat model of diabetes, animals were induced after being fed a diet with or without resveratrol. Rats on the resveratrol-supplemented diet showed a decrease in cellular apoptosis of the lens epithelium (Wang, 2015).

As previously discussed, autoimmunity can be caused by unchecked inflammation or the body's inability to recognize self. Indiscriminate of the cause, autoimmune responses are governed by several proteins, enzymes and cytokines; and retinal cells with induced inflammation show an increased expression of these factors. Kutty et al. showed that treatment with resveratrol blocked the expression of CXCL11 and decreased that of NF-κβ (2015). Resveratrol has also been shown to inhibit COX-1, COX-2, IL-6, IL-8, ICAM-1, SIRT-1, MCP-

1 and NF-κβ in retinal cells (Dugas, 2010; Pintea, 2011; Lancon, 2016; Bola, 2014). These various proteins, enzymes, and cytokines have all been linked to various ocular inflammatory diseases.

## Resveratrol and Uveitis

Resveratrol's effects are well documented in other ocular diseases, however its role in the pathology of uveitis is poorly understood. The most referenced study is that by Kubota et al. (2009) in which uveitis (EIU) was induced in male mice and oral supplementation of resveratrol was given for 5 days at 5, 50, 100 and 200mg/kg. The study showed a decrease in the expression of NF-κβ by increased SIRT-1 activity, inhibition of leukocyte adhesion, and a decrease in oxidative stress, all of which contribute to uveitis pathology. These studies have been repeated in order to further investigate NF-κβ and SIRT-1 inhibition in uveitis (Kubota, 2010). Resveratrol has shown great potential as a treatment for AU and EU as evidenced by the study of these diseases, and the cellular effects that resveratrol has exhibited in other diseases.

# CHAPTER III

# MATERIALS AND METHODS

The following protocols were used to obtain the data necessary to run statistical analysis. Protocols were constructed from a study of the literature pertaining to the subject areas.

## Cell Culture

### *Reagents*

Growth media was prepared by combining DMEM and F12 medias (Gibco) at a one to one ratio for a total of 500ml. To that 50ml of fetal bovine serum was added along with 5ml of pen/strep. Phosphate buffed saline (PBS) was prepared in both 10x and 1x concentrations. The premixed crystallizes buffer (Gibco) was added to a 1L bottle, filled to 1.0L with RO water, autoclaved and then stored at room temperature. The 1x concentration was made by transferring 100ml of the 10x solution to a sterile 1L bottle and filling it to 1.0L with RO water and stored at room temperature. The resveratrol (RV) (Sigma) was prepared by suspending 4.6mg in a few drops of 100% EtOH and queuing it to 20ml with sterile PBS yielding 20ml of a 1.0mM solution. It was then aliquoted in sterile 2.0ml microfuge tubes and stored at -20C.

*Thawing of frozen cells*

ARPE-19 cells (ATCC) were removed from frozen storage and immediately placed in a 37C water bath until the ice pellet thawed. As soon as the ice crystals melted the cells were pipetted into a sterile 25cm culture flask (Corning). 6ml of pre-warmed complete media was added drop by drop into the flask so as not to shock or lyse the cells. Cells were then incubated at 37C, and 5% CO2, and checked in 24 hours for attachment. If attachment occurred the media was replaced at that time.

*Passaging adherent cells*

After ARPE cells became confluent in the culture flask they were passaged with a 0.25% trypsin (Corning) in order to have enough cells for the experiment. Media was pipetted from the flask and 1ml of trypsin, that had been warmed in a 37C water bath, was added to the flask and gently rocked to wash the surface of the cells and remove any remaining media. The trypsin was removed, replaced with 2ml of fresh trypsin and the flask was placed in the incubator for 2 minutes. When the cells showed detachment from the flask 3ml of warmed media was added to the flask to inactivate the trypsin. The cells were then aspirated to dissociate any clumps and the back of the flask was rinsed with its own contents to remove all cells. The resulting suspension was transferred to four culture flasks and 6ml of media was added to each. Flasks were then placed back into the incubator and checked at the 24 hour mark for attachment. Media exchange then took place every 2 to 3 days to feed the cells until the flask was confluent.

## Pilot Study to Determine Resveratrol Dose Range

APRE cells were treated with the proposed resveratrol doses of 50µM, 100µM and 200µM to test for detachment before the experiment was started. Cells were trypsinized as previously described and dissociated into a single-cell suspension for counting. Cells were then counted using a hemocytometer, yielding a concentration of 3.05 x 10+E5, and then suspended in 18ml of complete medium. The prepared 1.0mM resveratrol was then plated on a six well cell culture plate (Corning), in duplicate, at 150µl, 300µl and 600µl to yield concentrations of 50µM, 100µM and 200µM respectively, in a final volume of 3.0ml. The cell suspension was then added to its appropriate resveratrol well at 2.8ml, 2.7ml and 2.4ml. The plate was then incubated for 24 hours and observed for detachment.

## ARPE Cell Treatment with Resveratrol

Each condition (N = 6) was prepared on a 6 well cell culture assay plate (Corning). There were three controls in the experiment; 1) untreated ARPE cells, 2) ARPE cells that were treated with 50µM of resveratrol for 24 hours to determine any effect on inflammatory response in normal cells, and 3) ARPE cells that were treated with TNF-α for 24 hours to verify induced inflammatory response. The experiment itself was a time/dose course of resveratrol at 12, 24 and 48 hours with the concentrations being 50µM, 100µM and 200µM of resveratrol.

Procedure for Passaging and Plating of ARPE Untreated Control Cells

Four flasks of confluent ARPE cells were used for the untreated control. Media was pipetted from the flasks and cells were trypsinized using standard cell culture technique. Cells were then transferred to a 50ml conical tube, and 1ml of the cell suspension was transferred to each culture flask with 6ml of warmed media to reseed the flasks.

1ml of the cell suspension from the conical tube was transferred to a microfuge tube, then 100ul from that tube was transferred to another sterile microfuge tube, and finally 10ul of the second microfuge tube was pipetted into a hemocytometer slide to come up with a cell count. The cell count was then used to calculate a cell count of 5 x 10+E6 cells per well in a final volume of 3ml per well. The plated untreated ARPE cells were then returned to the incubator for 24 hours. After 24 hours the cells were lysed in order to isolate total RNA.

Procedure for ARPE Control Cells treated with Resveratrol

Four flasks of confluent ARPE cells were used for the resveratrol treated control. Media was pipetted from the flasks and trypsinized using standard cell culture technique. Cells were then transferred to a conical tube, with 1ml of the cell suspension returned to each culture flask with 6ml of warmed media to reseed the flasks.

1ml of the cell suspension from the conical tube was transferred to a microfuge tube, then 100µl from that tube was transferred to another sterile microfuge tube, and finally 10µl of the second microfuge tube was pipetted into a hemocytometer slide to come up with a cell count. The cell count was then used to calculate a cell count of 5 x 10+E6 cells per well in a final

volume of 3ml per well. The volume of cell suspension was then transferred to a second conical tube and warm media was added to equal 18ml total.

150µl of resveratrol was added to each well along with 2.85ml of the cell suspension, for a total well volume of 3ml. The plated resveratrol treated ARPE cells were then returned to the incubator for 24 hours. After 24 hours the cells were lysed in order to isolate total RNA.

Procedure for ARPE Control Cells treated with TNF-α

Four flasks of congruent ARPE cells were used for the TNF-α treated control. Media was pipetted from the flasks and trypsinized using standard cell culture technique. Cells were then transferred to a conical tube, with 1ml of the cell suspension returned to each culture flask with 6ml of warmed media to reseed the flasks.

1ml of the cell suspension from the conical tube was transferred to a microfuge tube, then 100µl from that tube was transferred to another sterile microfuge tube, and finally 10µl of the second microfuge tube was pipetted into a hemocytometer slide to come up with a cell count. The cell count was then used to calculate a cell count of 5 x 10+E6 cells per well in a final volume of 3ml per well. The volume of cell suspension was then transferred to a second conical tube and warm media was added to equal 18ml total.

30ul of TNF-α suspended at 5ng/µl was added to each well along with 3ml of the cell suspension. The plated TNF-α treated ARPE cells were then returned to the incubator for 24 hours. After 24 hours the cells were lysed in order to isolate total RNA.

Procedure for ARPE Cells with TNF-α Induced Inflammation and Treated with Resveratrol in a Time/Dose Dependent Manner

Four flasks of congruent ARPE cells were used to plate each condition. Media was pipetted from the flasks and trypsinized using standard cell culture technique. Cells were then transferred to a conical tube, with 1ml of the cell suspension returned to each culture flask with 6ml of warmed media to reseed the flasks.

1ml of the cell suspension from the conical tube was transferred to a microfuge tube, then 100ul from that tube was transferred to another sterile microfuge tube, and finally 10µl of the second microfuge tube was pipetted into a hemocytometer slide to come up with a cell count. The cell count was then used to calculate a cell count of 5 x 10+E6 cells per well or a final volume of 3ml per well. The volume of cell suspension was then transferred to a second conical tube and warm media was added to equal 18ml total.

2.97ml of the cell suspension was placed in each well of the plate along with 30µl of the suspended TNF-α. The plate was placed in the incubator for 24 hours before treatment with resveratrol occurred. For the cells that were treated with 50µM of resveratrol 150µl of the media was removed from each well and replaced with 150µl of the prepared resveratrol. For the cells that were treated with 100µM of resveratrol 300µl of the media was removed from each well and replaced with 300µl of the prepared resveratrol. For the cells that were treated with 200µM of resveratrol 600µl of the media was removed from each well and replaced with 600µl of the prepared resveratrol. Plates were then returned to the incubator for 12, 24 or 48 hours. After the time course was reached cells were lysed in order to isolate the RNA.

## Cell Lysis and RNA Isolation

Total RNA was isolated from all samples using the RNaqueous-Micro Total RNA Isolation Kit (Invitrogen™). Plates were removed from the incubator at their appropriate time points and the culture media aspirated from each well. 300ul of cell lysis buffer was added to each well. The plate was then gently agitated to completely lyse all cells. Lysate was then aspirated with a pipette to thoroughly detach cells and disperse RNA into solution. 150µl of 100% EtOH was then added to each well and aspirated to mix thoroughly. Lysate containing detached cells from each well was then transferred to sterile micro filter columns and caps closed. Columns were centrifuged for 10 seconds at max speed till all lysate passed through and filtrate was discarded. 180ul of Wash Solution #1 was added to each tube and pulse spun in the centrifuge for 10 seconds with filtrate then being discarded. 180ul of Wash solution #2 was added and centrifuged for 10 seconds; this was repeated with the filtrate being discarded after each step. Columns were then centrifuged for one minute to remove all residual fluid before being transferred to sterile labeled elution tubes. 10µl of elution buffer, pre-heated to 75C, was added to each tube at the center of the filter cartridge and incubated at room temperature for one minute, before being centrifuged for 30 seconds in order to elute RNA into the tube. A second 10µl of elution buffer was added and the process repeated. RNA was then stored at -80 C until further use.

