RESEARCH PROPOSAL

STATEMENT OF PURPOSE

Study Rationale: Neovascular age-related macular degeneration (nAMD) is the leading cause of blindness in the developed world. Vascular endothelial growth factor (VEGF) is one of the key drivers of angiogenesis in nAMD, and current treatment focuses solely upon VEGF inhibition. However, despite maximal anti-VEGF therapy, 15% of patients lose vision, which totals 3 million people worldwide. Therefore, new non-VEGF therapeutic targets are an unmet clinical need for patients with anti-VEGF resistant nAMD.

VEGF primes endothelial cells for proliferation, branching, and morphogenesis by increasing the expression of Flt3. Flt3 is a receptor tyrosine kinase expressed in endothelial cells and macrophages in the retina. Flt3 binds to Flt3 ligand, which is expressed in hematopoietic cells, including monocytes and dendritic cells. The Flt3 internal tandem duplication mutation, termed Flt3-ITD, duplicates Exons 14-15, and results in ligand-independent activation of Flt3. Flt3-ITD mutations have been detected in acute myeloid leukemia (AML), and this mutation drives multiple signaling pathways, resulting in cellular proliferation and survival [1]. Flt3-ITD mutations are associated with a poor prognosis in AML, but multiple receptor tyrosine kinase inhibitors have been developed to inhibit Flt3 and Flt3-ITD signaling. If VEGF has already primed the endothelial cell and increased the expression of Flt3 during CNV pathogenesis, then anti-VEGF treatment will not affect Flt3 expression. *Our central premise is that inhibition of Flt3 will decrease choroidal angiogenesis, and since Flt3 is downstream of VEGF, this effect will be a useful adjunct to anti-VEGF therapy.*

Flt3 inhibitors have been developed as anti-cancer therapeutics, and are currently approved for the treatment of AML, renal cell carcinoma, hepatocellular carcinoma, and gastrointestinal stromal tumors. In our Preliminary Data, we show that the Flt3 inhibitor KW-2443 reduces choroidal angiogenesis by 47-80%. Importantly, this effect is far greater than inhibition of the VEGF receptor, which decreases choroidal angiogenesis by 20%. In confirmation of our hypothesis, $Flt3^{-/-}$ mice show less CNV area using the laser-induced mouse model. Nonetheless, several important questions remain unanswered: (1) what is the prevalence and clinical course of nAMD in patients taking anti-Flt3 therapeutics, (2) is Flt3 ligand necessary for laser-induced CNV, and (3) is Flt3 activation sufficient for increased CNV area. Based on published studies and our Preliminary Data, *we hypothesize that patients taking anti-Flt3 therapeutics will have improved response to anti-VEGF therapy compared to matched controls. Furthermore, we hypothesize that Flt3 ligand is necessary for CNV, and that constitutive Flt3 activation increases CNV area*

Specific Objective #1: Determine the prevalence of nAMD and response to anti-VEGF treatment in patients taking anti-Flt3 therapeutics. Since Flt3 inhibition reduces choroidal angiogenesis and laser-induced CNV, we hypothesize that patient undergoing anti-Flt3 therapy will have reduced nAMD prevalence and improved response to anti-VEGF therapy. We will perform a retrospective analysis of patients taking anti-Flt3 therapeutics to determine if Flt3 inhibitors affect the prevalence of nAMD compared to age matched historical controls. For patients with nAMD, we will assess how Flt3 antagonists affect visual acuity, retinal thickness on spectral-domain optical coherence tomography, and the number of required anti-VEGF injections compared to historical controls.

Specific Objective #2: Determine the role of Flt3 ligand and Flt3 during laser-induced murine CNV. Our preliminary data show that Flt3 inhibition reduces choroidal angiogenesis by 47-80%, and published studies show that Flt3 is necessary for laser-induced CNV. We hypothesize that Flt3 ligand is also necessary for CNV and Flt3 is sufficient for increased CNV area. We will test this hypothesis by subjecting wildtype, $Flt3^{-/-}$, and $Flt3L^{-/-}$ mice to laser-induced CNV. We anticipate that Flt3 and Flt3 ligand will be necessary for laser-induced CNV. We will next subject $Flt3^{ITD/+}$ and $Flt3^{ITD/ITD}$ mice, which display ligand-independent Flt3 activation, to laser-induced CNV. We expect that Flt3 activation will be sufficient to increase CNV area.

