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The Effect of Plasma Levels of Matrix Metalloproteinase-9 on Intracranial Aneurysm Progression

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THE EFFECT OF PLASMA LEVELS OF MATRIX METALLOPROTEINASE-9 ON INTRACRANIAL ANEURYSM PROGRESSION

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Table of Contents:

List of Abbreviations:

AA - aortic aneurysm
aSAH - aneurysmal subarachnoid hemorrhage
IA - intracranial aneurysm
CI - confidence interval
CTA - computed tomographic angiography
EC- endothelial cell
ECM - extracellular matrix
ELISA - enzyme-linked immunosorbent assay
EMR - electronic medical record
HIPAA - Health Insurance Portability and Accountability Act
HR - hazard ratio
IEL - internal elastic lamina
IRB - institutional review board
MCA - middle cerebral artery
mm - millimeter
MMP - matrix metalloproteinase
MRA - magnetic resonance angiography
OR - odds ratio
PHI - protected health information
RIA - ruptured intracranial aneurysm
ROS - reactive oxygen species
RR- relative risk
RT-PCR - real time polymerase chain reaction
SMD - standard mean deviation
TIMP - tissue inhibitor of matrix metalloproteinase
UIA - unruptured intracranial aneurysm
VSM - vascular smooth muscle
YNHHS - Yale New Haven Health System

List of Tables:

List of Definitions:

Anterior location: IA presence anterior to middle cerebral artery
IA Progression: 1 mm or greater increase in IA height, width, or length as compared to intake imaging assessment or development of SAH
Hypertension: Blood pressure > 140/90 or current antihypertensive medication administration or previous diagnosis of hypertension
MMP+: Greater than the 50th percentile of MMP-9 concentration of the study population
MMP-: Less than the 50th percentile of MMP-9 concentration of the study population
Posterior location: IA presence posterior to middle cerebral artery

Abstract

Intracranial aneurysms are a common vascular abnormality identified incidentally or after rupture leading to aneurysmal subarachnoid hemorrhage. However, accurately assessing the risk of aneurysmal disease progression is challenging. Here we propose a prospective cohort study to identify if elevated levels of matrix metalloproteinase-9, a molecular marker of vascular degeneration, in the peripheral blood samples of subjects with unruptured intracranial aneurysms correlate with aneurysm progression over a follow up period. This study aims to aid in identifying patients at increased risk of aneurysm progression in order to guide treatment decisions and ultimately decrease subarachnoid hemorrhage morbidity and mortality.

CHAPTER 1 - INTRODUCTION

1.1 - Overview of Aneurysmal Epidemiology, Significance, and Detection

Intracranial aneurysms (IAs) are acquired pathological expansions of the intracranial vasculature often presenting in adulthood as nontraumatic or aneurysmal subarachnoid hemorrhage (aSAH) secondary to aneurysmal rupture. It is estimated that between 3% and 6% of the United States population over the age of 30 harbors an IA with this incidence increasing with age.[1, 2, 3, 4, 5, 6] One quarter of these individuals have multiple IAs.[7] Several IA subtypes exist based on their morphology to include saccular, fusiform, and dissecting forms.[8] Between 80%-90% of all IAs are saccular, also known as berry aneurysms, due to their rounded appearance.[7] Saccular IAs are characterized by their thin or absent tunica media and internal elastic lamina and are found largely at arterial bifurcations near the circle of Willis at the base of the brain.[9,10,11]

aSAH secondary to rupture poses the most serious health effect of IAs, occurring with an estimated incidence of 30,000 per year in North America.[12] While most IAs do not progress to rupture, rupture carries with it a significant health burden. Despite advances in treatment, fatality is approximately 45% one month after rupture.[13] Greater than 40% of aSAH survivors never return to pre-morbid capabilities.[14] Of these, 10% - 20% are unable to function independently.[15] Identification of IAs prior to rupture is thus important, considering the high morbidity and mortality associated with aSAH.

IA detection is performed using magnetic resonance angiography (MRA), computed tomographic angiography (CTA), or invasive angiography. Despite the large number of people harboring IAs, approximately 50% of these aneurysms are found only after rupture.[16] Of the remaining 50% of IAs found prior to aSAH, small IAs measuring <

7mm are often asymptomatic and thus found incidentally. Larger IAs, accounting for 15% of all IAs, are more likely to cause symptoms leading to identification such as headaches, seizures, motor or sensory changes, or third nerve palsy.[17]

Screening via CTA or MRA is recommended in patients seen to be at increased risk of IA formation or rupture such as those with ≥2 family members with IA or SAH, conditions such as Polycystic Kidney Disease, Marfan's syndrome, type IV Ehlers-Danlos syndrome, neurofibromatosis type 1, alpha-1-antitrypsin deficiency, fibromuscular dysplasia, pheochromocytoma, and Klinefelter's syndrome, as well as congenital malformations such as coarctation of the aorta, bicuspid aortic valve, and intracranial arteriovenous malformations, as these patients generally follow a distinctly different treatment course and present at earlier ages.[18]

1.2: Treatment Options and Associated Risks

Treatment of identified IAs is accomplished in one of two ways, either via surgical intervention or observation. Both carry health risks as intervention carries the risk of invasive complications while observation places the patient at risk of aSAH.

Surgical treatment is generally accomplished either by open craniotomy with vascular clipping or endovascular coil embolization. Both surgical treatments are associated with non-trivial chances of complications. One large prospective cohort

demonstrated that clipping was associated with a severe functional disability of 17.5% at 30 days post-op and a 2.3% chance of death at post-op day 30.[19] These percentages increase in patients older than 50 years old.[18, 20] Craniotomy with clipping, as a more invasive treatment than endovascular coiling, is associated with more disability immediately after the procedure, most of which resolve over time.[21] Despite this, one large review reported a 13.7% chance of morbidity in clipping vs a 4.0% associated with coiling, with risk increasing with age. As such Endovascular coiling is generally seen as safer and is performed more commonly.[20] However, coiling is still associated with thromboembolic events, intraoperative IA rupture, and a greater need for re-intervention due to coil compaction.[21]

1.3 - Risk of Disease Progression

One of the main challenges in IA management is determining which IAs are at an increased risk of rupture and thus require intervention. IAs have a 0.95% risk of rupture per year according to a large prospective cohort of Japanese descent.[22] This risk has been found to be as high as 1.4% per year in a recent systematic review.[23] Physically, rupture is dependent on the interaction between wall tension versus wall strength as described by Laplace's Law which states that vascular wall tension increases as a function of vessel size, intravascular pressure, and wall thinness.[7, 24, 25, 26] The risk of SAH associated with IA rupture has thus historically been understood as a function of IA size, where larger IAs are more prone to rupture.[22, 27] Multiple studies have demonstrated higher overall risk for IA rupture in IAs >7mm.[7, 22, 28] However, size alone

is insufficient to accurately assess IA rupture risk as it is common for IAs under 7mm to rupture.[29] A more detailed discussion of IA rupture covariates is included in Chapter 2.

1.4 - Treatment Guidelines

Treatment decisions regarding IAs require careful weighing of the risk of rupture against the risk of surgical complications in the setting of age.[7] However, guidelines rely on expert opinion in the context of IA natural history. Nevertheless, these guidelines recommend that surgical treatment be advocated in patients with symptomatic or large IAs, especially in younger patients or IAs in the posterior circulation, as the risk of rupture is seen to outweigh the risks of intervention.[19, 27, 30, 31, 32] Because risk of aSAH increases with IA size while risk of surgical complications increases with age, guidelines recommend that surgical treatment is appropriate in patients under 60 with IAs > 7mm, preferably via endovascular coiling to reduce complication risks.[19] In patients over 60, intervention is usually recommended in IAs >12mm.

Conservative management via observation is advocated in asymptomatic IAs <7mm, and is composed of serial imaging with aggressive control of modifiable risk factors for aSAH such as reduction in smoking ,alcohol, and blood pressure.[5] The American Heart Association suggests that a follow up image at 6-12 months after discovery followed by a 12-24 month follow up image is reasonable.[18] Other sources recommend reimaging of small aneurysms every six months as they may be at higher risk of progression.[7] IAs measuring 7-10mm need to take into account the patient's age and comorbidities.[33] Despite these guidelines, conservative management retains a risk of sudden IA rupture. Further, assessment for IA growth requires expensive imaging

tests, highlighting the need for timely and cost-effective alternatives to assess for disease progression. Understanding the role inflammation plays in the disease progression may aid in these tasks.