## RNA Quantification

Total RNA was quantified by UV spectrophotometry. Absorption was measured at 260nm and 280nm, and the A260/A280 ratios were also calculated to determine purity of

samples. A ratio of 2.0 ± 0.1 was considered optimal. Absorption peaks at 260nm were observed for verification of RNA product. See Appendix A.

RNA GEL

RNA gel was prepared by heating 1g of agarose in 36ml of water until dissolved and then cooled to 60C. 5ml of 10X MOPS buffer was then added with 9ml of 37% formaldehyde. The gel cast was then assembled, a comb to form 25µl wells inserted, and the gel poured. After the gel polymerized, enough 1X MOPS buffer was added to cover the gel and the comb removed. Samples were prepared by mixing 1µg of the RNA, along with 8µl of formaldehyde loading dye and 2µl of EtBr. Samples were then heat denatured for 10-15 minutes at 70C. Gel was electrophoresed at 80V until the bromophenol blue had migrated 2/3 the length of the gel. The integrity of the RNA was verified by visualizing the distinct bands of the18S and 28S ribosomal subunits under UV light.

cDNA Synthesis

cDNA was synthesized from 500ng RNA from each sample using the Verso cDNA Synthesis Kit by Invitrogen® (Thermo-Scientific™). The appropriate volume of RNA was transferred to sterile PCR reaction tubes and DNase-free water was added to equal 12.0µl) A master mix containing 5X reaction buffer, dNTPs, polyT primer, and reverse transcriptase enzyme was prepared, and 8ul added to each reaction tube containing the 12µl of RNA plus water. The tubes were then pulse centrifuged and placed on a 42C heat block for 30 minutes. To stop the reaction the tubes were then placed on a second heat block at 95C for 2 minutes.

Samples were then placed in an ice bath for 5 minutes before being spun down again. A 1:10 dilution was made by adding 180µl of sterile DNase-free water and aliquoted in 50ul. During the aliquoting procedure 5ul of each sample was also pooled in one sterile tube to be used later to make a standard curve. cDNA was then kept at 4C for immediate use or -20C for long term storage.

Inflammatory Cytokine Arrays

RT2 Inflammatory Cytokine Arrays (Qiagen™) were purchased and run according to the manufacturers specifications. cDNAs for each condition (N = 6) were pooled and run as a single sample on each plate. A 96 well plate containing 84 gene specific primers for inflammatory cytokines was run for each of the controls and each of the time/dose dependent conditions to quantify expression of inflammatory responses using the CFX96 Touch Real-Time PCR system (Bio-rad). A master mix was prepared with 1250µl of 2X SYBR Mastermix (Qiagen), 100ul of the cDNA template and 1150ul of RNase-free water for each condition. 25µl of this mixed was transferred to each well of the array plate and then carefully sealed with the cap strips provided. The plate was then centrifuged for one minute at 1000g at room temperature to remove bubbles. The plate was then placed in the Bio-Rad instrument and run for 40 cycles by the following protocol: 3 min @ 95 C hot start; 30 sec @ 60 C, and 30 sec @ 95 C x 40 cycles; 1 min @ 95 C; plus a final dissociation curve.

## Primer Test

Gene specific primers were either designed using Primer Design software or identified in the published literature, and purchased through IDT Technologies™. All primers were first tested by running a standard curve with cDNA samples to verify a single amplicon and optimize time and temperature parameters for qPCR. Sequences for all primer pairs are included in Appendix C.

## Verification of Candidate Genes by Quantitative PCR

Five genes were chosen for verification based on the change in expression observed from the Inflammatory Cytokine arrays. The criteria for selection was based on a minimum 1-fold difference, indications of a time- or dose-dependent effect, and relevance to the study. The condition(s) with the most significant expression change, for each gene, were then run on individual PCR plates with gene specific primers to precisely quantify the data, enable statistical analysis, and validate the evidence of dose or time effect. For each plate a standard curve was constructed from a 4 x five-fold dilution series of pooled cDNA samples by adding 2.0µl of each dilution in triplicate to individual wells. Each plate also contained 2.0µl in duplicate for each untreated control, treated TNF-A control and treated RV control sample. The remaining wells contained cDNAs from the conditions of interest to quantify and compare their differences in expression. To these wells 20µl of the gene primer master mix was added for a total well volume of 22µl. The plate was then placed in the thermocycler and run according to the following protocol: 3 min @ 95 C hot start; 30 sec @ 60 C, and 30 sec @ 95 C for 40 - 55

cycles; 1 min @ 95 C; plus a final dissociation curve. Ribosomal 18S subunit was also run for all samples as an internal control and for data normalization.

## Statistical Analysis

Statistical Analysis was done using the Prism 6 statistical analysis program (GraphPad™) to run 1-Way ANOVA, Tukey's and independent T-tests. All data charts for individual gene expression were generated by the same program.

# Chapter IX

# Results

## Pilot Study

The pilot study was done to determine the dose range of resveratrol treatment on control ARPE cells both to avoid cell detachment and to observe the effect of resveratrol on cell viability. The 50μM and 100μM wells showed minimal to no detachment, while the 200μM wells showed 50% detachment. Trypan blue was used to assess cell viability in the detached cells and showed the viability was greater than 75%. A dose range of 50, 100 and 200μM resveratrol was deemed acceptable based on these results and supported by the literature (Boocock, 2007; Donnelly, 2004; Lasa, 2012; Wang, 2010)

## RNA Purity

All samples quantified by UV spectrophotometry fell within the optimal A260/A280 ratio range of 2.0 ± 0.1. See Appendix A

RNA Gel

The RNA gel was run to check the integrity of the TNF-α-C control. Under the UV transiluminator the RNA was shown to be intact, as the 18S and 28S ribosomal bands were clearly visible with the 28S band being twice as intense as the 18S band. The 2:1 ratio is used to show that the RNA is intact, were a smear across the gel would indicate degraded RNA.

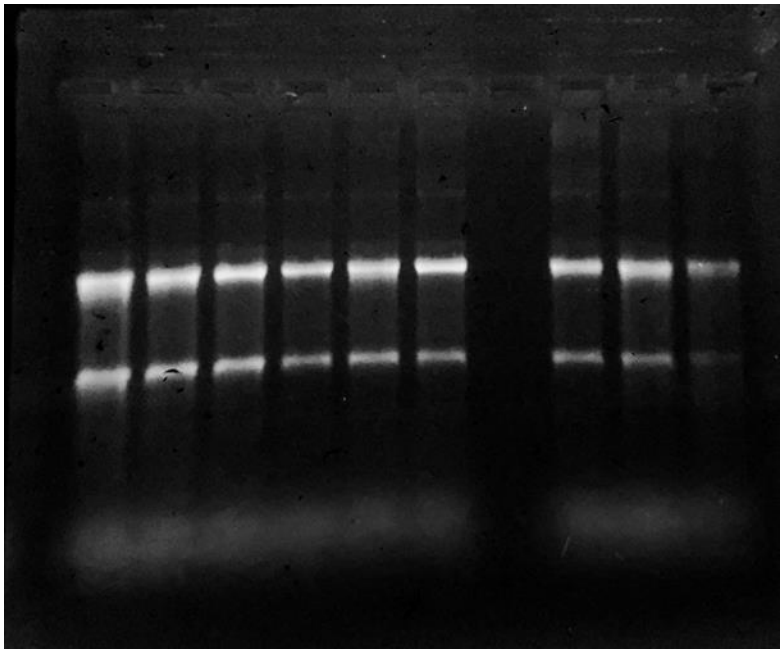

Figure 2 RNA Gel

Inflammatory Cytokine Arrays

The gene array assays yielded a preliminary list of genes which were differentially expressed relative to the controls, across all treatment groups. Candidate genes for follow-up quantitative PCR validation were selected based on their difference in fold-change compared to controls. The criteria for selection was a plus or minus 1-fold difference in relative copy

number. This list was then filtered according to relevance to uveitis and whether a dose- or time-dependent effect was observed.

Five genes were ultimately selected for validation by qPCR.

| GenBank # | Abbreviation | Scientific Name | Fold Change* |
|---|---|---|---|
| NM_002982 | CCL2 | Chemokine (C-C Motif) Ligand 2 | + 2.06 |
| NM_052872 | IL17F | Interleukin 17F | - 0.81 |
| NM_033439 | IL33 | Interleukin 33 | + 1.36 |
| NM_003376 | VEGFA | Vascular Endothelial Growth Factor A | + 3.94 |
| NM_000639 | FASLG | Fas Ligand (TNF Superfamily, member 6) | + 0.41 |

Table 1. Genes selected for validation. *increase or decrease from Control Cq value to TNF Cq value.

Quantitative PCR

All candidate genes were successfully quantified according to MIQE recommendations. Amplification results were validated by observation of single dissociation peaks. See Appendix B.

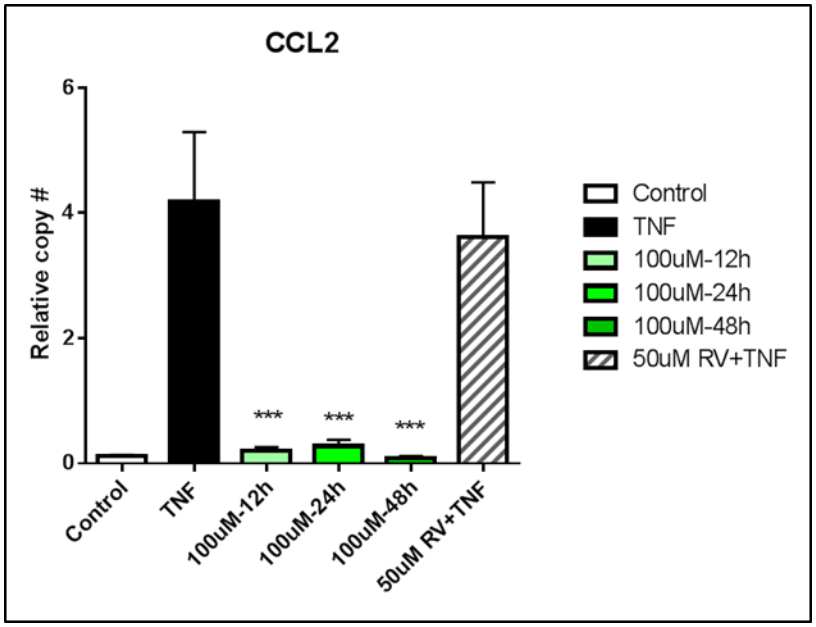


Fig. 3. CCL2 expression. CCL2 expression returned to control levels after resveratrol treatment. Tukey's multiple comparison test showed a significant down-regulation of CCL2 in cells treated with 100uM RV, at 12, 24, and 48h, compared to TNF-α. Pre-treatment with 50uM RV for 24h did not attenuate CCL2 up-regulation when cells were then exposed to TNF-α. [Bars represent Mean ± S.E.M.; *** = $p<0.001$] N=6