Clinical Relevance*:* Our focus on angiogenesis pathways downstream of VEGF holds great promise for the 2.5 million patients who do not respond to anti-VEGF therapy. Flt3 ligand – Flt3 signaling is under-investigated in the eye and could be a novel therapeutic target for treatment resistant patients.

BACKGROUND AND SIGNIFICANCE:

Neovascular age-related macular degeneration (nAMD) is the most common cause of irreversible vision loss in the developed world. Vascular endothelial growth factor (VEGF) is a key driver of angiogenesis during the development of choroidal neovascularization (CNV), the key pathology of nAMD. Intravitreal anti-VEGF injections improve visual acuity by 10-15 letters [2,3]. However, continued therapy is expensive with an annual cost of $4.6 billion in the US [4], and frequent injections increase the risk of endophthalmitis [5]. Additionally, 15% of patients demonstrate no improvement in visual acuity despite monthly anti-VEGF treatment [6]. Although 15% of patients appears small, this totals over 2.5 million people today and is estimated to include 4.3 million people by the year 2040 [4]. For these millions of patients, an unmet need exists for alternative therapies.

VEGF is upstream of several angiogenic factors which direct vascular tube morphogenesis and sprouting in endothelial cells. Human endothelial cells will proliferate, sprout, and assemble into vascular tubes in the presence of a few cytokine factors. VEGF and fibroblast growth factor 2 (FGF2) prime endothelial cells for sprouting and vascular tube formation by increasing the expression levels of 5 receptors: Flt3, c-Kit, colony stimulating factor receptor (CSFR), C-X-C chemokine receptor 4 (CXCR4), and interleukin 3 receptor alpha (IL-3Ra) [7]. Once primed by VEGF and FGF2, endothelial cells will proliferate, sprout, and assemble into vascular tubes in the presence of IL-3, stromal derived factor 1 alpha (SDF-1a, ligand for CXCR4), and one of the following 3 ligands: Flt3 ligand (Flt3L), stem cell factor (SCF, ligand for c-Kit), or macrophage colony stimulating factor (M-CSF, ligand for CSFR) [7]. Therefore, VEGF and FGF2 prime endothelial cells for vascular tube morphogenesis and sprouting, which is directed by IL-3, SDF1a, and either Flt3 ligand, SCF, or M-CSF (Fig 1). Among these 5 cytokines, it is unclear which is most important, and it is unknown if this pathway exists in ocular angiogenesis and choroidal endothelial cells. Furthermore, anti-VEGF treatment reduces choroidal neovascularization (CNV) size by 33% initially, but CNV size rebounds and remains unchanged despite monthly treatment [8]. We hypothesize that these endothelial cells are already by primed by VEGF, and anti-VEGF won't regress the CNV membrane because these downstream angiogenic receptor levels remain elevated despite anti-VEGF therapy.

Figure 1. Schematic diagram illustrating endothelial cell (EC) priming by VEGF and FGF2 for morphogenesis and sprouting, which is directed by other cytokine factors.

The Flt3 ligand – Flt3 axis is critical for hematopoiesis. Flt3 is a receptor tyrosine kinase which demonstrates significant sequence homology to c-Kit, CSFR, and platelet-derived growth factor receptor (PDGFR) [1]. Flt3$^{-/-}$ mice display impaired B-cell populations, but develop normally with no known endothelial cell phenotypes [9]. Flt3 is expressed in endothelial cells and in macrophages in the retina [10]. Flt3 ligand is a transmembrane protein, and Flt3L$^{-/-}$ mice demonstrate deficiencies in B-cell, NK-cell, and dendritic cell populations [11]. There are no reports or investigation of Flt3 ligand expression in the choroid or retina. The Flt3 internal tandem duplication mutation, termed Flt3-ITD, duplicates Exons 14-15, and results in ligand-independent activation of Flt3. Flt3-ITD mutations have been detected in acute myeloid leukemia, and this mutation drives multiple signaling pathways, resulting in cellular proliferation and survival [1]. Flt3-ITD mutations are associated with a poor prognosis in acute myeloid leukemia, but multiple receptor tyrosine kinase inhibitors have been developed to inhibit Flt3 and Flt3-ITD signaling. These Flt3 antagonists have been additionally successful in the treatment of renal cell carcinoma, hepatocellular carcinoma, and gastrointestinal stromal tumors.