1.5 - Aneurysmal Histology and Development

Arterial walls are composed of 3 layers from innermost to outermost. The innermost layer, the tunica intima, is composed of an endothelial cell layer, a subendothelium composed of the extracellular matrix proteins collagen, elastin, and ground substance. The internal elastic lamina composed of collagens and elastins.[34] The Tunica media is composed of vascular smooth muscle cells (VSMCs) and the external elastic lamina composed of elastin and collagen fibers. Elastin fibers stretch while collagen fibers provide rigid support with little ability to stretch prior to failure. The outermost layer is the tunica externa composed of collagen fibers, fibroblasts, and the vasa vasorum. Additionally, while the tunica media contains an external elastic lamina in most arteries throughout the body, the cerebral vasculature does not contain this additional elastic layer.[34] The lack of an external elastic lamina in cerebral arteries lends them more prone to aneurysm formation as the elastic lamina and collagen fibers provide resistance to stretch related deformation and structural strength respectively.[24, 35]

Histological examinations of IA reveal several pathological changes in these three arterial layers.[36] The most widely observed is the loss of the internal elastic lamina and atrophy of the tunica media is considered a hallmark of the disease and forces the remaining collagen fibers of the tunica externa to maintain the bulk of aneurysmal

structural integrity.[3, 9, 24, 36, 37] These findings are often found in the presence of fibrotic changes and infiltration of leukocytes into the remaining arterial wall. Leukocytes within IA walls secrete inflammatory chemoattractants and enzymes such as matrix metalloproteinases (MMPs)which are known to degrade the arterial wall components.[38, 39]

These histological changes are caused by pathologic blood flow near arterial bifurcations which expose vascular endothelial cells to two increased wall stresses.[40] The first is cyclic transmural stress caused by arterial pulsations related to blood pressure, the other is wall shear stress, a frictional force generated as blood flows past the arterial wall through the vessel lumen. Increases in either, through hypertension or via non laminar flow at arterial bifurcations and curvatures, is noxious to endothelial cells and causes the release of inflammatory adhesion molecules, reactive oxygen species, and complement system activation.[3, 7, 39] This endothelial damage and its resulting inflammatory response begins a process of vascular remodeling.[8]

The remodeling process requires a balance between cell proliferation, cell apoptosis, and extracellular matrix reconstruction via a variety of factors, enzymes, and second messengers.[39, 41, 42] In IA development, tissue breakdown outpaces tissue construction as leukocyte infiltration leads to VSMC apoptosis and progressive tunica media thinning.[39] Leukocytes secrete proteases such as (MMPs) that act to both breakdown extracellular matrix proteins such as collagen and elastin and induce further macrophage infiltration.[3,24] The degradation of arterial wall layers is believed to be catalyzed by these increased MMPs.[43] Loss of the internal elastic lamina, ECM, and

VSMC layer results in weakening of the vascular wall, allowing for pathologic bulging of the IA under arterial pressure.

1.6 - Overview of Matrix Metalloproteinases

MMPs are a family of peptidases containing a zinc ion catalytic domain.[57] MMPs are expressed intra or extracellularly in their inactive form and require activation to begin cleavage of their respective substrates.[44] Currently there are over 28 individual MMPs grouped into six families according to their main substrates although a large amount of overlap between families exists.[45, 46] Several inhibitors of MMPs, named tissue inhibitors of metalloproteinases (TIMPs), also exist. These enzymes and their inhibitors are necessary for normal physiological processes such as cell migration, wound healing, and tissue remodeling as well as being implicated in many pathogenic processes such as cancer metastasis, multiple sclerosis, and aortic disease. Their role in IA is believed to be a runaway process of ECM breakdown as collagen and elastin are common substrates for several MMPs such as MMP-2, MMP-9, and MMP-12.[46] MMP-9, also known as gelatinase B, is a proteolytic enzyme for collagen types 4, 5, 11, and 14, as well as elastin, gelatin, and several other matrix proteins.[47] This implicates MMP-9 in the degradation of major components of vascular ECM and internal elastic lamina, ultimately contributing to IA pathology.

Circulating plasma levels of MMPs including MMP-9 are increased following both IA rupture as well as other forms of stroke. A dose response relationship has been described between MMP-9 levels and poor outcome following stroke.[48] Additionally, other studies have demonstrated increased plasma levels of MMP-9 and decreased

levels of its inhibitors in aSAH patients as compared with controls.[49] The utility of peripheral levels of MMP-9 are further demonstrated as they correlate with NIHSS scores following stroke.[49] MMP-9 levels have also been demonstrated to predict ischemic stroke.[50] However, as MMP levels, and MMP-9 in particular, are integral components of tissue remodeling, it follows that these enzymes would be increased following various events resulting in tissue loss and subsequent remodeling. This paper is instead interested in if MMP-9 levels can be used prognostically prior to massive tissue damage. A more complete review of MMP-9 in aneurysmal disease is included in Chapter 2.

1.7 - Statement of the Problem

Intracranial aneurysms are a potentially deadly arterial malformation. However, assessment of IA progression is challenging, expensive, and incompletely understood. As such there is a need for better disease progression risk stratification in conservatively managed patients. MMP-9 activity is implicated in IA pathogenesis and progression, but it is unclear if this enzyme can be used prognostically as a biomarker for disease progression.

1.8 - Goals and Objectives

To establish if elevated MMP-9 concentration in peripheral venous blood samples of patients with conservatively managed IAs is associated with IA disease progression over a follow-up period.

1.9 - Hypothesis

There will be a statistically significantly greater proportion of subjects with IA disease progression over the follow-up period among subjects with an unruptured intracranial aneurysm (UIA) diagnosed within the last 3 years who are found to have a plasma MMP-9 level greater than the 50th percentile among subjects within the study group as compared to those with a plasma MMP-9 level less than the 50th percentile when controlling for other risk factors.

1.10 – Relevant Definitions

1) Elevated MMP-9 level is defined as theMMP-9 level being greater than the 50th percentile of MMP-9 levels among subjects.
2) Disease progression is IA growth or identification of aSAH during the study period.
3) Growth is defined as any increase greater than 1 mm in aneurysmal length, width, or height upon follow-up CTA or MRA.
4) Follow up period is defined as one year after plasma MMP-9 level measurement.

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CHAPTER 2 - REVIEW OF THE LITERATURE

2.1 - Search Criteria

We conducted searches of PubMed, Scopus, Ovid, and Cochrane Library between January 2021 and April 2021. Searches were conducted using combinations of MeSH keywords such as intracranial aneurysm, cerebral aneurysm, brain aneurysm, berry aneurysm, saccular aneurysm, mmp-9, gelatinase b, 92-kda gelatinase, matrix metalloproteinase, growth, increased size, volume, subarachnoid hemorrhage, SAH, bleed, and intracranial hemorrhage. We also examined the references lists of all studies. Preference was given to articles published within the last 10 years, however all pertinent studies, reviews, and meta-analyses written in or translated to English were included. Relevant non-human studies were included due to a relative lack of human studies related to the exposure and outcome.

2.2 - Literature Review Introduction

This search demonstrated that MMP-9 presence in the IA wall is a cornerstone of IA development and progression as well as significant in post rupture pathology. Further, our literature review demonstrates the multifaceted and complex nature, highlighting the many covariates at work in this complex disease. Whether plasma levels of MMP-9 found in peripheral blood samples are associated with IA progression as we have defined it is less clear, mainly due to a relative lack of studies on this

subtopic. Widely quoted yet older and smaller studies suggest this is not the case while newer studies, at times, suggest the opposite. No studies investigating plasma MMP-9 levels associated with IA growth were found.

2.3 - Review of MMP-9 in Aortic Aneurysm

Interest in this topic traditionally stems from studies suggesting that MMPs are implicated in aortic aneurysm, which, while not the specific focus of our proposed study, deserves brief mention here. A meta-analysis of studies reporting differential protein expression in subjects with abdominal aortic aneurysm (AAA) spanning 106 studies found that there was a significant increase in circulating MMP-9 levels as compared to controls (SMD 0.67 ng/ml; $p=0.002$; $I^2 = 91\%$)[1]. MMP-9 activity within aortic tissue was also increased significantly. Included in this analysis is the 2013 case control study consisting of 120 subjects with AAA diagnosed between 1996 and 2007 and 78 controls without aneurysmal disease. Plasma samples analyzed via enzyme linked immunosorbent assay (ELISA) techniques revealed that MMP-9 levels were higher in subjects with AAA vs controls ($p<0.05$).[2] Interestingly, this difference remained significant in the analysis of subjects with repaired AAA compared to those without repair suggesting that increased MMP-9 levels remain elevated until repair ($p<0.05$), in agreement with other studies[3]. Further, levels of MMP-9 mRNA demonstrated a 16.3-fold increase in aortic aneurysmal tissues as compared to the aortic samples of controls[2]. Another study found that elevated plasma levels of MMP-9 had a positive predictive value of aortic aneurysm presence of 92.3% if levels were found to be >87.8 ng/mL ($p<0.05$)[4]

2.4 - Review of MMP-9 in IA Animal Models

Much of the knowledge about MMP-9 in IA, however, stems from animal models of the disease. Animal studies have demonstrated MMP-9 plays a significant role in IA formation and growth, as well as the protective role of exogenous inhibitors of MMP-9 in IA. The important role of MMP-9 in IA progression has been demonstrated in studies using MMP analogs such as elastases and collagenases, as well as MMP inhibitors.[5, 6] In one study, IAs were induced in rats via hypertension and injection of elastase.[5] Treatment with doxycycline, a nonselective MMP inhibitor, in doses of 40 mg/kg/day reduced IA formation from 70% to 10% ($p<0.05$) and to 40% in MMP-9 knockout mice ($p<0.05$). Conversely, other animal models demonstrate more severe IA formation via MMP disinhibition in TIMP deficient mice relative to control animals.[7]