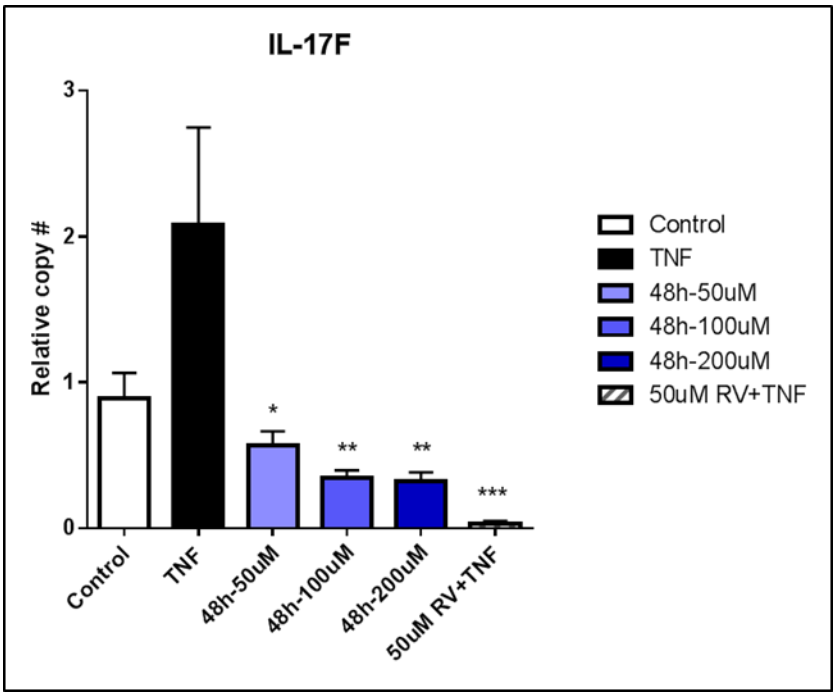


Fig. 4. IL-17F expression. IL-17F expression returned to control levels after resveratrol treatment. Down-regulation of IL-17F was dose-dependent in cells treated for 48h exposure, plateauing at 100uM (Tukey's multiple comparison test). Pre-treatment with 50uM RV for 24h followed by TNF-α inhibited IL-17F up-regulation. [Bars represent Mean ± S.E.M.; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$] N=6

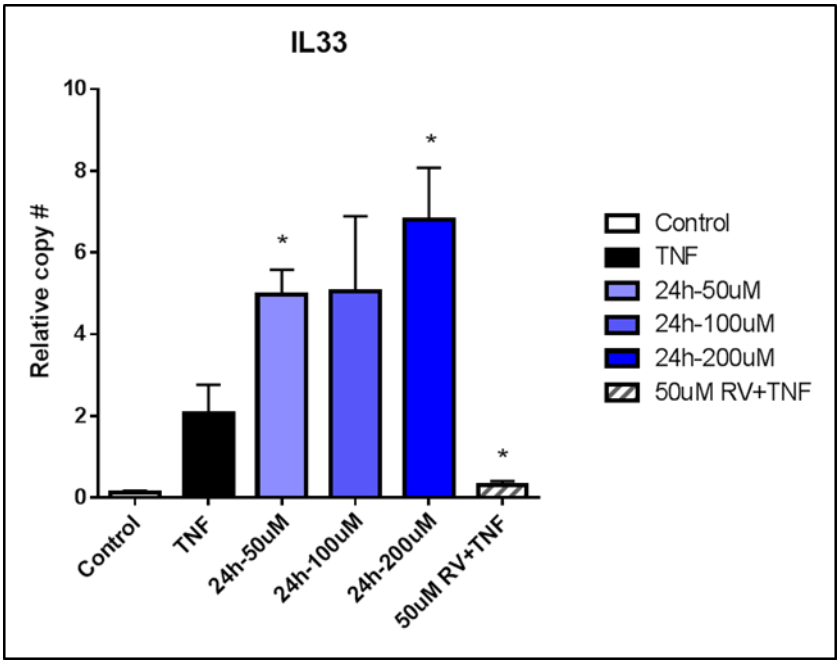


Fig. 5. IL-33 expression. One-way ANOVA showed the means were significantly different ($p<0.01$). Individual t-test also showed significant up-regulation of IL33 in 50uM and 200uM RV v. TNF-α, at 24h exposure (promoting chemotaxis?). Pre-treatment with 50uM RV for 24h did not induce this response in IL33. [Bars represent Mean ± S.E.M.; * = $p<0.05$] N=6

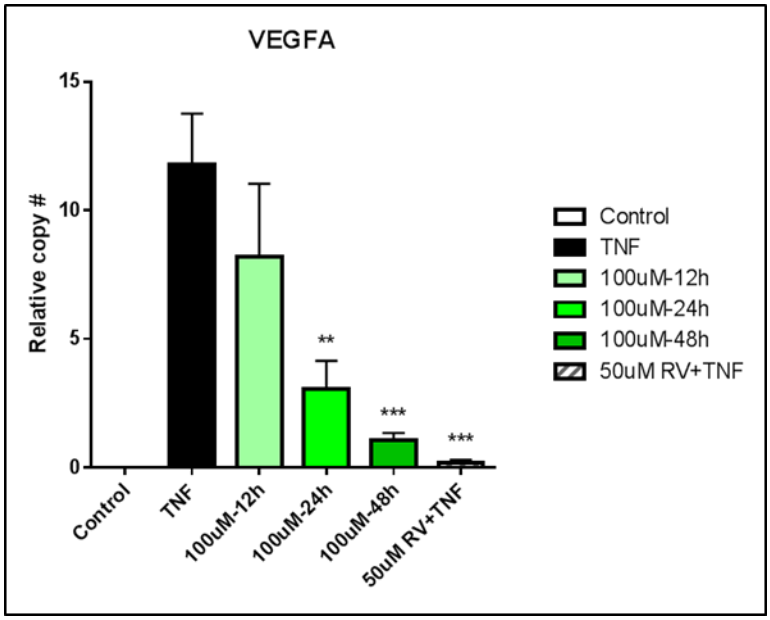


FIG. 6. VEGFA expression 1. Time course of VEGFA expression with 100uM RV. Time course of 100uM RV treatment shows significant down-regulation of VEGFA compared to TNF-α. Pre-treatment with 50uM RV followed by TNF-α inhibited up-regulation of VEGFA. [Bars represent Mean ± S.E.M.; ** = $p<0.01$; *** = $p<0.001$] N=6

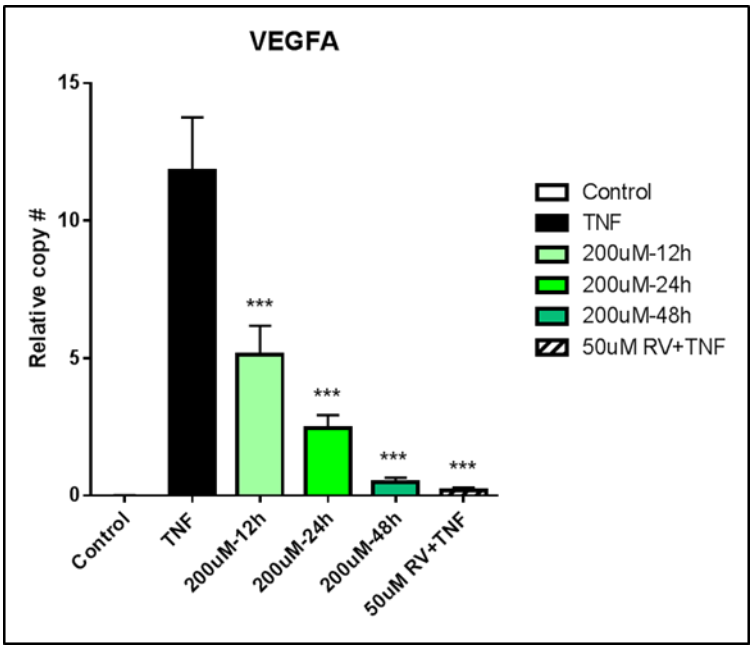


Fig. 7. VEGFA expression 2. Time course of VEGFA expression with 200uM RV. Treatment with 200uM RV showed significant down-regulation of VEGFA expression compared to TNF-α that is time-dependent. Pre-treatment with 50uM RV for 24h followed by TNF-α attenuated up-regulation of VEGFA. Two-way ANOVA showed a significant interaction of dose and time ($p<0.01$). [Bars represent Mean ± S.E.M.; * = $p<0.05$; *** = $p<0.001$] N=6

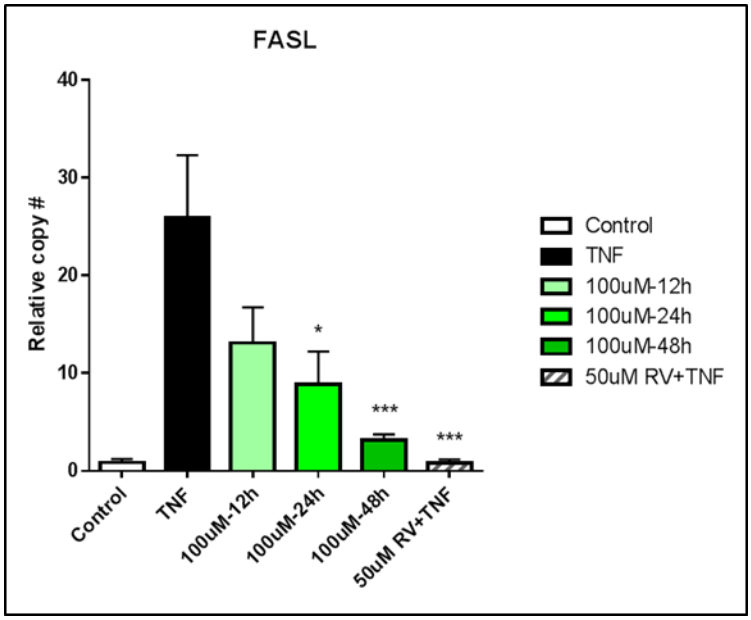


Fig. 8. FASL expression. Tukey's multiple comparison test showed a significant time-dependent down-regulation of FASL in cells treated with 100uM RV, at 24h and 48h, compared to TNF-α. Pre-treatment with 50uM RV for 24h followed by TNF-α inhibited up-regulation of FASL. [Bars represent Mean ± S.E.M.; * = $p<0.05$; *** = $p<0.001$] N=6

# CHAPTER X

# DISCUSSION

Five candidate genes were selected for validation by quantitative PCR (qPCR) based on the results of the cytokine arrays. All five genes play a role in either human or equine uveitis, or demonstrate an alternative interaction with resveratrol in other studies.