PRELIMINARY DATA:

The choroidal sprouting assay is an *ex vivo* model of angiogenesis. Briefly, mouse eyes are enucleated, the anterior segment is removed, and the lens and retina are pulled free from the posterior eye cup. The remaining retinal pigment epithelium (RPE), choroid, and scleral complex is dissected into 1 x 1 mm pieces, plated into Matrigel, and cultured [12]. This model studies endothelial cell angiogenesis in the context of pericytes, RPE, and choroidal macrophages. To initially validate this model in our hands, we treated choroidal explants with DMSO vehicle and the receptor tyrosine kinase inhibitors: apatanib and sunitinib. Representative images are shown in Fig 2A-C on Day 7. We chose these two inhibitors because of their efficacy for the VEGF receptor 2, where apatanib is more effective than sunitinib. We found that apatanib and sunitinib reduced choroidal sprouting growth area by 20% ($p<0.01$) and 45% ($p<0.0001$), respectively (Fig 2D). Although apatanib is relatively specific for VEGF receptor 2, sunitinib additionally inhibits PDGFR-beta, c-Kit, and Flt3. In order to determine which of these 3 receptors mediate the potent anti-angiogenic effects of sunitinib, we next investigated motesanib and KW-2443. Motesanib inhibits c-Kit and PDGFR-beta while KW-2443 inhibits Flt3. We found that motesanib had no effect upon choroidal angiogenesis while KW-2443 reduced growth area by 47% ($p<0.0001$, Fig 2E). *These data suggest that Flt3 is critically important for choroidal angiogenesis, and that this effect is independent and more significant than VEGFR2 inhibition.* In agreement with our hypothesis, Flt3-/- mice demonstrate 40-50% decreased laser-induced CNV area [10]. However, this study did not report the age or sex of the mice. Furthermore, many critical questions remained unanswered. Specifically, (1) is the Flt3 ligand necessary for CNV? (2) is Flt3 activation sufficient to increase CNV area? (3) what is the prevalence and clinical course of nAMD in patients taking anti-Flt3 therapeutics? These preliminary studies suggest that Flt3 inhibition could be a potential new therapeutic for exudative AMD, but many important questions require further investigation.

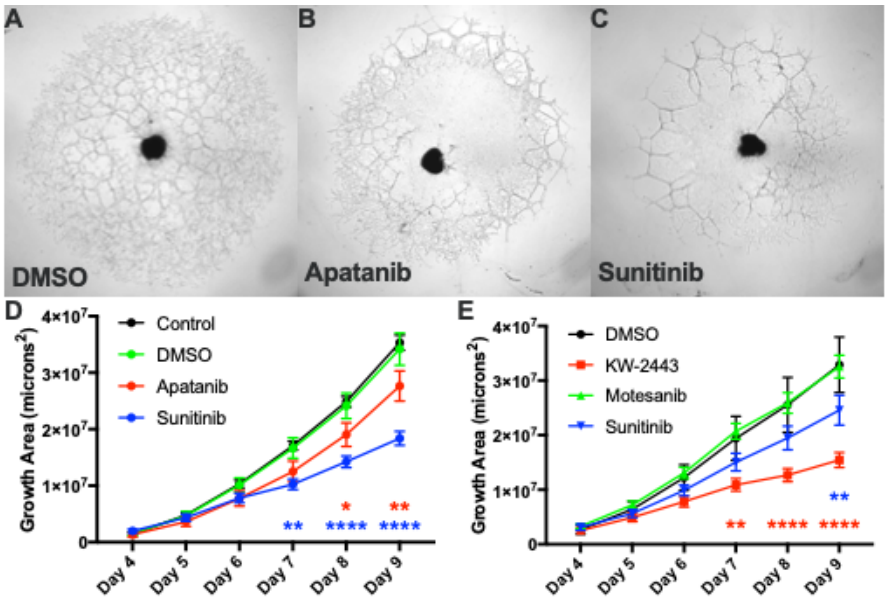

Figure 2. Flt3 is necessary for angiogenesis in the choroidal explant assay. Representative images of DMSO **(A)**, apatanib **(B)**, and sunitinib **(C)** treated choroidal explants on Day 7. **(D-E)**: Growth curves of receptor tyrosine kinase inhibitor (1 μM) treated choroidal explants (N = 4-6). *p<0.05, **p<0.01, ****p<0.0001.

RESEARCH PLAN: As our proposed plan involves both a retrospective clinical study component (Objective #1) and a mouse modeling component (Objective #2), we will comment on the plans for each separately below.

Specific Objective #1: Determine the prevalence of nAMD and response to anti-VEGF treatment in patients taking anti-Flt3 therapeutics.