A 2007 paper demonstrates several core features of MMP-9 in the pathogenesis of IA using a rat model.[8] Investigators induced IAs via high salt diet, left carotid artery and bilateral posterior renal artery ligations with resultant hypertensive flow through the Circle of Willis according to a standard IA induction protocol. The anterior cerebral artery/olfactory artery bifurcation of these animal subjects were then harvested at one ($n=11$) or three months ($n=10$) post IA induction. MMP-9 tissue levels were then assessed using real time polymerase chain reaction (RT-PCR) demonstrating increases in MMP-9 at 3 months post induction vs age matched, non-IA induced control subjects ($p<0.01$) expressed as a ratio vs beta actin, a non-MMP sensitive component of arterial walls in agreement with many other studies on the topic.[9, 10] Further, MMP activity was assessed in a separate cohort ($n=10$) given therapeutic levels (50 mg/kg/day) of

Tolylsam, a nonspecific competitive inhibitor of MMP-2, -9, and -12 vs a control group (n=20) after IA induction. 90% of control rats developed both gross (advanced) IA bulging and IEL discontinuity, with the remainder developing only discontinuity of the IEL. In comparison, 50% of the experimental group developed advanced IAs and IEL discontinuity, while the remaining 50% demonstrated only IEL discontinuity. While neither the incidence of IA nor the degree of hypertension (control group:160.7±21.1 mm Hg; n=20; experimental group:161.2±12.2 mm Hg; n=10; $p>0.05$) were different between the two groups, the rate of developing advanced IA changes was significantly lower in the experimental group ($p=0.013$). The number of MMP producing cells within the IA wall was also not significantly different between the groups (control group:5.4±1.5; n=14; tolylsam group:5.1±1.3; n=10, $p>0.05$). In situ zymography for gelatinase activity was demonstrated in the control rats and not found to be present in the treatment group. Overall, this study demonstrates several core features of IA disease such as the presence of MMP-9 in IA tissues, the protective role that inhibition of MMPs can play, as well as the importance of other risk factors for IA development such as hypertension.

The role of MMP-9 in the pathogenesis of IAs was further explored in animal models demonstrating an increased level of MMP-9 mRNA by RT-PCR in rats with induced IAs. [11, 12] In a 2020 paper, investigators attempted to examine the relative concentrations of vascular degeneration substances via mRNA level analysis of 36 rats with induced IAs relative to sham-operated rats. [11] Significant findings include higher MMP-9 mRNA and a higher MMP-9/TIMP-2 mRNA ratio expressed in arbitrary units ($p<0.01$), especially in the posterior cerebral circulation, in the experimental group vs

the sham control group. This finding was supported by other studies where investigators found that the total number of IAs formed and their rates of rupture were significantly increased in rats that underwent IA induction with associated increases in the mRNA levels of MMP-9 and MMP-9/TIMP2 ratios ($p<0.01$) vs sham-operated rats expressed as mean and standard deviations in arbitrary units (alpha=0.05; power = 0.8; effect size of 0.4). [12] These findings suggest that absolute levels of MMP-9 as well as the relative level of activated MMP-9 play important roles in IA formation.

While the literature demonstrates that MMPs are strongly implicated in aneurysmal pathogenesis and progression, there remains significant ambiguity regarding the utility of MMP-9 measurements in blood samples of subjects with IA. There are obvious mechanistic components to the relationship of MMP-9 and IA disease as described above, however the relationship between MMP-9 and IA progression over time requires further investigation.

2.5 - Blood Sampling of MMP-9 in IA Disease

This literature review found relatively few studies investigating the relationship between MMP-9 concentration in the blood of human subjects with IAs and IA disease progression despite the mechanistic relationship between them. Indeed, no papers were found which assessed for increased size of UIA over a follow up period in relation to MMP-9 levels. Instead MMP-9 concentrations were only related to IA presence or rupture. Further, the papers that were found often present conflicting or contradictory results.

Our search of the literature found several review articles stating that peripheral blood levels of MMP-9 in relation to IA disease would be a fruitless course of

investigation. [13, 14, 15] This appears to be largely due to the findings of a single, widely quoted paper from 1997 *by* Kim et al. [9] Here the experimenters attempted to compare MMP-9 levels in peripheral plasma samples in human subjects with known IAs (n=6) vs those with no known IAs (n=6). The characteristics of each subject such as age, gender, IA size, comorbid conditions, etc., are not presented. Results are presented qualitatively as approximately the same visual appearance between control and experimental samples for the activity of MMP-9 via gelatin zymography. The small sample size, ambiguity of both control and IA group characteristics, as well as the qualitative nature of result reporting should call into question the validity of this study. Nevertheless, this study is widely quoted in the argument against the utility of peripheral blood sampling in the assessment of IAs and likely contributed to reduction of interest in the topic of assessing peripheral MMP-9 levels in IA disease.

The few studies directly measuring peripheral blood levels of MMP-9 following Kim's watershed paper demonstrate a large degree of variability in conclusions. However, we have attempted to make it clear that these papers fail to distinguish if the increase in MMP-9 found in subjects with RIA causes IA rupture or is caused by IA rupture. One such study is a 2018 paper titled *"Levels of MMP-9 in patients with intracranial aneurysm: Relation with risk factors, size and clinical presentation"* by Rojas et al. [16] In this case-control study investigators attempt to evaluate the relationship between plasma MMP-9 levels and the risk factors that are associated with ruptured and unruptured IAs. To do this, peripheral blood samples were taken from 282 subjects with IA and 286 age and sex matched control volunteers. Samples were assessed by ELISA. In contrast to Kim's 1997 paper, Rojas et al. thoroughly describe the

characteristics of the study participants, including smoking, sex, age, hypertension, family history of IAs, IA rupture status, and IA size. The results of the analysis demonstrate increased MMP-9 levels, unfortunately expressed as unitless numbers, in the patient group as a whole vs control (190.5 +/- 5.4 vs 149.7 +/- 4.7; $p<0.0001$), the ruptured group vs control ($p<0.0001$) and the unruptured group vs control ($p<0.0006$). Interestingly, no difference in MMP-9 levels were found between RIA and UIA ($p=0.4792$), suggesting that MMP-9 levels do not change significantly post IA rupture, in contrast to findings of other papers discussed below. Instead, results suggest MMP-9 levels can act as an indicator of IA presence regardless of rupture status. Further analysis suggests that while MMP-9 levels of subjects with RIAs taken are significantly higher than the control group, this difference is significant only in those subjects with RIA measuring <12mm ($p = 0.008$) and >25mm ($p = 0.047$), with RIA measured between 12 and 25 mm having a corresponding p-value of 0.062. This is even more true of the UIA group as only the group with UIAs measuring <7mm being statistically significant ($p = 0.0071$). All other UIA size groupings did not correlate with a significant difference in MMP-9 levels when compared to controls. This may be due to sub-optimized sampling methods as evidenced by the fact that only 23 subjects were included in the 12-25mm RIA group, and the fact that the UIA group contained only 75 subjects, 32/75 (42.6%) of those subjects having an IA <7mm.

In contrast to these findings, several studies have found significant differences in serum MMP-9 concentrations between ruptured and unruptured IAs groups.[17, 18] A 2007 paper titled *"Matrix metalloproteinases and tissue inhibitors of metalloproteinases expression in human cerebral ruptured and unruptured aneurysm"* by Jin et al

redemonstrated localized increases of MMP-2 and MMP-9 within IA walls and serum samples in subjects with RIAs as compared with UIAs [19]. To do this, IA tissue samples were collected from 30 patients undergoing IA clipping. These 30 patients were split into 2 equal groups of 15 each by rupture status (15 RIA, 15 UIA). These samples were subsequently analyzed for MMP/TIMP mRNA ratio levels using RT-PCR and compared to MMP-2, MMP-9, and TIMP-1,-2,and -3 serum levels using ELISA. Results demonstrate that MMP-9 mRNA levels were significantly associated with RIA vs UIA (RIA: 5.21 +/- 0.87; UIA: 1.69 +/- 1.00, expressed as a numberless unit relative to the enzyme GADPH, $p = 0.05$). MMP-2 mRNA levels were also significantly higher in the RIA group. The serum level branch of this study demonstrated significant ($p<0.02$) increases in MMP-9 and MMP-2 in RIA vs UIA as well (MMP-9: 1066 +/- 43 vs 120 +/- 27 ng/mL; MMP-2: 1047 +/- 33 vs 110 +/- 26 ng/mL respectively; $p<0.02$). Immunohistostaining demonstrated increased levels of MMP-2, -9, TIMP-1, -2, -3 in the RIA group relative to the UIA group as well. Despite these findings, the only conclusion that can be reliably drawn is that MMP levels increase after rupture as it is unclear if these elevations were the result or cause of rupture. Further loss of utility of the study is realized as the study failed to take confounding variables or IA risk factors into account in either data presentation or data analysis. As such, the prognostic utility of the study cannot be assessed, nor can its conclusions easily inform our own hypothesis.

Despite these ambiguities and methodological shortcomings, similar conclusions were found in a small case-control study evaluating the plasma levels of MMP-9 assessed via ELISA in human subjects with IA; 3 of which ruptured (mean diameter: 28mm) and 4 of which did not rupture (mean diameter: 13.5), vs a healthy control [20].

Venous plasma levels of MMP-9 were higher in IA subjects as compared to controls ($p<0.01$) as well as higher in subjects with RIA and larger IAs ($p<0.05$), however exact concentrations, means, and standard deviations are not stated. All IA subjects underwent surgical clipping which allowed IA samples to be taken and assessed for tissue expression of MMP-9. Here results were similar as above, with MMP-9 tissue levels assessed via western blot analysis found to be significantly higher in RIAs vs UIAs ($P<0.01$). This study's use of a control group helps improve its internal validity in contrast to Jin, et al. However, its external validity is marred by its small sample sizes and poor data reporting.