## Vascular Endothelial Growth Factor (VEGF)

In healthy cells VEGF is responsible for recruiting the formation of new blood vessels and muscle tissue. TNF-α induces overexpression of VEGF which promotes cancer metastasis and vascular disease in other tissues (Hancock, 2017). VEGF can also be chemotactic for macrophages, granulocytes and leukocytes in the eye; with excessive retinal leukocyte attachment being one of the leading causes of uveitis in both human and equine systems (Rutar, 2016; Curto, 2016). The presence of VEGF has been confirmed in several uveitis studies of the aqueous humor, tears, uveitis induced human retinal pigmented epithelial (HRPE) cell lines, and in rabbit and mouse models (Decker, 2013; Fine, 2001; Carreno, 2016; Ha, 2014; Nagineni, 2014).

Nagineni et al. observed that resveratrol suppressed the expression of VEGF in HRPE cells at doses as low as 10μM for 24 hours, following induced inflammation (2014). This study focused on VEGF's tendency to cause retinal vascular pathologies like vascular leakage and neovascularization when over expressed, as in the case of AMD. The authors also suggested that

resveratrol could be effective in treating diabetic retinopathy, which if left unchecked becomes an autoimmune disease leading to uveitis. Ha et al. looked specifically at HRPE cells and mice in which inflammation had been induced with TNF-α. VEGF expression was significantly reduced when muscadine grape polyphenols were administered in doses ranging from 25 to 100μg/mL (2014). In addition, several ARPE-19 cell lines also showed reduced VEGF secretion when treated with 25 – 100μg/mL resveratrol after induced inflammation (Lacon, 2016; Losso, 2010).

Pharmaceutically there are anti-VEGF drugs on the market that are used in autoimmune diseases, including uveitis, in order to target the vascular component of the disease (Kurup, 2009). The drug is delivered to the eye by injection, every one-to three-months at the ophthalmologist's discretion. Side effects of anti-VEGF drugs include systematic high blood pressure leading to thrombo-embolic effects such as stroke, myocardial infarctions and vascular death (Moshfeghi, 2016). Oral supplementation of resveratrol showed clinical improvement in the retina of patients with AMD in two separate studies (Richer, 2009, 2013). Additional studies of this type are needed to increase the data base, broaden our overall knowledge of resveratrol's action, and elucidate its potential as an anti-VEGF intervention in ocular disease.

This study demonstrated that ARPE-19 cells treated with resveratrol following induced inflammation showed suppressed VEGFA expression, which was both time- and dose-dependent. Cells treated with 100uM resveratrol for 12, 24 and 48h showed a linear decline in VEGFA expression which was highly significant at 24 and 48h. Cells treated with 200μM resveratrol showed a significantly greater down regulation which was also linear, indicating an interaction of time and dose. Cells treated with 50μM resveratrol for 24h prior to TNF-α completely blocked the up-regulation of VEGFA induced by the immune response. These data

suggest that resveratrol has great potential as an anti-VEGF treatment for ocular and other diseases.

## Interleukin-33

Interleukin-33 (IL-33) is a cytokine found in endothelial cells and is released after cell injury, alerting the immune system to trauma or infection in the damaged tissue (Liew, 2016). Recent studies show that IL-33 has two modes once activated depending on tissue type. It can increase inflammation by recruiting Th2 cells and neutrophils in lung tissue, while in the skin this is seen in conjunction with inducing TNF-α production. However, in the peritoneal cavity IL-33 activation induces B cells to produce IL-10 which is an anti-inflammatory cytokine (Hardman, 2016). IL-33 differentiation has also been described as antigen dependent. In cases of bacterial infection IL-33 takes on the role of being antibacterial through recruitment of macrophages and neutrophils. In the case of inflammatory diseases, tissue specificity has been the determining factor in the expression of IL-33 in the immune response (Liew, 2016).

Barbour et al. published a study on uveitis in 2016 which documented the role of IL-33 as that of autoimmune suppressor, and should be considered as an option for treatment. IL-33 was first assessed in the retina of uveitis-induced mice to confirm its pathology in the disease; and a later trial using IL-33 as a pretreatment pharmaceutical resulted in pretreated mice showing reduced pathological scores and significantly less retinal inflammation. A third study utilized transgenic mice that were negative for the IL-33 receptor ST2. The ST2-/- mice showed no response to treatment with IL-33 after inflammation was induced, whereas the control mice treated with IL-33 showed minimal inflammation of the retina. In addition, spleens of both

treated and control mice were examined to identify IL-33's role in further cytokine production of uveitis. Mice treated with IL-33, when compared to controls, showed significantly less expression of IFN-ϒ and IL-17, two cytokines known to be produced during uveitis (2014).

There is a lack of literature on the interaction between IL-33 and resveratrol, and even less on its interaction with uveitis. Another question that arises from the research is the interaction between IL-33 and IL-17. IL-17 is recognized as the major inflammatory cytokine in both human and equine uveitis, and resveratrol has been shown to down regulate its expression in auto immune diseases (Malalana, 2015; Asadi-Samani, 2017). This poses the question as to whether IL-17 down regulation is due to resveratrol itself, increased expression of IL-33 due to resveratrol, or a combination of both pathological pathways.

In this study cells treated with TNF-α showed a small up-regulation of IL-33, indicating an effort by the cells to launch a counter response to inflammation. Cells treated first with TNF-α, followed by resveratrol, showed a significantly enhanced up-regulation of IL-33 which was dose-dependent. This suggests that resveratrol itself is an effective anti-inflammatory agent by its active promotion of an anti-inflammatory pathway. Pre-treatment with resveratrol followed by TNF-α suppressed up-regulation of IL-33 in response to induced inflammation. This conforms with the mechanism of IL-33 activity to attenuate the development of uveitis as proposed in the Barbour study.

Chemokine Ligand 2

Chemokine ligand 2 (CCL2) is a chemokine that is secreted by monocytes, macrophages, dendritic cells, neurons, astrocytes and microglia. Expression of CCL2 is regulated by platelet

derived growth factor (PDGF) or TNF-α which is triggered by cellular injury (Antoniades, 1991; Wegscheider, 2005). CCL2 is chemotactic for basophils and monocytes; with monocytes being responsible for several autoimmune diseases including RA, psoriasis, atherosclerosis and uveitis, as well as neuroinflammatory diseases such as Alzheimer's (Hancock, 2017).

CCL2 has been shown to be involved in the development of uveitis, regardless of its cause, and maintains elevated expression throughout the disease (Curnow, 2005). Carreno et al. found that CCL2 is expressed in 75% of both uveitis individuals and healthy controls. This demonstrates the autoimmune nature of uveitis once the underlying cause has triggered the disease (2016). Polymorphisms of the CCL2 gene result in an increase in acute anterior uveitis at a 2:1 ratio over controls with a single copy of the gene. Identifying genetic predisposition could potentially aid in early diagnosis and open the door for studies in gene therapy specific to uveitis (Wegscheider, 2005).

Resveratrol's effects on the expression of CCL2 have been studied in retinal pigmented epithelial cells that have had an inflammatory response induced. Kutty et al. showed that pretreating cells with 50μM of resveratrol significantly reduced the expression of CCL2 post inflammation as opposed to no pretreatment (2015). Studies of resveratrol's effects on CCL2 expression have also been done in other tissue and cell models. Tung et al. observed the effect of resveratrol in mouse liver at three stages of development. Their findings showed that resveratrol not only decreased CCL2 expression in the older population, but also decreased TNF-α expression across all three age groups (2015). Pretreatment with resveratrol was also tested in RAW2647 and THP-1 cells at varying concentrations, before inflammation was induced. RAW cells showed a significant decrease in CCL2 expression at 50μM and THP-1 cells at 25μM (Schwager, 2017).

In this study post-inflammation treatment with 100uM resveratrol resulted in significant down-regulation of CCL2 which was consistent across all time exposures. This study showed no significant difference in CCL2 expression by pretreating with resveratrol. Although there was a slight down regulation of the gene with pretreatment this was not significant. These data support previous reports of resveratrol's effect on CCL2 after post-inflammation treatment, however CCL2 did not show the same response as the other genes to pre-treatment. A search of the literature shows the Kutty study being the only data source for CCL2 expression in ARPE cells that have been treated with resveratrol prior to inflammation. Additional studies are therefore warranted to better understand the precise effect resveratrol has on CCL2 signaling.

Fas ligand

Fas ligand (FASL) is a member of the tumor necrosis factor (TNF) receptor family and is expressed by T lymphocytes. It induces apoptosis by forming what is known as the death-inducing signaling complex (Hancock, 2017). Cells in the immune privileged eye also secrete FASL and destroy any lymphocytes that infiltrate the area. Lymphocytes have also been shown to release FASL which leads to the activation of IL-8 and nuclear factor κβ (NF-κB). IL-8 is known to be chemotactic for granulocytes and facilitate angiogenesis, while NF-κβ is known to regulate expression of inflammatory genes. (Sugita, 2000).

FASL's role in uveitis is thought to be that of lymphocyte chemotaxis and increased expression of inflammatory cytokines through NF-κβ. In a study of FASL levels in the AH of eyes with and without active uveitis, those eye that were considered active had a statistically higher amount of FASL over those eyes in the quiescent state of uveitis. The study suggested

that FASL had a regulatory role in the inflammation flare ups that are part of the etiology of uveitis (Sugita, 2000). The association of RPE and monocytes has been shown to cause a significant increase in FASL expression from the monocytes. RPE cells are located between the choroid and photoreceptors and secrete FASL as part of their role in the immune privilege of the eye. The interaction of monocytes and RPE cells in the eye is unusual unless pathological changes have occurred from disease, inflammation or trauma. Once RPE cells and monocytes interact with one another monocytes begin to express FASL, resulting in RPE cell death (Hettich, 2014).

The interactions between resveratrol and FASL are documented in other tissues, while those interactions in tissues specific to uveitis have been few. Resveratrol's effect on cancer cells has been documented in numerous studies showing that resveratrol elicits FASL apoptotic signaling in malignant cells (Wu, 2016; Leischer, 2016; Mulakayala, 2013). Resveratrol has been shown to down-regulate FASL, therefore reducing inflammation in other autoimmune disease etiologies including rheumatoid arthritis and atherosclerosis (Marino, 2015; Ruan, 2013).

With FASL reported as the cause of cell death in RPE cells and evidence showing resveratrol down-regulates FASL in autoimmune diseases, experiments specifically focusing on the interaction between the two in uveitis are warranted. This study showed that resveratrol reduced FASL expression in a time/dose and pretreatment course, in ARPE cells that had inflammation induced with TNF-α. The most significant effect was seen in 100μM concentrations and was enhanced as exposure time increased. Pre-treatment with resveratrol was significantly effective in attenuating the up-regulation of FASL after TNF-α exposure. This supports the existing data from studies of other autoimmune diseases.

Interleukin 17F

Interleukin 17F (IL17F) is produced by Th-17 helper cells after stimulation by the inflammatory cytokine interleukin-23. Expression of IL-17F causes the recruitment of monocytes and neutrophils, along with the production of other cytokines, chemokines and prostaglandins from other cell types, including epithelial cells. It is the dysregulation of the Th17 helper cells that is responsible for IL-17F's role in autoimmune diseases (Hancock, 2017; Park, 2005).