Rationale: Since Flt3 inhibition reduces choroidal angiogenesis and laser-induced CNV, we hypothesize that patient undergoing anti-Flt3 therapy for cancer treatment will have reduced nAMD prevalence and improved response to anti-VEGF therapy.

Study Design and Methods: Patient data will be identified by the Northwestern EDW system. We will search for patients taking the following medications: sunitinib, sorafenib, cabozantinib, midostaurin, or gilterinib. We will then screen the patients charts for a documented eye exam at Northwestern Medicine. Inclusion criteria will include: (1) patients taking Flt3 antagonist medications as part of routine clinical practice or a clinical trial, and (2) patients with a documented eye exam at Northwestern Medicine. Exclusion criteria will include patients ≤ 45 years of age, pregnant, or incarcerated. Data will be collected using the EDW system for all patients satisfying the inclusion/exclusion criteria between 01/01/2007 and 04/1/2019. We will collect the following information: unique patient identifier, age, sex, ocular diagnoses, cancer type treated with Flt3 antagonists, Flt3 antagonist used, time frame taking the Flt3 antagonist, visual acuity (VA), central subfield thickness (CST) on optical coherence tomography, type of anti-VEGF injection, number of anti-VEGF injections, date of anti-VEGF injections, medical record number, and date of birth. Comparison will be made before, during, and after Flt3 medication treatment if possible, and to historic controls from published clinical trials in nAMD.

Statistical Plan: We propose to identify at least 100 patients with AMD of any type, and 10 patients with nAMD. We compare prevalence of nAMD for patients on Flt3 antagonists to published historical controls including published Medicare databases[13] and the Beaver Dam Eye Study[14]. Comparisons will be made using the chi-square test. For patients with pre-existing nAMD who begin a Flt3 antagonist, we will compare pre-Flt3 antagonist VA, CST, and number of anti-VEGF injections to post-Flt3 treatment VA, CST, and number of anti-VEGF injections using a paired Student's t-test or repeated measures ANOVA. For patients taking a Flt3 antagonist who are diagnosed with nAMD, we will compare VA, CST, and number of injections to published clinical trials, specifically the CATT and HARBOR trials. Data will be analyzed using Student's unpaired t-test.

Anticipated results, potential pitfalls, and alternative approaches: We expect that, when compared to historic age-matched controls, the prevalence of nAMD will be lower in patients taking Flt3 antagonists. We also expect that taking Flt3 antagonists will improve VA, reduce CST, and reduce the number of required anti-VEGF injections. The primary pitfall in the retrospective clinical study would be a lack of sufficient patients meeting the inclusion criteria, which would hamper the study's ability to draw any statistically rigorous conclusions. An alternative strategy would be to increase the patient population from which we are drawing. To do this, we will partner with Andrew Browne, MD PhD at University of California-Irvine, who has expressed interest in collaborating on this project. Inclusion of this second patient population would allow us to pool data and increase or statistical power.

Timeline: Since our IRB is approved, we expect to begin extracting data from the EDW upon receipt of funds. We expect to be able to search through the data and finish the statistical analysis within 6 months.

Specific Objective #2: Determine the role of Flt3 ligand and Flt3 during laser-induced murine CNV. *Rationale:* Our preliminary data demonstrate that Flt3 inhibition potently inhibits choroidal sprouting angiogenesis by 50-80% (Fig 3). In confirmation of our preliminary data, $Flt3^{-/-}$ mice show reduced CNV area by 40-50% compared to wildtype controls [10]. This study, however, was limited because it did not investigate Flt3 ligand nor determine if Flt3 is sufficient to increase CNV area. In this Aim, *we will test whether Flt3L is necessary for laser-induced CNV and if Flt3 is sufficient for CNV.* These experiments will demonstrate the utility of future therapeutics targeting the Flt3L – Flt3 signaling for nAMD-like disease in mice.

Study Design and Methods: We will primarily use the laser-induced CNV mouse model to determine the effects of the Flt3 ligand – Flt3 pathway on angiogenesis. We will perform and analyze experiments on both male and female mice, but since there are sex differences in laser-induced CNV, the data will be analyzed independently. Based upon prior data using the laser-induced CNV model[4, 5], our sample size calculation estimates that 10 mice will be needed per group to detect a 50% reduction in laser-induced CNV. We will repeat each experiment three times to confirm reproducibility.