A 2018 paper adds further confusion to the role of MMP-9 in IA disease.[21] In this paper the investigators carried out a population based case-control study where all adult men and women of the city of Malmo, Sweden were invited to undergo baseline examinations and peripheral blood draws to assess if various MMP levels would correlate with spontaneous SAH over a follow up period from intake spanning 1991-1996 until SAH, death, emigration away from Sweden, or December 31, 2010, whichever occurring first. Baseline examination included BP measurement, BMI, waist circumference, smoking and alcohol habits, and current medications taken. Peripheral blood samples were drawn and assessed for white blood cell count, MMP-1, -2, -3, -7, -9, -10, and -12. In total 79 cases with a median time from baseline to SAH of 8.5 years (0.4-18.7 years) were compared to 261 controls matched for age and sex. MMP-9 levels were found to be higher in cases of SAH as compared with controls, but they failed to reach statistical significance in multivariate analysis (adjusted OR 1.06; 96%CI, 0.79-1.41; $p=0.713$). Only MMP-7 was significantly associated with an increase in SAH

frequency when adjusting for confounders (OR 1.64; 95% CI, 1.19-2.27; p=0.0026). . While this study suggests that peripheral sampling of blood to assess for MMP-9 is not associated with SAH, it is unclear if the cases in this study included SAH stemming from IAs as no imaging of cerebral vasculature was done during subject intake. Indeed, it is not clear if vascular imaging was completed at all, or if it was, what proportion of cases had undergone this assessment of IA presence as it is not readily clear how diagnosis of SAH was identified in the cases. As such, it is not known if the cases had IAs during the only assessment of MMP levels during intake or if these cases developed IAs after MMP assessment, if at all. As such, this study does not present data directly impacting or related to our own when scrutinized to a higher degree.

2.6 - Review of Confounder Variables

Several variables may affect either MMP-9 activity or IA progression. In general, these confounders can be categorized as affecting IA rupture, IA growth, or MMP functioning. The following review summarizes the studies most closely related to the investigations of these topics.

2.6.1 - Confounding Variables Affecting IA rupture

Lesion and subject dependent variables related to increased risk of IA progression has been a topic of study for many decades. It is generally established that IA size, location, shape, and multiplicity play significant roles, as well as subject age, sex, ancestry, smoking, SAH history, and hypertension status all contribute to IA rupture. Indeed even IA growth itself may predict rupture.[22] One large meta-analysis

spanning 19 previously published studies and 6556 UIAs followed over 26,122 patient years demonstrated that statistically significant risk factors for rupture were age >60 (RR 2.0; 95% CI, 1.1-3.7), female gender (RR 1.6; 95% CI, 1.1-2.4), Japanese or Finnish descent (RR 3.4; 95% CI, 2.6-4.4), IA size >5 mm (RR 2.3; 95% CI, 1.0-5.2), IA location in the posterior circulation aneurysm (RR 2.5; 95% CI, 1.6-4.1), and the presence of a symptomatic aneurysm (RR 4.4; 95% CI, 2.8-6.8).[23]

These findings agree with other large-scale reviews such as the 2014 systematic review of 6 prospective studies and 8382 participants with non-traumatic SAH as the primary outcome and hazard ratios (HR) representing a 5-year IA rupture risk. This study included hypertension as a risk factor, as well as stratified IA size with risk of SAH. Specifically, hypertension history or presence (HR 1.4; 95%CI 1.1-1.8) IA size 7-9.9 mm (HR 2.4; 95%CI 1.6-3.6) IA size 10 mm-19.9 mm (HR 5.7; 95% CI 3.9-8.3) IA size >20 mm (HR 21.3; 95% CI 13.5-33.8). If multiple IAs were present in a single subject, the largest IA was used in analysis without individual IA analysis. Results are comparable to the above reports for IA location in the anterior cerebral arteries (HR1.7; 95% CI 1.1-2.6), posterior comm artery (HR 2.1; 95% CI 1.4-3.0) posterior arteries (HR 1.9; 95% CI 1.2-2.9) relative to the Middle cerebral artery (MCA), and subject geographic location of Japan (HR 2.8; 95% CI 1.8-4.2) or Finland (HR 3.6; 95% CI 2.0-6.2) relative to North America when controlling for other covariates using multivariate Cox regression analysis.[24] While the presence of any prior smoking history was not reported in multivariate analysis, this definition appears to be a limited means of determining the importance of smoking history, and is in contrast to other studies suggesting that smoking increases relative risk of aSAH to as high as 7.3 when

controlling for other variables.[25] Instead, operationalization via pack-years likely represent better methods of determining the importance of smoking on IA rupture. Furthermore, familial history of aSAH and aneurysm growth over time were not assessed, the latter of which is particularly important to our study. Additionally, 4 of the 6 studies used in this paper included studies with subjects who underwent clipping or coiling of IAs during the follow-up period excluding them from inclusion in risk calculations.[26, 27, 28, 29] Selection bias found in exclusion of these subjects with a potentially higher rupture risk may have skewed results by removing subjects with more volatile lesions from data analysis. Furthermore, 6,486 of the 8,382 included subjects were of Japanese descent (77.38% of all subjects), highlighting a source of selection bias. Indeed, the above meta-analysis includes a widely quoted prospective cohort study that followed 5720 adult Japanese subjects with a total of 6697 newly diagnosed IAs measuring greater than 3mm.[29] Relative to aneurysms that were sized 3 to 4 mm, the hazard ratios (HR) for rupture were significantly increased for IA size categories 7 to 9 mm (HR 3.35; 95% CI, 1.87-6.00; p<0.01), 10 to 24 mm (HR 9.09; 95% CI, 5.25-15.74; p<0.01), and 25 mm or larger (HR 76.26; 95% CI, 32.76-177.54; p<0.01). HRs for IAs 5 to 6 mm were not statistically significant (HR 1.13 (95% CI, 0.58-2.22; p = 0.71). IAs in the posterior and anterior communicating arteries were more likely to rupture as compared to IAs in the middle cerebral artery (HR 1.90; 95% CI, 1.12-3.21; p= 0.02; HR 2.02; 95% CI, 1.13-3.58; p = 0.02, respectively). Irregularly shaped IAs were also more likely to rupture (HR 1.63; 95% CI, 1.08-2.48; p = 0.02).

One retrospective cohort study of subjects with UIA demonstrated that risk factors for rupture following the multivariate analysis were IA growth (OR 55.9; 95% CI

4.47-700.08; p=0.002) and multilobulated IA shape (OR 17.4; 95% CI, 1.52-198.4; p=0.022) demonstrated via serial CTA.[22] These findings suggest that the limited definition of IA progression of either rupture or growth is misleading as a risk factor for IA rupture is IA growth. IA growth, therefore, requires its own dedicated discussion.

2.6.2 - Confounding Variables Affecting IA Growth

Several risk factors of IA growth have been proposed, many of which are the same risk factors for IA rupture such as IA size, location, and shape as well as subject characteristics such as hypertension, smoking, age, and sex. Of these only IA size, shape, multiplicity, and location as well as subject smoking status and hypertension are substantiated via meta-analysis. As such, in the absence of MMP exposure designation, IA growth is mainly lesion rather than subject specific. For example, one systematic review including 4972 UIAs included in 18 studies concluded that irregular IA shape, posterior location, IA size greater than or equal to 5mm, and the presence of multiple IAs in a single subject were associated with relative risks (RRs) of 2.32 (95% CI, 1.46-3.68; I^2 =91%), 1.94 (95% CI, 1.32-2.38; I^2=57%) 2.56 (95% CI, 1.93-3.39; I^2 =98%) and 2.04 (95% CI, 1.56-2.66; I^2 =90%) respectively.[30] Other parameters such smoking at baseline (RR=2.04; 95% CI, 1.56-2.66; I^2=90%) and hypertension (RR=2.32; 95% CI, 1.46-3.68; I^2=91%), were statistically significant, though high I^2 values may discredit these findings somewhat. This review included a retrospective cohort study which found that the only risk factor for IA growth measured via serial MRI upon multivariate analysis was original IA size >8mm (OR=7.25; 95% CI, 1.96-27.1; P=0.003).[31] Other variables

such as smoking, hypertension, IA multiplicity, and IA location were either not statistically significant or not included in multivariate analysis.

Generally, these agree with the findings reported in a systematic review and meta-analysis of IA growth, in which 23 studies and 7208 subjects were included. Larger initial aneurysm sizes (OR 2.73; 95%CI, 2.21-3.36; p<0.00001) and smoking history (OR 1.45; 95% CI, 1.07-1.98; p = 0.02) were associated with increased odds of IA growth.[32] Of the studies included in these systematic reviews, one multi-centered prospective cohort of 557 non-Japanese subjects followed over a mean of 2.7 years assessed risk factors for IA growth as measured via serial CTA or MRA. Multivariate analysis via Cox logistic regression showed that size 7 mm-9.9 mm (HR 4.14; 95% CI 2.04-8.37), 10 mm-19.9 mm (HR8.06, 95% CI 3.89-16.72), >20 mm (HR12.92, 95% CI, 4.11-40.62) relative to size <7 mm predicted growth rather than rupture during a 5 year follow up period.[33] Interestingly this study did not find significant increases in hazard ratios for IA growth associated with hypertension, age >70, history of previous SAH, or IA location.