IL-17F was reportedly found in the eyes of ERU horses (Regan, 2012; Simeonova, 2016) and was previously discussed in the pathology of ERU in the literature review section. IL-17 has been found, in both humans and mice, in the AH, VH and tears of uveitis patients (Amadi-Obi, 2007; Carreno, 2016; Chi, 2008, 2016; Wan, 2016) as well in ARPE cell lines with induced inflammation (Chen, 2011, 2014; Ke, 2009). The roll of Th17 cells and their regulation of autoimmune disease, including uveitis, has also been extensively documented (Horai, 2011; Liang, 2012; Qu, 2013; Yoshimura, 2009).

Resveratrol's effect on Th-17 cells and IL-17 was discussed in the literature review, under resveratrol's effects on autoimmune diseases. However the literature is vague and sparse in support of utilizing resveratrol to inhibit IL-17 expression and secretion, specifically in uveitis. One recent study reports resveratrol's downregulation of IL-17 in the ocular disease diabetic retinopathy (Liu, 2016), and there is an emerging interest in using resveratrol to down regulate Th-17 in autoimmune diseases (Lanzilli, 2012; Petro, 2011; Wang, 2015; Yao, 2015).

The link between ERU and IL-17 has been well established, in addition to resveratrol's ability to down-regulate IL-17 and Th-17. These findings warrant further investigation of

resveratrol as a potential therapeutic for ERU. In this study resveratrol significantly down-regulated the expression of IL-17F in inflammation-induced ARPE cells when incubated for at least 48 hours. The three concentrations of resveratrol used in this time course all showed a significant effect in lowering IL-17 expression, with 100μM and 200μM having the greatest effect. Pretreatment with resveratrol blocked the up-regulation of IL-17 seen in TNF-treated cells, lowering expression of IL-17 to less than that of the control. Considering the eye's need for protection from infiltrates, studies would have to be done to ensure this low level of IL-17 expression would be healthy for the eye.

Pretreatment with Resveratrol

Four of the five of the genes that were selected in this study showed a significant effect of pre-treatment with 50 μM resveratrol before inflammation was induced with TNF-α. A review of the literature indicates that pretreatment with resveratrol may have promising potential in ocular studies. Resveratrol injected into the eye in adult rats for 5 days, followed by induced inflammation on the third, results in less retinal thinning compared to control rats injected with saline (Vin, 2013). Oral supplementation for four months with resveratrol showed enhanced oxidative markers, weight loss and superoxide dismutase activity in the retinas of diabetic rats (Soufi, 2012). Another study using oral resveratrol pre- and post-neovascular lesion showed a decrease the expression of VEGF in mouse retinas (Khan, 2010). When resveratrol was added to RPE cell lines in concentrations of 20, 50, and 100μM before exposure to $H_2O_2$, it showed a significant protective effect on cell viability at all three concentrations (Pintea, 2011). Kubota et al. conducted two studies in which resveratrol was used in pretreatment. The 2009 study showed

that pretreatment with varying concentrations of resveratrol for 5 days in mice caused a suppression of retinal leukocyte adhesion and expression of MCP-1 (a chemokine associated with CCL2 expression) and NF-κβ. The 2010 study showed that orally pretreating mice with 50mg/kg of body weight per day for 5 days significantly decreased light-induced retinal dysfunction and AP-1 activation, which leads to cellular apoptosis (Kubota, 2009).

This study, in conjunction with data reported in the literature, warrant a long term study focused on testing resveratrol as a pretreatment for ERU. If successful, it would be highly preventative for those horses who are genetically predisposed to the disease as well as those who are at risk for Leptospirosis. Administering resveratrol in high doses has not been found to be harmful to animals, making daily oral treatment a possibility for the appropriate candidates (Juan, 2002).

Resveratrol and Corticosteroid Use

As previously discussed in the literature review for ERU, current corticosteroid treatments have adverse side effects which owners and equine practitioners would prefer to avoid. Outside of the physical and behavioral effects, rules in competitive equine sports prohibit the use of any steroid, as it is known to be a performance enhancing drug (FEI Rulebook, AQHA Rulebook). Thus any competitive horse with ERU would require an alternative anti-inflammatory agent in order to compete. The availability of resveratrol as a treatment in these situations would ensure that the animal was still eligible to compete, and thus retain its value.

For those horses in which corticosteroid use is the only viable option, co- treatment with resveratrol may not only reduce the amount of steroid needed but also be protective against the

side effects. Sadarani et al. reported that when resveratrol was given with dexamethasone not only were all inflammatory parameters significantly reduced over dexamethasone alone, but there was a protective effect from the side effects of dexamethasone (2015). In addition, when examining the role of MMP-9 activity (degradation of extracellular matrix) in human monocyte (U937) cells 10μM resveratrol showed a statistically similar outcome as 10μM dexamethasone (Yi-Tang, 2003). Further studies on the co-treatment of corticosteroids and resveratrol to reduce the symptoms of ERU are warranted as the evidence supports it as a viable treatment route.

## Conclusions

Resveratrol effectively attenuates the up-regulation of key inflammatory cytokines in response to induced immune response by TNF-α. Resveratrol also effectively blocks recruitment of key immune response factors when administered prior to inducing inflammation. VEGF expression has been documented in autoimmune diseases, including uveitis; the results of this study show that using resveratrol prophylactically has the potential to decrease the severity of the disease. The cytokines and immune responses that were studied have been linked in the literature to uveitis in both the equid and in humans. This lends itself to the growing body of evidence that resveratrol is a viable option in the treatment of uveitis.

## Limitations

Initially the project proposed the use of equine retinal primary cell lines over human ARPE cells. However, even with collaboration from local veterinary sources, the necessary supply of primary tissue could not be supported. There was also the concern that all animals used would not be genetically the same, thus leading to inconsistent and uninterpretable data. A search of available sources produced no known permanent cell lines for equine retina, and therefore a human retinal line (ARPE-19) was substituted. This is consistent with the standard use of animal and invertebrate models to study human systems and pathologies in the laboratory.

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# APPENDIX

## Appendix A. RNA Purity

| **Total RNA – Dose Response at 12h*** | | |
|---|---|---|
| | **ng/ul** | **260/280** |
| T+R 50-1 | 308.86 | 2.07 |
| T+R 50-2 | 291.74 | 2.04 |
| T+R 50-3 | 171.23 | 2.15 |
| T+R 50-4 | 112.11 | 1.90 |
| T+R 50-5 | 239.99 | 2.06 |
| T+R 50-6 | 126.15 | 1.94 |
| T+R 100-1 | 208.02 | 2.04 |
| T+R 100-2 | 152.65 | 2.07 |
| T+R 100-3 | 191.06 | 2.02 |
| T+R 100-4 | 143.75 | 2.09 |
| T+R 100-5 | 181.34 | 2.06 |
| T+R 100-6 | 172.64 | 2.06 |
| T+R 200-1 | 751.95 | 2.07 |
| T+R 200-2 | 334.4 | 2.07 |
| T+R 200-3 | 343.93 | 2.03 |
| T+R 200-4 | 245.64 | 2.04 |
| T+R 200-5 | 266.17 | 2.04 |
| T+R 200-6 | 433.89 | 2.08 |

*Quantification values and absorption ratios for ARPE-19 cells treated with TNF-a followed by either 50, 100 or 200μM resveratrol (N = 6) for 12 hours. [T+R (50,100,200) = TNF-treatment followed by (50,100,200 μM) resveratrol]

| **Total RNA – Dose Response at 24h*** | | |
|---|---|---|
| | ng/ul | 260/280 |
| T+R 50-1 | 94.06 | 2.11 |
| T+R 50-2 | 237.21 | 2.00 |
| T+R 50-3 | 276.63 | 2.04 |
| T+R 50-4 | 297.96 | 2.01 |
| T+R 50-5 | 188.88 | 2.01 |
| T+R 50-6 | 401.85 | 2.02 |
| T+R 100-1 | 279.28 | 2.03 |
| T+R 100-2 | 256.79 | 1.91 |
| T+R 100-3 | 149.83 | 1.94 |
| T+R 100-4 | 132.78 | 1.93 |
| T+R 100-5 | 106.32 | 1.94 |
| T+R 100-6 | 104.25 | 1.97 |
| T+R 200-1 | 216.01 | 2.03 |
| T+R 200-2 | 206.38 | 1.97 |
| T+R 200-3 | 320.54 | 2.02 |
| T+R 200-4 | 104.5 | 1.91 |
| T+R 200-5 | 186.5 | 1.98 |
| T+R 200-6 | 181.61 | 1.98 |

*Quantification values and absorption ratios for ARPE-19 cells treated with TNF-a followed by either 50, 100 or 200μM resveratrol (N = 6) for 24 hours. [T+R (50,100,200) = TNF-treatment followed by (50,100,200 μM) resveratrol]

| **Total RNA – Dose Response at 48h*** | | |
|---|---|---|
| | ng/ul | 260/280 |
| T+R 50-1 | 383.11 | 2.05 |
| T+R 50-2 | 293.49 | 2.09 |
| T+R 50-3 | 408.17 | 2.04 |
| T+R 50-4 | 140.03 | 2.01 |
| T+R 50-5 | 172.49 | 2.06 |
| T+R 50-6 | 212.63 | 2.05 |
| T+R 100-1 | 199.01 | 2.07 |
| T+R 100-2 | 98.52 | 2.05 |
| T+R 100-3 | 158.39 | 2.04 |
| T+R 100-4 | 169.09 | 2.02 |
| T+R 100-5 | 91.43 | 1.98 |
| T+R 100-6 | 67.09 | 1.91 |
| T+R 200-1 | 101.94 | 1.93 |
| T+R 200-2 | 97.09 | 1.94 |
| T+R 200-3 | 84.54 | 2.10 |
| T+R 200-4 | 48.41 | 2.04 |
| T+R 200-5 | 33.93 | 1.97 |
| T+R 200-6 | 49.77 | 1.97 |

*Quantification values and absorption ratios for ARPE-19 cells treated with TNF-a followed by either 50, 100 or 200μM resveratrol (N = 6) for 48 hours. [T+R (50,100,200) = TNF-treatment followed by (50,100,200 μM) resveratrol]

| **Total RNA- Untreated Control*** | | |
|---|---|---|
| Controls | ng/2ul | 260/280 |
| C1 | 193.12 | 2.04 |
| C2 | 232.41 | 2.00 |
| C3 | 272.07 | 2.05 |
| C4 | 228.50 | 2.02 |
| C5 | 275.37 | 2.02 |
| C6 | 275.00 | 2.02 |