Sub-Aim 1: Is Flt3 ligand necessary for laser-induced CNV? In this Sub-Aim, we will use $Flt3L^{-/-}$ mice to determine if Flt3 ligand is necessary for laser-induced CNV. Wildtype C57BL/6J and $Flt3L^{-/-}$ mice aged 10-12 weeks will be subjected to laser-induced CNV as previously described [15]. We will use $Flt3^{-/-}$ mice as our positive control. All three mouse strains are currently at Northwestern University animal facilities. Separate experiments will be performed on male and female mice, N=10 per group. On Day 14, we will perform fluorescein angiography followed by posterior eye cup harvest for immunofluorescence staining and quantification of CNV area.

Statistical Analysis: Data will be analyzed using one-way ANOVA followed by Bonferroni post-hoc test.

Anticipated results, potential pitfalls, and alternative approaches: Because $Flt3^{-/-}$ and $Flt3L^{-/-}$ mice have comparable hematopoietic cell phenotypes, we expect that $Flt3^{-/-}$ and $Flt3L^{-/-}$ mice will have similar laser-induced CNV phenotypes. Therefore, we expect that $Flt3L^{-/-}$ mice will demonstrate reduced CNV area compared to wildtype controls and equal CNV area to $Flt3^{-/-}$ mice. If we do not see reduced CNV area in $Flt3L^{-/-}$ mice compared to wildtype controls, there could be functional redundancy in the eye. However, we believe this is unlikely because of the similarities between $Flt3^{-/-}$ and $Flt3L^{-/-}$ mice in immune cell development. An alternative approach would be intravitreal Flt3 ligand blocking antibody injection compared to isotype control. Given our expertise and experience with the laser-induced CNV model and knockout mice, we do not anticipate any technical challenges or pitfalls.

Sub-Aim 2: Is Flt3 sufficient for laser-induced CNV? The Flt3-ITD knock-in mouse carries the internal tandem duplication of amino acids 596 – 602 at the endogenous Flt3 locus [16]. The Flt3-ITD protein shows ligand-independent constitutive activation, and is available from Jackson Labs. We will cross $Flt3^{ITD/+}$ x $Flt3^{ITD/+}$ to generate $Flt3^{+/+}$ littermate controls, $Flt3^{ITD/+}$ heterozygotes, and $Flt3^{ITD/ITD}$ homozygous mice. Laser-induced CNV will be performed on 10-12 week old mice (N=10 per group). Experiments will be performed on male and female mice, and the data will be analyzed independently. On Day 14, we will perform fluorescein angiography followed by posterior eye cup harvest for immunofluorescence staining and quantification of CNV area.

Statistical Analysis: Data will be analyzed using one-way ANOVA followed by Bonferroni post-hoc test.

Anticipated outcomes, potential pitfalls, and alternative approaches: The Flt3-ITD protein causes ligand-independent constitutive Flt3 activation and results in cellular migration and proliferation, both key cellular aspects of endothelial angiogenesis. Thus, we expect that $Flt3^{ITD/ITD}$ mice will demonstrate the largest CNV area compared to $Flt3^{+/+}$ wildtype mice. Additionally, we anticipate that $Flt3^{ITD/+}$ mice will show an intermediate phenotype between homozygous $Flt3^{ITD/ITD}$ mice and $Flt3^{+/+}$ wildtype mice. A potential pitfall is that $Flt3^{ITD/ITD}$ mice get myeloproliferative disease between 1-4 months of age [16]. An alternative approach is to create bone marrow chimera mice where wildtype bone marrow is given to $Flt3^{ITD/ITD}$ mice. These mice will still express Flt3-ITD protein in endothelial cells, but will have normal bone marrow and will not develop myeloproliferative disease. These experiments will demonstrate that Flt3 is sufficient to increase CNV area.

Timeline: Wildtype C57BL/6J, $Flt3^{-/-}$ and $Flt3L^{-/-}$ mice are all currently at Northwestern University and breeding in the animal care facilities. Experiments are performed at the age of 10-12 weeks of age, and take 2 weeks to complete. We anticipate beginning experiments in 3 months, and completion of experiments in 9 months of time. $Flt3^{ITD/+}$ mice are available from Jackson labs via cryo-recovery. It takes 3 months for recovery of mice, 3 months for establishing the colony, and 6 months to complete experiments. We anticipate completing these experiments in 12 total months. Experiments will be performed in Dr. Lavine's laboratory by myself, Dr. Lavine's technician Steven Droho PhD, and Dr. Lavine.

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