2.6.3 - Confounding Variables Affecting MMPs

While we have taken care to describe confounding variables that may affect the primary outcome of our proposed study, time must be taken here to review possible confounders of our study's independent variable, summarized as either exogenous or endogenous inhibitors of MMPs. Exogenous inhibitors of MMP-9 include Doxycycline, Statins, and Aspirin, all of which are relevant to our study as they are commonly prescribed medications for conditions with a high prevalence in the United States.

Endogenous inhibitors of MMP-9, such as TIMPs discussed in previous sections, are to be considered an intrinsic property of each subject's unique physiology and would require their own serological assays and analysis to measure and understand. As such they are outside the scope of this study. Therefore, we will restrict the discussion of MMP inhibitors here to include only exogenous MMP inhibitors as they are well known and feasibly accounted for in our study population.

Statins are cited in the literature as effectively lowering the levels or activity of MMP-9 in animal models with associated protection against IA progression. Both simvastatin and pitavastatin administration are correlated with significant reductions in IA size, IEL thickness, MMP mRNA, and macrophage numbers within the IA wall in several animal trials. [34, 35.] However, a meta-analysis of 10 randomized, placebo controlled human trials found no significant change in plasma MMP-9 levels with either <12 weeks of statin therapy (SMD: -0.31; 95% CI, -0.81-0.19; p = 0.226) or >12 weeks statin therapy (SMD: 0.15; 95% CI, -.91 to 0.60; p = 0.689).[36] As such, there is no large scale evidence to date that statin use will contribute significant confounding to our proposed study. Despite this, we will nonetheless gather information about statin use in our study population through intake and outcome assessment forms as these medications are commonly prescribed and easily accounted for.

Antibiotics of the tetracycline class, specifically doxycycline, have also been quoted in the literature as having a protective effect on aneurysmal progression through an indirect MMP inhibitory mechanism. [37] One double-blind, placebo controlled trial of 44 subjects receiving 100mg of doxycycline twice daily for 6 months following AA repair vs placebo, matched for multiple characteristics, found that plasma MMP-9 levels were

significantly decreased below pretreatment baseline (-11.8% +/- 12.5%; p <0.06) as compared to the placebo group (206% +/- 98.5%; p <0.04) with the difference between the two groups also being statistically significant (p <0.009).[38] This has been substantiated in at least one additional AAA study.[39] However, a larger randomized placebo controlled trial of 286 subjects with AAA found that average AAA growth as assessed by ultrasonography was greater in the group receiving 100mg of doxycycline daily as compared to the placebo control group over an 18 month period (0.8mm; 95% CI. 0.1-1.4 mm; p = 0.016)[40] Other studies have found that a daily dose of 150mg of doxycycline given over 18 months slows AAA growth compared to placebo (1.5mm growth vs 3.0mm growth; p=0.01)[41] Generally, however, these studies fail to adjust for confounding variables, fail to account for compliance, and/or have small sample sizes, all of which call into question the validity of reported findings.[42] No studies were found examining the effect of doxycycline in human IA disease, nor were any meta-analysis found analyzing the body of information on doxycycline in AAA disease. Despite this, while doxycycline in the general population is not likely taken over the extended time period as found in the above studies, its use in the immediate time before or during our study may represent an easily identified source of confounding that should be accounted for in our statistical analysis.

Lastly, Aspirin has been suggested to be protective against IA progression through an MMP-9 inhibitory mechanism.[43] Meta-analysis of 5 studies consisting of 19,222 human subjects found that aspirin administration reduced the risk of non-traumatic or aSAH (OR 0.51; 95% CI, 0.34-0.76; p = 0.001), though the exact dose and frequencies are not reported unambiguously.[44] One case-control study used in this

analysis compared aspirin use in 4701 subjects with 1,302 (27.7%) cases of aSAH vs UIA found that daily dosages of 81 mg of aspirin decreased risk of rupture (OR 0.60; 95% CI, 0.45-0.80; p <0.05) with a further reduction in rupture risk with increased doses to 325 mg daily (OR 0.65; 95% CI, 0.53 -0.81; p<0.01) when adjusting for confounders such as age, sex, race, HTN, heart disease, and atrial fibrilation.[45] A large case control study using ISUIA data to assess the protective role of aspirin use in IA using 58 subjects with aSAH compared to 213 IA matched controls found that subjects who used aspirin at least 3 times weekly had lower odds of IA rupture compared to those who never take aspirin upon multivariate analysis using conditional logistic regression.[46] Aspirin use is defined in terms of frequency per week, but no dosages are presented. Another large meta-analysis of 10 studies consisting of 1,107,616 subjects reported an increase in aSAH only in a subgroup that used aspirin for less than 3 months (OR 1.697; 95% CI, 1.175-2.452; p = 0.005).[47] Despite this, the available data supports the notion that aspirin administration offers a protective role against aSAH overall. As such we will collect data on aspirin use immediately before and during our study as well as include it in our data analysis given the potentially large potential for confounding.

2.7 - Review of Relevant Methodology

The following section reviews methodologies relevant to our proposed study. A more detailed discussion of our study's methods is found in Chapter 3.

2.7.1 - Study Design

Our proposed study is designed as a prospective cohort study to examine the effects of MMP-9 levels in peripheral plasma samples on IA disease progression over a

follow up period. The choice to design our study as a prospective cohort stemmed from the lack of such studies with our exposure and outcome of interest in human subjects. Preceding studies completed on the topic have been prospective cohort designs with our outcome of interest but without assessment of our exposure of interest,[27, 29] retrospective cohort studies,[24, 33, 48, 49, 50, 51] again without assessment of our exposure, case-control studies using a limited definition of IA progression,[16, 19] or small scale randomized control trials performed using non-human subjects.[5, 6, 7, 9,10, 52, 60, 62] This lack of studies of the prospective cohort design using our outcome definition limits our ability to assess the prognostic value of MMP-9 measurements via literature review alone. This study will thus be the first prospective cohort study to our knowledge with our outcome of interest related to MMP-9 levels. While we recognize that randomized control studies are the gold-standard of study design, it would be impossible to randomize our exposure of interest as this exposure is an intrinsic property unique to each subject's physiology. Additionally, randomized administration of exogenous MMP-9 would be highly unethical as a breach of the beneficence principle. Further, a retrospective study design would likely be underpowered as MMP levels are generally not included in previous studies.

2.7.2. - Sampling of Study Population

The population we are interested in is composed of subjects with previously identified IAs that have been conservatively managed. Identification of the sample population will be made via searching the Yale New Haven Health system patient databases for subjects recently diagnosed with an UIA as we are interested in IA progression rather than IA development or genesis. Sampling of the population will then

occur via consecutive sampling, with each subject contacted and recruited from most recently imaged IA to most distantly imaged IA until our requisite sample size is met. This is in contrast with other studies on the subject, largely because these previous studies did not include reimaging of IAs as part of their outcome assessment or due to their retrospective study design. Despite this lack of precedence, we feel as though this method of population sampling is the most cost-effective manner to assess for IA progression as it limits the number of resources required to obtain baseline imaging as well as the amount of time variation between baseline imaging and outcome imaging. Previous prospective cohorts on the topic enrolled subjects as they were diagnosed with IA, largely via incidentally identifying IAs. This approach to enrollment is highly time consuming, with one study taking 4 years to recruit 448 subjects.[27] One Japanese study prospectively recruited a large number of subjects over a shorter period, however this was likely only possible due to aggressive IA screening protocols common in Japan[29] As such we feel as though this sampling method minimizes time and economic costs required for sampling to take place, as well as sampling bias while still maximizing the validity or fidelity of the data being gathered, while working within the framework of established domestic guidelines. Despite this, sampling bias may still be introduced by this method as it favors a sample with smaller, and thus perhaps more stable IAs as intervention decisions, while ultimately up to the patient, is advocated for larger IAs. As such, our study will attempt to draw conclusions about IAs from a sample that may be intrinsically more stable as less stable lesions will be treated and consequently excluded from our study.

2.7.3 - Inclusion and Exclusion Criteria

The inclusion and exclusion criteria of our proposed study have been designed to be consistent with those of the majority of previous studies and reviews mentioned in chapter 1.[53, 54] Generally these aim to exclude subjects who likely do not have MMP driven pathogenesis of their IAs such as those with connective tissue disorders, subjects with inherited disorders or malformations known to increase risk of IA, as well as those that cannot complete the process needed for outcome measurements, or those with surgically treated IAs. A further discussion of our study's inclusion and exclusion criteria can be found in Chapter 3.

2.7.4 - Exposure Determination

Our primary exposure will be plasma levels of MMP-9 collected from the peripheral venous vasculature and assessed by the ELISA method. This method of measurement was chosen because of its use in prior studies, ease of acquisition, and minimal associated risks as our study is interested primarily in developing a feasible prognostic biomarker for IA progression validated by traditional imaging methods.[13, 16, 38, 55, 56] Despite this, our literature review demonstrated that many studies preferred to assess the presence of a variety of MMPs in vascular walls themselves by other techniques such as tissue zymography largely due to subject populations undergoing surgical treatment of IAs or from non-human subject harvests.[57, 58, 59, 60, 61, 62, 63, 64] However, as discussed above, these studies necessitate surgical intervention in order to acquire tissue samples and thus cannot be included in our conservatively managed study population. Further, our literature review did not find studies which attempted to define normal or healthy concentrations of MMP-9. Indeed, sparingly few studies were

found which assessed MMP-9 concentrations in healthy controls.[9, 16, 20, 21] Even with such control values, it is impossible to determine if these are normal or healthy concentrations, or how an unhealthy concentration should be defined given this lack of large-scale data. As such, our exposure determination will be defined from within our study population relative to itself as greater than or less than the 50th percentile concentration of MMP-9 within the sample population.