*Quantification values and absorption ratios for untreated control ARPE-19 cells. [C = untreated controls]

| **Total RNA- Resveratrol Treated Controls*** | | |
|---|---|---|
| Resveratrol | ng/2ul | 260/280 |
| R1 | 76.56 | 2.07 |
| R2 | 202.80 | 2.04 |
| R3 | 176.97 | 2.07 |
| R4 | 145.94 | 2.10 |
| R5 | 202.17 | 2.05 |
| R6 | 196.32 | 2.12 |

*Quantification values and absorption ratios for Resveratrol treated control ARPE-19 cells. [R = resveratrol-treated controls]

| Total RNA- Treated with Resveratrol than TNF* | | |
|---|---|---|
| TNF/Resv | ng/2ul | 260/280 |
| R+T1 | 152.56 | 2.12 |
| R+T2 | 283.03 | 2.11 |
| R+T3 | 177.90 | 2.11 |
| R+T4 | 133.97 | 2.17 |
| R+T5 | 154.62 | 2.17 |
| R+T6 | 163.09 | 2.14 |

*Quantification values and absorption ratios for ARPE-19 cells pre-treated with Resveratrol before TNF. [R+T = resveratrol pre-treatment followed by TNF controls]

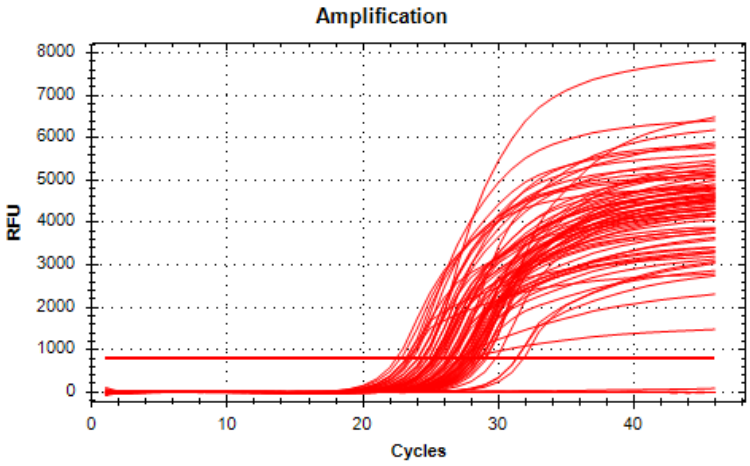
| **Total RNA- TNF-C*** | | |
|---|---|---|
| | ng/ul | 260/280 |
| T1 | 231.8 | 2.06 |
| T2 | 206.5 | 2.06 |
| T3 | 154.3 | 2.05 |
| T4 | 108.8 | 2.04 |
| T5 | 130.3 | 2.05 |
| T6 | 128.5 | 2.03 |

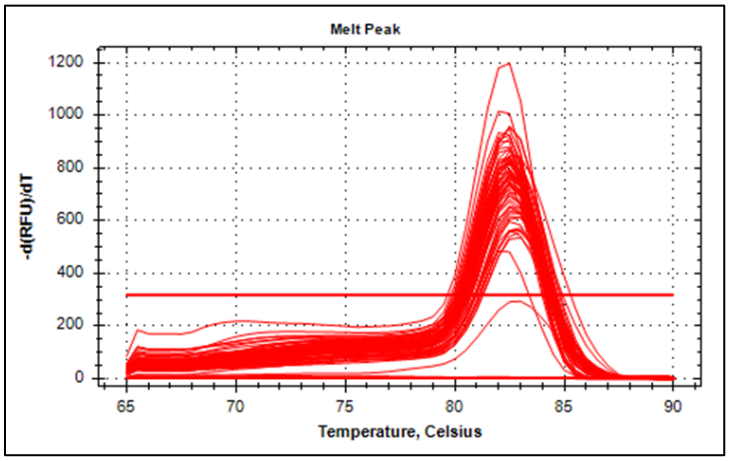
*Quantification values and absorption ratios for ARPE-19 cells treated with TNF. [T= TNF treated cells]

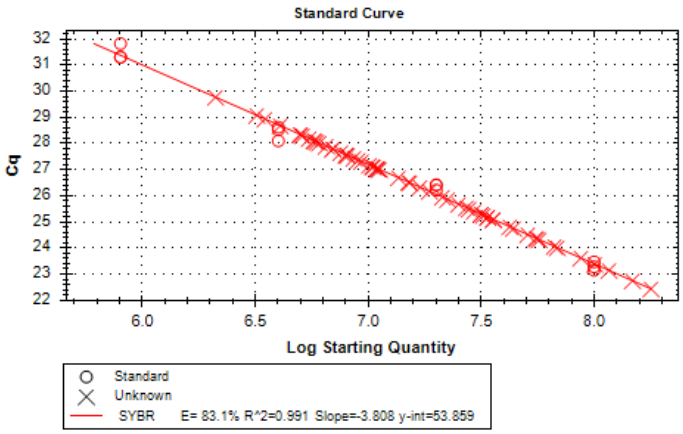
Appendix B  Validation Data for Quantitative PCR-Amplification

Amplification Curves, Dissociation Peaks and Standard Curves for validated genes.

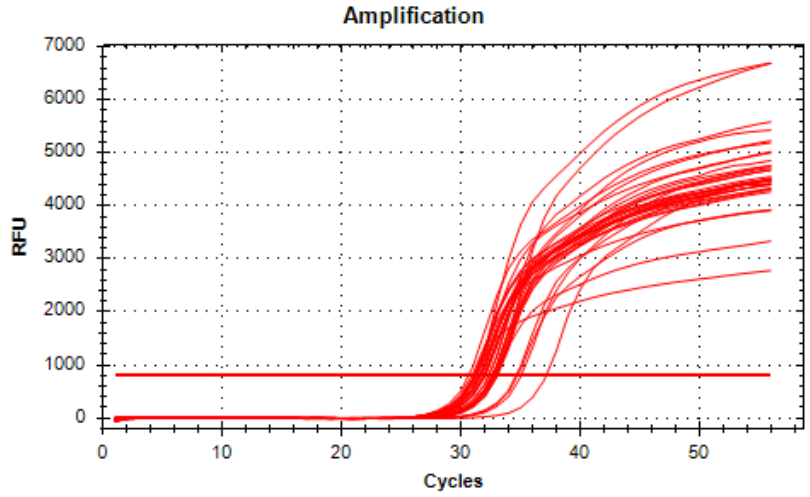
***Chemokine (C-C motif) Ligand 2***

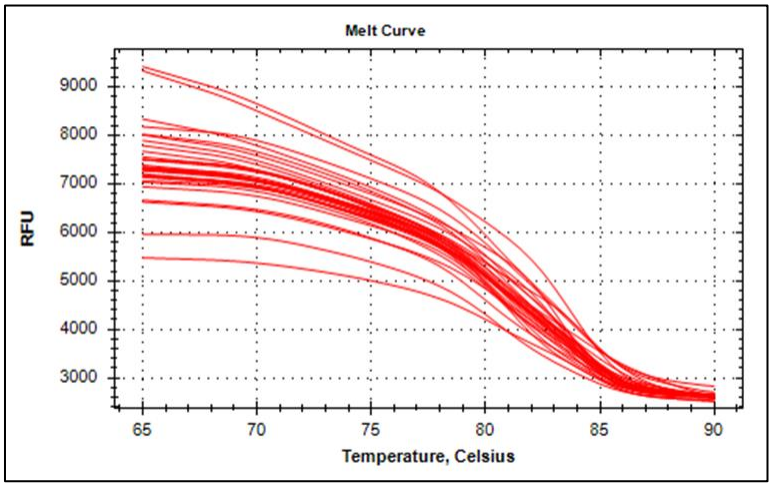


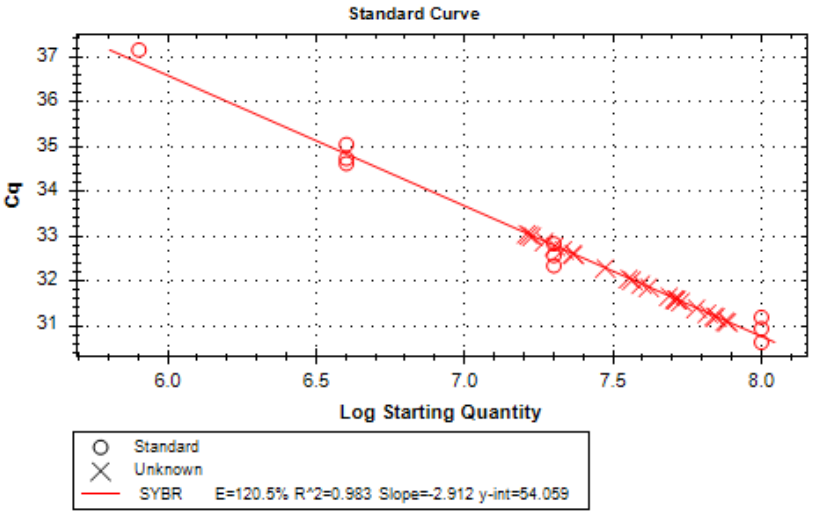



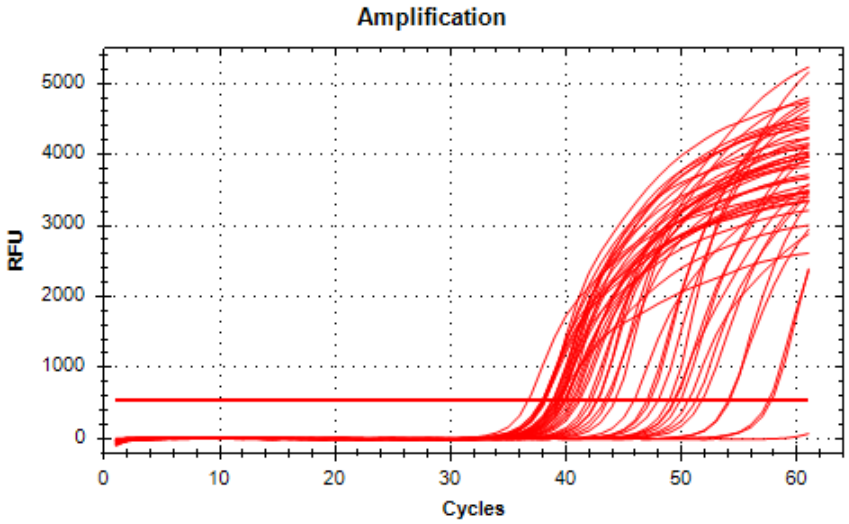
## *Interleukin 17F*

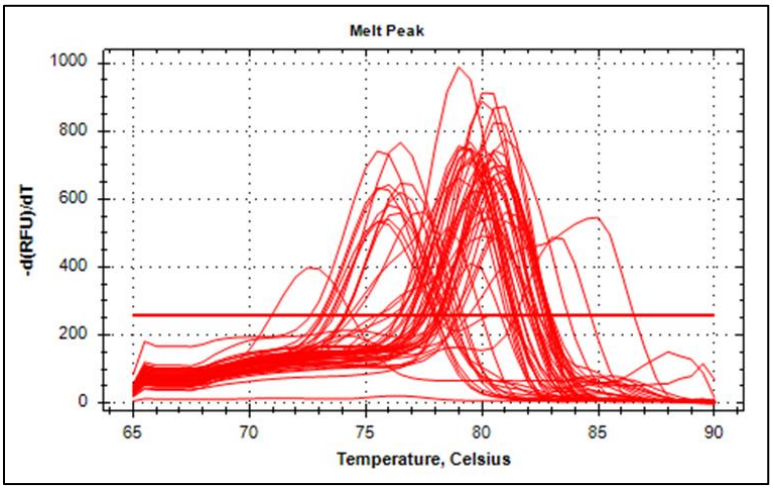


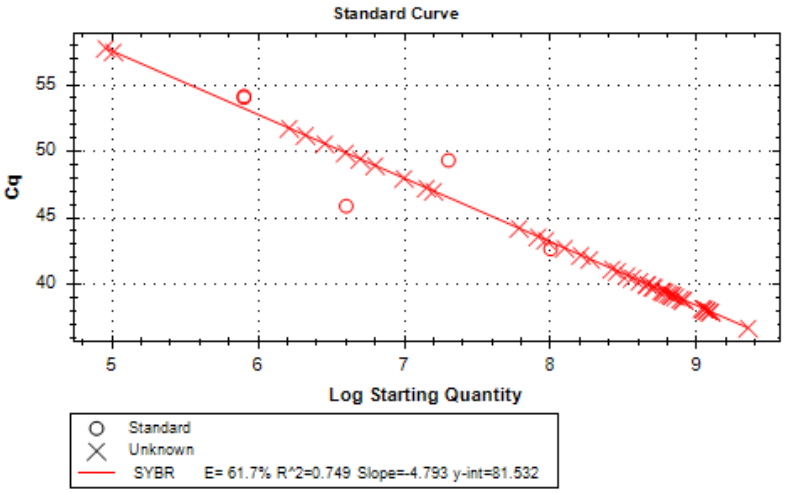



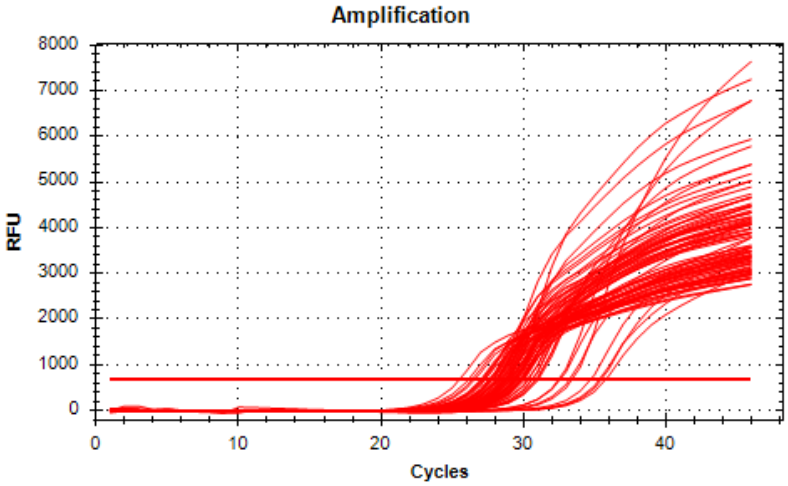
## *Interleukin 33*

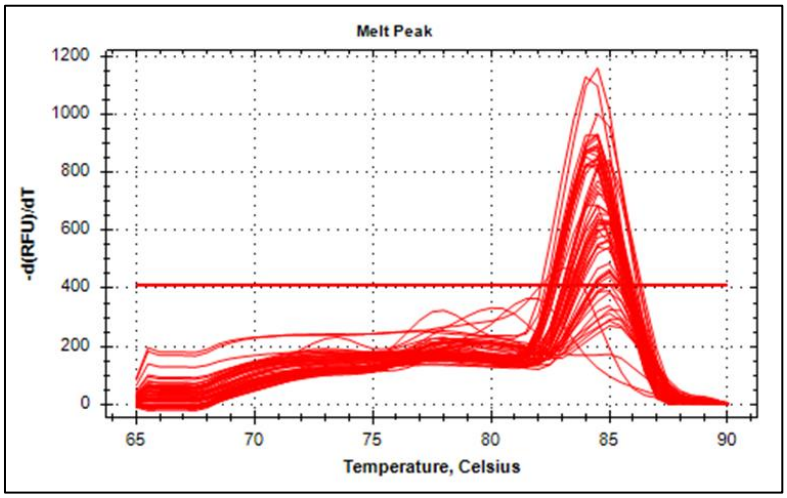


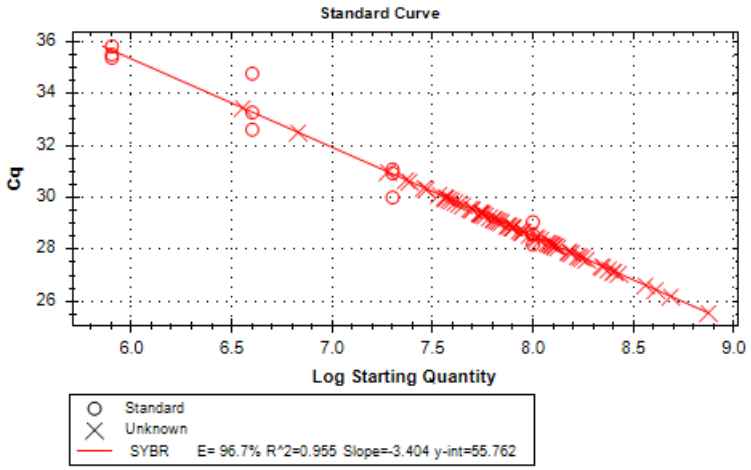



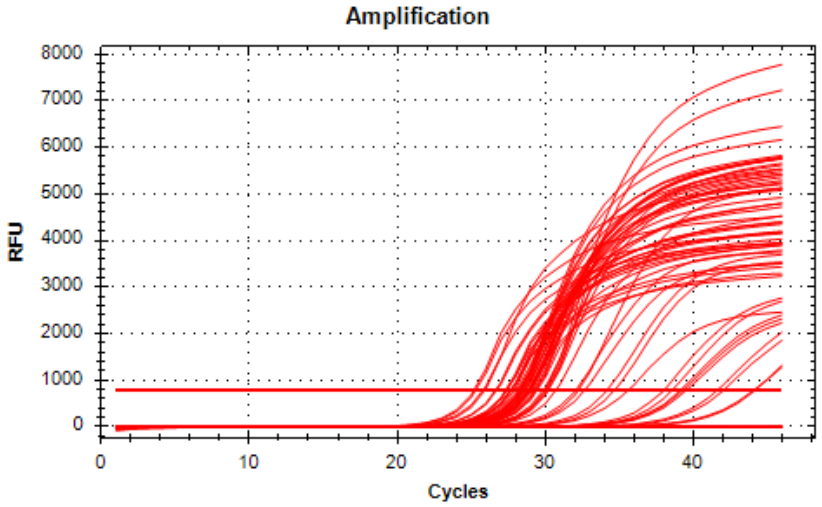
## *Vascular Endothelial Growth Factor A*

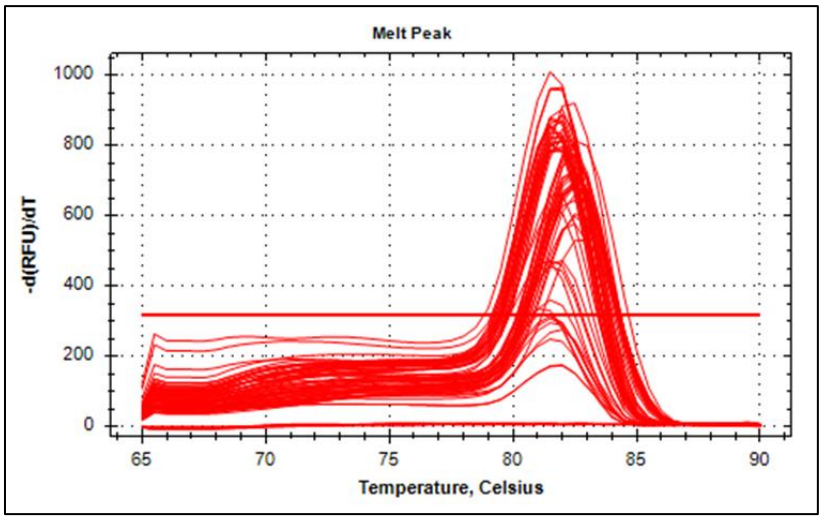


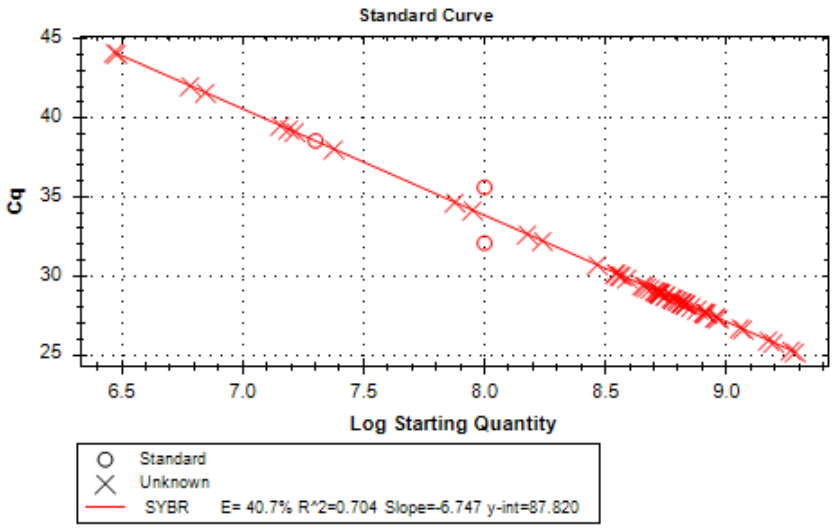



***Fas Ligand (TNF Superfamily, member 6)***



Appendix C List of Gene Primers

| Primer ID | Sequence |
|---|---|
| **IL 17F-F'** | 5’ - AGC GCA ACA TGA CAG TGA AG – 3’ |
| **IL-17F-R'** | 5’ - GTG TAA TTC CAG GGG GAG GT – 3’ |
| | |
| **FASL-F'** | 5’ - GCA GCC CTT CAA TTA CCC AT – 3’ |
| **FASL-R'** | 5’ - CAG AGG TTG GAC AGG GAA GAA – 3’ |
| | |
| **IL-33-F'** | 5’ - TCC TTG CTT GGC AGT ATC CA – 3’ |
| **IL-33-R'** | 5’ - TGC TCA ATG TGT CAA CAG ACG – 3’ |
| | |
| **VEGFA-F'** | 5’ - ACT TTC TGC TGT CTT GGG TG – 3’ |
| **VEGFA-R'** | 5’ - CTG CAT GGT GAT GTT GGA CT – 3’ |
| | |
| **CCL2-F’** | 5’ - CCC CAG TCA CCT GCT GTT AT – 3’ |
| **CCL2-R’** | 5’ - GAG TTT GGG TTT GCT TGT CC – 3’ |