2.7.5 - Outcome Definitions

The outcome of interest in this study is IA progression defined as either a 1mm or greater increase in IA size in any dimension as assessed by CTA or MRA, or development of SAH. As in previous decisions regarding study design, our definition of growth was chosen as it agrees with the majority of other studies on the topic and is the most easily verified.[29, 31, 33, 65, 66, 67, 68] Despite this, a number of other growth definitions have been used including a 2mm or greater increase in any dimension or a 1.5X increase in diameter.[22, 48, 67, 69, 70] Occasionally investigators will include “obvious change in shape” or the development of a bleb in the aneurysmal wall, however qualitative assessments of IA progression are to be avoided in our study as they are less easily verified and can require majority consensus of a panel of radiologist for outcome designation.[51] We also chose to include the development of SAH in our outcome assessment as this is seen as the most important and obvious outcome related to our study.

An additional aspect of our study design is the inclusion of a single neuroradiologist used to make outcome measurements. While this may be seen as introducing the risk of information bias perhaps avoided by using a panel of radiologists

for outcome designations as previously mentioned, studies on the topic do not support the notion of high intra-observer variability when making these types of assessments Indeed, one study comparing intra and inter-observer differences in IA measurements noting an intraclass correlation coefficient (ICC) of 0.97 when measuring IA height and 0.98 when measuring IA width using CTA (95% CI), when a value of 1 indicates perfect agreement.[70] Similar results were found when using MRA in the same study. Interobserver ICC by comparison are only slightly worse at 0.96 and 0.98 via CTA for IA height and width respectively and 0.95 and 0.94 via MRA for height and width respectively. Our study thus chose to avoid unnecessary complications in outcome assessment made by multiple observers in favor of a single observer as the quality of data obtained by either method is equivocal.

Further, we chose to assess IA progression via either CTA or MRA as long as the outcome assessment is the same as the most recent imaging technique for the subject in question. Again, while this may appear to introduce information bias due to the difference in imaging modality between subjects, a review of the available literature does not support this notion. When comparing IA measurements made by 4 independent radiologists using CTA and MRA against measurements made by invasive angiography, a combined average variance of +16.1% (p=0.03) for CTA and -15.9% (p=0.03) for MRA was found.[72] Similar results have been reported by other investigators.[73] Thus, while significant bias may be found in interchanging imaging modalities for any one subject and different modalities over or underestimate IA size, this bias will be significantly avoided by making outcome assessments in the same

modality as baseline measurements and our study is interested in change of IA size over time rather than total IA size.

2.7.6 - Sample Size and Statistical Significance

Although the theoretical link exists between MMP-9 and IA progression, in addition to the number of studies mentioned above that support this relationship, we cannot deny the heterogeneity of conclusions on the topic that, while not suggesting a protective role of MMP-9 on IAs, may still exist. As such we have chosen to utilize a two-tailed calculation to be able to detect this relationship in either direction. We will follow an analysis based on an alpha of 0.05 and a beta of 0.20 as this most closely follows the statistical methods as outlined in the above literature review. Further, while IAs are relatively common in the population at large, most of these lesions are undiagnosed, forcing us to identify subjects retrospectively via patient databases to meet our need for a sample size of 282 subjects. Our complete sample size calculation can be found in Appendix C.

2.8 - Conclusion

This review demonstrated that our exposure of interest has been an ongoing topic of exploration for greater than 20 years. While initial, low-quality studies suggested that MMP-9 concentration in blood samples would be a conceptual dead end, renewed interest in the topic seems promising as multiple animal studies have demonstrated interesting pathological findings related to the exposure as well as a small number of human studies with similarly intriguing results.

This review also demonstrated the relative lack of prospective cohort studies with our outcome of interest as we defined it, despite several large-scale prospective cohort studies relating risk factors for IA rupture over time. Despite this, several studies have attempted to expand this limited definition of IA progression to include IA growth, though these have largely been of a retrospective design. As such, ours is the first study of its kind to assess for a more complete definition of IA progression as it relates to MMP-9 exposure via a prospective study design. These retrospective studies, nonetheless, have highlighted many important risk factors and confounders for IA rupture and growth which will need to be accounted for in our proposed analysis below.

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CHAPTER 3 - STUDY METHODS

3.1 - Study Design

This prospective study will retrospectively identify subjects who have had at least one intracranial aneurysm identified for the first time via CTA or MRA within the last 3 years to determine the association between plasma MMP-9 concentration in peripheral venous blood of subjects with UIA and IA disease progression over a one-year follow-up period. A consent form to participate in the study will be presented to each subject after identification from the Yale New Haven Health (YNHH) system patient database. The consent form will be reviewed with the patient and signed to enroll the subject in the study. Demographic and pertinent medical data will be extracted via chart review and an intake survey. A copy of our intake survey can be found as part of our information form in Appendix A. These subjects will next have plasma MMP-9 concentration measured once at the time of or soon after enrollment and then be prospectively followed for one year starting on the date of their blood draw at which time they will have a repeat imaging assessment of their intracranial vasculature to assess for aneurysmal growth. Choice of imaging modality upon follow up will be the same modality as was used in the subject's most recent imaging study in order to avoid mismeasurements related to alternating modalities. As repeat imaging every 1-2 years is recommended by national guidelines, all subjects included in the study are expected to have recent imaging with which to compare our outcome imaging with. While this may introduce variation in the timeline between baseline and outcome imaging measurements, this variation will be

minimized as compared to previous studies as discussed above while still maximizing our study's feasibility and internal validity. There are no interventions related to this study.

3.2 - Study Population and Sampling

This study's population of interest is limited to subjects found within the YNHH system with an intracranial aneurysm diagnosed within the last 3 years who have chosen an observational treatment course. Criteria for exclusion will include prior aneurysmal surgical treatment, prior history of subarachnoid hemorrhage, history of blood clotting disorders such as Von Willebrand's Disease, Hemophilia, Factor 5 Leiden, or concurrent anticoagulation therapy, an inability to tolerate IV contrast as defined as previous contrast induced nephropathy, a history of intravenous contrast induced anaphylaxis, or a history of renal insufficiency, conditions associated with unstable IAs such as Polycystic Kidney Disease, Marfan's syndrome, type IV Ehlers-Danlos syndrome, neurofibromatosis type 1, alpha-1-antitrypsin deficiency, fibromuscular dysplasia, pheochromocytoma, Klinefelter's syndrome, and congenital malformations such as coarctation of the aorta, bicuspid aortic valve, and intracranial arteriovenous malformations as these patients generally follow a distinctly different treatment course, present at earlier ages, or have a different underlying pathology of their IAs.[1] We will also be forced to exclude subjects who have had baseline imaging only via traditional angiography as we are not willing to repeat this method of outcome assessment because it carries additional risks associated with its invasive nature. We will not

exclude patients based on IA size or other indications for IA surgical treatment if the subject consents to accepting the risk of choosing conservative treatment.

Sampling will take place via peripheral venous blood once at a Yale University affiliated site. Quantification of plasma MMP-9 levels will be made via Enzyme-linked immunosorbent assay (ELISA).[2] Follow up imaging will take place one year after this blood sample is collected and compared to the most recent aneurysm image taken prior to the start of the study period. All aneurysm measurements will be made by a single neuroradiologist blinded to subject identifiers and exposure status. The use of a single neuroradiologist may introduce an element of bias unique to this single observer, however this intra-observer variation has been found to be minimal by previous studies discussed above. Further, the use of a single observer will avoid inter-observer differences in measurements and evaluation.

The population under study is composed of subjects with IAs demonstrated via MRA or CTA who have elected for a nonoperative treatment course. Blood sampling will occur soon after enrollment. Repeat imaging will occur one year after blood collection. Subjects will be split into exposure or non-exposure (control) groups based on the presence or lack of increased MMP levels as defined by being above or below the 50th percentile of MMP blood concentration within the study group.

TABLE 1: *Eligibility Criteria*

Inclusion Criteria	**Exclusion Criteria**
IA diagnosed < 3 years prior	Aneurysmal hereditary syndromes
Elected for conservative treatment	Prior IA surgical treatment
Age greater than 18	Bleeding disorders

Most recent IA imaging by CTA or MRA	Current anticoagulation therapy
	Cardiovascular congenital malformations
	Prior aSAH
	Inability to tolerate IV contrast

3.3 - Recruitment

Recruitment will be made via phone and email contact with eligible subjects found by using YNHH patient databases. We plan to recruit subjects with at least one IA identified within the last 3 years who have elected for conservative management. Retrospectively identifying subjects will minimize recruitment time. Each site will be assigned a research assistant who will provide information about the study to subjects and clinicians, enroll eligible patients, perform intake assessments, and obtain informed consent.