## Appendix D Qiagen® RT$^2$ Profiler PCR Array-Human Inflammatory Cytokines and Receptors

| Position | GenBank | Symbol | Description |
|---|---|---|---|
| A01 | NM_004757 | AIMP1 | Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1 |
| A02 | NM_001200 | BMP2 | Bone morphogenetic protein 2 |
| A03 | NM_001735 | C5 | Complement component 5 |
| A04 | NM_002981 | CCL1 | Chemokine (C-C motif) ligand 1 |
| A05 | NM_002986 | CCL11 | Chemokine (C-C motif) ligand 11 |
| A06 | NM_005408 | CCL13 | Chemokine (C-C motif) ligand 13 |
| A07 | NM_032965 | CCL15 | Chemokine (C-C motif) ligand 15 |
| A08 | NM_004590 | CCL16 | Chemokine (C-C motif) ligand 16 |
| A09 | NM_002987 | CCL17 | Chemokine (C-C motif) ligand 17 |
| A10 | NM_002982 | CCL2 | Chemokine (C-C motif) ligand 2 |
| A11 | NM_004591 | CCL20 | Chemokine (C-C motif) ligand 20 |
| A12 | NM_002990 | CCL22 | Chemokine (C-C motif) ligand 22 |
| B01 | NM_005064 | CCL23 | Chemokine (C-C motif) ligand 23 |
| B02 | NM_002991 | CCL24 | Chemokine (C-C motif) ligand 24 |
| B03 | NM_006072 | CCL26 | Chemokine (C-C motif) ligand 26 |
| B04 | NM_002983 | CCL3 | Chemokine (C-C motif) ligand 3 |
| B05 | NM_002984 | CCL4 | Chemokine (C-C motif) ligand 4 |
| B06 | NM_002985 | CCL5 | Chemokine (C-C motif) ligand 5 |
| B07 | NM_006273 | CCL7 | Chemokine (C-C motif) ligand 7 |
| B08 | NM_005623 | CCL8 | Chemokine (C-C motif) ligand 8 |
| B09 | NM_001295 | CCR1 | Chemokine (C-C motif) receptor 1 |
| B10 | NM_001123396 | CCR2 | Chemokine (C-C motif) receptor 2 |
| B11 | NM_001837 | CCR3 | Chemokine (C-C motif) receptor 3 |
| B12 | NM_005508 | CCR4 | Chemokine (C-C motif) receptor 4 |
| C01 | NM_000579 | CCR5 | Chemokine (C-C motif) receptor 5 |
| C02 | NM_004367 | CCR6 | Chemokine (C-C motif) receptor 6 |
| C03 | NM_005201 | CCR8 | Chemokine (C-C motif) receptor 8 |
| C04 | NM_000074 | CD40LG | CD40 ligand |
| C05 | NM_000757 | CSF1 | Colony stimulating factor 1 (macrophage) |
| C06 | NM_000758 | CSF2 | Colony stimulating factor 2 (granulocyte-macrophage) |
| C07 | NM_000759 | CSF3 | Colony stimulating factor 3 (granulocyte) |
| C08 | NM_002996 | CX3CL1 | Chemokine (C-X3-C motif) ligand 1 |
| C09 | NM_001337 | CX3CR1 | Chemokine (C-X3-C motif) receptor 1 |
| C10 | NM_001511 | CXCL1 | Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha |
| C11 | NM_001565 | CXCL10 | Chemokine (C-X-C motif) ligand 10 |
| C12 | NM_005409 | CXCL11 | Chemokine (C-X-C motif) ligand 11 |

| Position | GenBank | Symbol | Description |
|---|---|---|---|
| D02 | NM_006419 | CXCL13 | Chemokine (C-X-C motif) ligand 13 |
| D04 | NM_002090 | CXCL3 | Chemokine (C-X-C motif) ligand 3 |
| D05 | NM_002994 | CXCL5 | Chemokine (C-X-C motif) ligand 5 |
| D06 | NM_002993 | CXCL6 | Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2) |
| D07 | NM_002416 | CXCL9 | Chemokine (C-X-C motif) ligand 9 |
| D08 | NM_000634 | CXCR1 | Chemokine (C-X-C motif) receptor 1 |
| D09 | NM_001557 | CXCR2 | Chemokine (C-X-C motif) receptor 2 |
| D10 | NM_000639 | FASLG | Fas ligand (TNF superfamily, member 6) |
| D11 | NM_000605 | IFNA2 | Interferon, alpha 2 |
| D12 | NM_000619 | IFNG | Interferon, gamma |
| E01 | NM_001558 | IL10RA | Interleukin 10 receptor, alpha |
| E02 | NM_000628 | IL10RB | Interleukin 10 receptor, beta |
| E03 | NM_002188 | IL13 | Interleukin 13 |
| E04 | NM_000585 | IL15 | Interleukin 15 |
| E05 | NM_004513 | IL16 | Interleukin 16 |
| E06 | NM_002190 | IL17A | Interleukin 17A |
| E07 | NM_013278 | IL17C | Interleukin 17C |
| E08 | NM_052872 | IL17F | Interleukin 17F |
| E09 | NM_000575 | IL1A | Interleukin 1, alpha |
| E10 | NM_000576 | IL1B | Interleukin 1, beta |
| E11 | NM_000877 | IL1R1 | Interleukin 1 receptor, type I |
| E12 | NM_000577 | IL1RN | Interleukin 1 receptor antagonist |
| F01 | NM_021803 | IL21 | Interleukin 21 |
| F02 | NM_145659 | IL27 | Interleukin 27 |
| F03 | NM_000588 | IL3 | Interleukin 3 (colony-stimulating factor, multiple) |
| F04 | NM_033439 | IL33 | Interleukin 33 |
| F05 | NM_000879 | IL5 | Interleukin 5 (colony-stimulating factor, eosinophil) |
| F06 | NM_000564 | IL5RA | Interleukin 5 receptor, alpha |
| F07 | NM_000880 | IL7 | Interleukin 7 |
| F08 | NM_000584 | IL8 | Interleukin 8 |
| F09 | NM_000590 | IL9 | Interleukin 9 |
| F10 | NM_002186 | IL9R | Interleukin 9 receptor |
| F11 | NM_000595 | LTA | Lymphotoxin alpha (TNF superfamily, member 1) |
| F12 | NM_002341 | LTB | Lymphotoxin beta (TNF superfamily, member 3 |
| G01 | NM_002415 | MIF | Macrophage migration inhibitory factor (glycosylation-inhibiting factor) |
| G02 | NM_005746 | NAMPT | Nicotinamide phosphoribosyltransferase |

| Position | GenBank | Symbol | Description |
|---|---|---|---|
| G04 | NM_000582 | SPP1 | Secreted phosphoprotein 1 |
| G05 | NM_000594 | TNF | Tumor necrosis factor |
| G06 | NM_002546 | TNFRSF11B | Tumor necrosis factor receptor superfamily, member 11b |
| G07 | NM_003810 | TNFSF10 | Tumor necrosis factor (ligand) superfamily, member 10 |
| G08 | NM_003701 | TNFSF11 | Tumor necrosis factor (ligand) superfamily, member 11 |
| G09 | NM_003808 | TNFSF13 | Tumor necrosis factor (ligand) superfamily, member 13 |
| G10 | NM_006573 | TNFSF13B | Tumor necrosis factor (ligand) superfamily, member 13b |
| G11 | NM_003326 | TNFSF4 | Tumor necrosis factor (ligand) superfamily, member 4 |
| G12 | NM_003376 | VEGFA | Vascular endothelial growth factor A |

Appendix E Abbreviations

| Abbreviation | Full Name |
|---|---|
| AH | Aqueous Humor |
| AMD | Age-related Macular Degeneration |
| AMPK | Activated Protein Kinase |
| AU | Autoimmune Uveitis |
| AM549 CELLS | Adenocarcinoma Human Alveolar Basal Epithelial Cells |
| ARPE-19 | Human Retinal Pigment Epithelium Cells |
| BRB | Blood Retinal Barrier |
| CCL-2 | Chemokine Ligand 2 |
| COX-1 | Cyclooxygenase 1 |
| COX-2 | Cyclooxygenase 2 |
| CRALBP | Cellular Retinaldehyde-binding Protein |
| CRP | C-Reactive Proteins |
| CXCL11 | C-X-C Motif Chemokine 11 |
| DC | Dendric Cell |
| EIU | Endotoxin-induced Uveitis |
| ERU | Equine Recurrent Uveitis |
| EtOH | Ethanol |
| FASL | Fas Ligand |
| HCT116 CELLS | Human Colon Carcinoma Cell Line |
| Hep 3B CELLS | Human Hepatoma Cell Line |
| Hep G2 CELLS | Human Liver Cancer Cell Line |
| HIV | Human Immunodeficiency Virus |
| NO | Nitric Oxide |
| HRPE CELLS | Human Retinal Pigment Endothelial Cells |
| HT-29 | Colon Cancer Cell Line |
| H2O2 | Hydrogen Peroxide |
| H411E | Rat Hepatoma Cell Line |
| ICAM-1 | Intercellular Adhesion Molecule 1 |
| IL-1B | Interleukin 1B |
| IL-2 | Interleukin 2 |
| IL-6 | Interleukin 6 |
| IL-8 | Interleukin 8 |
| IL-10 | Interleukin 10 |
| IL-17A | Interleukin 17A |
| IL-17F | Interleukin 17F |
| IL-21 | Interleukin 21 |
| IL-22 | Interleukin 22 |

| | |
|---|---|
| IL-23 | Interleukin 23 |
| IL-33 | Interleukin 33 |
| IRBP | Iron-responsive Element Binding Protein |
| MCF-7 | Breast Cancer Cell Line |
| MCP-1 | Monocyte Chemoattractant Protein 1 |
| NF-κβ | Nuclear Factor κβ |
| PDGF | Platelet-derived Growth Factor |
| p53 | Tumor Protein p53 |
| RAW2647 | Murine Macrophage Cell Line |
| ROS | Reactive Oxygen Species |
| RPE CELLS | Retinal Pigment Endothelial Cells |
| RV | Resveratrol |
| SIR 1 | Surtuin 1 |
| SIR 2 | Surtuin 2 |
| TGF-β | Basic Fibroblast Growth Factor |
| TH17 | T Helper cell 17 |
| THP-1 | Human Leukemia Monocyte Cell Line |
| TNF-α | Tumor Necrosis Factor- Alpha |
| VEGF | Vascular Endothelium Growth Factor |
| VH | Vitreous Humor |
| UV | Ultraviolet |