3.4 - Subject Protection and Confidentiality

We will obtain Yale University's Institutional Review Board approval prior to recruitment of subjects. Study personnel will access all participant electronic medical records (EMRs) on university-approved, encrypted, and secure electronic devices after completion of Health Insurance Portability and Accountability Act (HIPAA) training and Yale Human Subjects Protection training. Protected health information (PHI) not in electronic form will be stored within a locked cabinet in the locked office of the principal investigator to which only direct research staff will have access. All PHI will be disposed of in a secure manner after the study is completed.

Participants will be required to grant written, informed consent in order to participate in the study. An example of the informed consent form can be found in the Appendix B. Study personnel will explain the consent form in a simple manner to allow participants to have the opportunity to ask questions and discuss concerns prior to consenting. The consent form contains a study description, duration of participation, and potential risks and benefits of the study in a style that is intelligible to a 6th grade reading level. It will be available in English with translation to other languages if needed. Interpreter services will be utilized as needed for other languages. For those who are unable to read, informed consent will be obtained after an oral presentation with a third-party present to ensure all information is accurately represented.

3.5 - Study Variables and Measures

3.5.1 - Independent Variable

The independent variable or exposure of this study will be MMP-9 levels measuring greater than the 50th percentile among study participants with an equal number of participants in each group. This measurement will be taken once starting at the onset of the study period. MMP-9 exposure status is measured once as they are assumed to be present on a chronic basis. Samples will be collected from the peripheral venous system at or below the level of the subject's antecubital fossa into a 4.5mL tube with ethylenediaminetetraacetic acid (EDTA) each month. The plasma will be separated from the total blood and stored frozen at -80 degrees C until analysis. Samples will then be sent to a participating laboratory where plasma MMP-9 levels will be measured using ELISA-DuoSet ELISA, DY911, R & D Systems.

3.5.2 - Primary Dependent Variable:

The primary dependent variable is IA disease progression as defined as IA growth greater than 1mm in any dimension or development of aSAH during the follow-up period. This definition agrees with previous studies discussed above in an attempt to establish outcome homogeneity. We will not include the formation of a bleb or change in IA shape in our outcome definition as this represents a subjective determination that will likely result in an increase in IA size and is thus better evaluated by this objective measurement. Determination of this growth will be made by a single, blinded neuroradiologist. Determination of IA rupture will be made by the attending radiologist at the presenting institution if a diagnosis of aSAH is made.

3.5.3 - Confounding Variables

Known confounders or secondary variables that contribute to aneurysmal growth as identified in the above literature review such as age, baseline aneurysmal size, female sex, hypertension status, smoking history, medications, family history, previous aSAH will be collected at intake through a combination of intake survey, radiological assessment of baseline images, and chart review. A summary of these variables and how we plan to test them can be found in Table 2.

3.6 - Methodology Considerations

3.6.1 - Blinding of Exposure

Subjects will not be informed of their exposure status until after the study is completed, as it is unclear if exposure carries any risk. Exposure status will also not be released to any clinical teams as this may unnecessarily influence treatment decisions. Exposure status will be blinded from the neuroradiologist assessing our outcome by assigning subjects a randomly generated numerical code and removing any potential identifiers or exposure status information.

3.6.2 - Blinding of Outcome

Information related to disease progression assessed as our outcome upon study completion will be considered medically necessary information and will likely influence treatment decisions. As such, subjects will be offered the option to obtain the results of their imaging at the time of their outcome assessment in which to share with their treatment teams in order to facilitate proper treatment decisions. In this way, there will be no blinding of outcomes related to this study. This is not seen as a threat to the validity of the study as the study is considered complete upon outcome image acquisition.

3.6.3 - Assignment of Intervention

Subjects will be assigned to exposure or non-exposure groups based on average MMP-9 level. The unexposed group is defined as those subjects whose MMP-9 level is below the 50th percentile of the total cohort. While this method of exposure assignment may be seen as introducing a form of sampling and information bias as it is unknown

how MMP-9 levels will be distributed within the sample, no normal or healthy level of MMP-9 concentration can be found in the literature to which compare to.

3.6.4 - Adherence

It is believed that recruited subjects will be highly motivated to remain in the study due to the severe nature of their condition and the established need for periodic monitoring. However, it is critical to the study that subjects attend their appointments for blood sampling and reimaging. As such, patients will be reminded of their appointment one week in advance to the date of their appointments. However, if the initial appointment is missed, the subject will be contacted via phone and scheduled for blood draw at their earliest convenience but not to exceed one month after their missed appointment in order to minimize deviations from our proposed study timeline. If the follow up appointment is missed the subject will be contacted by phone and email to schedule outcome imaging up to one week after their missed appointment in order to minimize variations in outcome assessment timelines. If a subject is unable to reschedule appointments in this time frame they will be dropped from the study. We recognize this may introduce a degree of compliance bias. However, we expect adherence to be extremely high as a result of our study design, namely that we have defined our outcome of interest to include those subjects who experience an IA rupture who would otherwise not be able to be assessed for IA growth.

3.6.5 - Monitoring of Adverse Events

It is possible that subjects experience aSAH or symptoms warranting reevaluation of their IA during the study period. If this should occur the subject should call 911 to get evaluated as soon as possible and follow all recommendations of his or her care team. This will likely include urgent neurovascular imaging via CT scan to assess for aSAH. If repeat imaging shows aSAH or marked increase in IA size warranting surgical intervention, then the subject will be included in the study as the outcome of interest has been demonstrated, with no other intervention required from the study administrators. These patients should undergo whichever procedure is recommended by their care team. If imaging occurs during the follow-up period that does not demonstrate IA progression, but the subject undergoes IA surgical or endovascular treatment, then the subject will be dropped from the study in agreement with previous study protocols as above. If repeat imaging occurs during the follow up period that does not show IA progression and the subject does not undergo IA intervention, the subject will still be included in the study if they can undergo repeat imaging at the end of the follow up period.

3.6.6 - Data Collection

Subjects will fill out an intake survey to assess patient demographics such as age, sex, height, weight, smoking history, hypertension status, family history, allergies, medications, and presence of IA symptoms, among other parameters. A complete example of the intake survey can be found in Appendix A. The electronic medical record will be referenced to gather aneurysmal specific characteristics such as size, location, most recent imaging, most recent imaging modality. MMP-9 levels will be collected once

starting as soon as possible after consenting. Outcome data via imaging will be collected one year after the last exposure measurement is collected by a single neuroradiologist blinded to subject identifiers and exposure status. While a single observer introduces a potential for intra-observer information bias, multiple observers have the risk of introducing their own inter-observer information bias. Further, data suggests that this intra-observer bias is generally minimized as described in Chapter 2.

3.6.7 - Sample Size Calculation

The proposed sample size is 282 subjects calculated using *Power and Precision 4 Software* and the UCSF Sample Size Calculator.[3, 4] Several assumptions were required to arrive at this sample size. It was assumed that all subjects with intracranial aneurysms could be placed into two groups, either the elevated MMP-9 group (MMP+), or the non-elevated MMP-9 group (MMP-). Further it was assumed that growth rates will be increased in the MMP+ group as compared to the MMP- group, although this parameter was not specifically measured in previous studies assessing growth risk. Thus, any study describing the proportion of subjects with growing IAs by a variety of variables contains a mixture of MMP+ and MMP- subjects, where MMP+ subjects contribute to the proportion of growing IAs at a higher rate, and MMP- subjects, while still contributing to the proportion of growing IAs depending on their co-variables for growth, grow slower or less frequently and thus decrease the proportion of growing IAs as a whole.[5 ,6] The conclusion of these studies thus acts as an average proportion of growing IAs in subjects with elevated and non-elevated MMP levels. Our estimated effect size is then found by assuming that the proportion of growing IAs in the

unmeasured MMP+ subjects of previous studies will be 5% higher than the total number of growing IAs growing due to other risk factors for growth, while the MMP- group will be 5% lower than the total but still grow according to their own non-MMP risk factors for growth, for an effect size of 10%. The proportion of aneurysms growing in one year was assumed to be 9% based on work by Villablanca demonstrating that 18% of aneurysms grew over a period of 2.2 years.[6] Thus, we assume that 4% of MMP- subjects will progress and 14% of MMP+ subjects will progress over one year. Setting the power of our proposed statistical analysis at 80% for a two-sided hypothesis, with a confidence interval of 95%, with the above described effect size of 10% allows us to arrive at the sample size of 256 subjects. The additional 26 subjects proposed are to account for an estimated 10% loss to follow up over the one-year study period. Since mortality is an expected complication associated with our outcome of interest of disease progression, subject mortality will not affect our study population size and thus need not be factored into our sample size calculation.

3.6.8 - Statistical Analysis

The characteristics of our sample population will be analyzed by univariate analysis to assess for common factors influencing IA progression as summarized in Table 2. Bivariate analysis will be conducted using the Chi-squared test of significance to assess for aneurysm progression, defined dichotomously as either present or not present, in the setting of MMP-9 levels with a two tailed test for significance set as $p<0.05$. Final models will be adjusted for known confounders, identified as categorical variables, reaching a significance level of $p<0.05$ using multiple logistic regression to

assess if these confounders share an association with our exposure of interest as established by preceding studies mentioned above. While a potential for selection and information bias exists in defining exposure status according to percentiles defined within the study group, normal or healthy levels of MMP-9 have not been established in the population at large, making it difficult to define high or low levels in other ways. Sensitivity analysis will be completed on our sample's MMP-9 level distribution to identify an alternative exposure cutoff associated with IA progression.

Table 2: *Subject and IA Characteristics with Statistical Tests*

Subject Characteristics	**MMP - Group**	**MMP+ Group**	**p-value**
Age	mean ± SD	mean ± SD	ANOVA
Sex at Birth	n (%)	n (%)	Chi-square
Pack Years	mean ± SD	mean ± SD	ANOVA
Hypertension	n (%)	n (%)	Chi-square
Japanese Ancestry	n (%)	n (%)	Chi-square
Finish Ancestry	n (%)	n (%)	Chi-square
Doxycycline Use	n (%)	n (%)	Chi-square
Aspirin Use	n (%)	n (%)	Chi-square
Statin Use	n (%)	n (%)	Chi-square
IA Characteristics			
IA Size (most recent)	mean ± SD	mean ± SD	ANOVA
MCA IA Location	n (%)	n (%)	Chi-square
Posterior IA Location	n (%)	n (%)	Chi-square

Anterior IA Location	n (%)	n (%)	Chi-square
IA Multiplicity	n (%)	n (%)	Chi-square
Previous IA Growth	n (%)	n (%)	Chi-square

3.7 - Timeline and Resources

Pending Institutional Review Board (IRB) approval, we will retrospectively identify and recruit subjects with an UIA first diagnosed within the last 3 years over a period of 6 months. During recruitment, peripheral blood sampling of subjects to determine exposure status will begin. Repeat imaging will occur one year after blood sample collection, allowing the study to be completed within 18 months. After this we will interpret the results and report our findings.

Study personnel will include one principal investigator, Dr. Kevin Sheth, one co-principal investigator, Peter Stampfel, to coordinate blood sample collection and analysis, intake survey administration, electronic medical record data collection, and statistical analysis upon study conclusion, and one neuroradiologist to obtain outcome measurements.

References

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Chapter 4 - Conclusion

4.1 - Advantages

Our study has several strengths including the use of a prospective design, expanded definition of IA progression, an exceptional ethical profile, and an efficient subject recruitment

We propose a prospective cohort with repeat imaging obtained over shorter periods relative to previous studies. This will allow us to compare our exposure to a gold standard of disease monitoring in a way uncommonly employed in previous studies. Our study's prospective design also allows us to include a more comprehensive definition of IA progression. As other studies have avoided acquiring repeat imaging and thus were only able to define IA progression as IA rupture, they failed to acquire data on IA growth and thus the risk of IA rupture in UIAs. Our prospective design with repeat imaging on outcome assessments allows us to include RIAs and growing but currently UIAs within our definition of disease progression.

Additionally, our study design is exceptionally ethical as it fits within the recommended IA monitoring guidelines established by the AHA. As such it does not require significant deviation from standard of care. Further, the only deviation from standard of care is a peripheral venous blood draw and does not represent any

meaningful risk to the subjects. The only subjects included are those who have chosen conservative treatment. This minimal deviation from standard of care also suggests that our study design has maximized its feasibility while still acquiring high quality data. IA imaging is time consuming and expensive, leading many previous studies to avoid a prospective design, instead opting for retrospectively collected data. However, this has necessarily led to lower quality data as follow up imaging has been acquired on a highly variable timeline.

Retrospectively identifying subjects with IAs to be included in our prospective cohort also reduces the amount of time that would be needed to recruit subjects as they are identified upon presentation as is done in traditional prospective cohort studies. Further, using recently acquired images that are independently evaluated by our blinded neuroradiologist reduces the time and cost of acquiring our own intake/baseline imaging.

4.2 - Disadvantages

Despite these advantages as well as our significant efforts to optimize our study design, we acknowledge that this study has several shortcomings. Perhaps the main shortcoming of this study is that it measures a single MMP. While we have attempted to choose the MMP with the strongest evidence for its contribution to IA disease, it is likely that it is only one of many enzymes contributing to the formation and progression of IA lesions. Thus, future studies may find it fruitful to expand their IA markers to include the enzymes, second messengers, and inflammatory cells likely to be found implicated in IA progression upon a broader search of the scientific literature. Additionally, several studies have demonstrated the importance of endogenous inhibitors of MMPs. Further

studies on the topic may find it worthwhile to include TIMP levels and MMP/TIMP ratios into their exposure determination.

While it is well established that MMPs as a family of enzymes play an important role in the formation and progression of aneurysms, some authors have suggested that aneurysm growth is episodic.[1, 2] This study will then only succeed in taking a snapshot in time via its short assessment period for MMP-9 exposure. This will likely lead to under or overestimating exposure levels and outcomes. As such, the short period of exposure evaluation proposed in this study likely represents a narrow time window which increases the chance that any one subject will be misplaced in the wrong exposure group. Ideally, MMP-9 levels will be drawn as regularly as possible to avoid this misclassification of exposure, however it is unlikely that subjects will be willing to adhere to such numerous blood collections. Our study attempted to maximize its feasibility at the expense of absolute validity.

Lastly, there was a lack of reliable information related to the calculation of our effect size. This required us to make several assumptions that, while not wholly unjustifiable, are possibly inaccurate. As such, our study is at risk of being overpowered or underpowered.

4.3 - Health Significance

Intracranial aneurysms are incredibly common, extremely dangerous, and difficult to monitor via traditional means of MRA or CTA. Further, our collective understanding of the pathological processes at work is still in development. These factors, when combined, leave many patients with this condition at significant risk for deadly or

debilitating consequences. The development of an easily acquired alternative to disease progression as proposed in this study would be invaluable to many individuals harboring IAs as they may decrease more invasive and expensive disease progression assessments.

References

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Appendices

Appendix A: Subject Information Form

Yale University

Section I: To be completed by Subject

Name:_____ Sex at Birth:_____ DOB:_____ Age:_____
(dd/mm/yy)

Phone Number:_____ Email:_____

May we leave a voice message for you? Yes____ No____

Address:_____

City:_____ State:_____ Postage Code:_____

Have you ever been diagnosed with high blood pressure? Yes____ No____

Have you ever been diagnosed with kidney dysfunction? Yes____ No____

Do you use tobacco? Yes____ No____ If yes, how much per day? _____

Are you of Japanese ancestry? Yes____ No____

Are you of Finish ancestry? Yes____ No____

Have you ever had a reaction to intravenous or IV contrast? Yes____ No____

Do you or any members of your family have any of the following conditions:

Polycystic Kidney Disease: Yes_____ No_____ Marfan Syndrome: Yes_____ No_____

Ehlers-Danlos Syndrome: Yes_____ No_____ Klinefelter's syndrome: Yes_____ No_____

Neurofibromatosis type 1: Yes_____ No_____

Alpha-1-antitrypsin deficiency: Yes_____ No_____

Fibromuscular dysplasia: Yes_____ No_____ Pheochromocytoma: Yes_____ No_____

Have you ever been diagnosed with coarctation of the aorta OR bicuspid aortic valve

OR intracranial arteriovenous malformations? Yes_____ No_____

Have you ever been diagnosed with a bleeding disorder?: Yes_____ No_____

Have you ever been diagnosed with a blood clotting disorder?: Yes_____ No_____

If yes, what is the bleeding OR blood clotting disorder named?: _____

Have you ever had brain OR aneurysm surgery? Yes_____ No_____

If yes, when?:_____

Section II: To be Completed ONLY by Research Staff upon Intake

Subject MRN:_____ Subject Identification Code:_____

Date of Blood Draw:_____ MMP-9 Level (ng/ml):_____
(dd/mm/yy)

Heart Rate:_____ Blood Pressure:____/____ Respirations:_____ O2 Sat:_____
Height (cm):_____ Weight (Kg):_____

Medication	**Dose**	**Frequency**

Allergic To	Effect

Date of most recent cerebral vascular imaging:_____

Imaging type: CTA:___ MRA:___ Number of IAs:_____

IA symptoms present?:Yes__ No__

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

Section III: To be Completed ONLY by Research Staff upon Study Completion

Heart Rate:_____ Blood Pressure:____/____ Respirations:_____ O2 Sat:_____

Height (cm):_____ Weight (Kg):_____ IA symptoms?:Yes____ No____

Incidence of SAH during study period? Yes____ No____

Incidence of subject death during study period? Yes____ No____

If yes, when_____ Cause of death:_____

Imaging type: CTA:___ MRA:___ Date of Imaging:_____
(dd/mm/yy)

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

IA Identification Number:_____ IA H x L x W(mm):___x___x___ IA location:_____

Appendix B: Informed Consent Form

Yale University

Informed Consent form for adults participating in the study titled "The Effect of Plasma Levels of Matrix Metalloproteinase-9 on Intracranial Aneurysm Progression"

Title of Study: The Effect of Plasma Levels of Matrix Metalloproteinase-9 on Intracranial Aneurysm Progression
Principal Investigator: Kevin Sheth, MD
Co-Principal Investigator: Peter Stampfel
Affiliation: Yale University School of Medicine and Yale New Haven Health System

This Informed Consent Form has two parts:

- **Information Sheet (to share information about the research with you)**
- **Certificate of Consent (for signatures if you agree to take part)**

You will be given a copy of the full Informed Consent Form

PART I: Information Sheet

Introduction

I am Peter Stampfel, a Physician Associate student at the Yale School of Medicine. My team and I are doing research on a disease called intracranial aneurysms which can cause bleeding inside a person's brain. We are inviting you to participate in our research because you have been found to have this disease but have decided with your doctor to not have surgery. We are planning to invite nearly 300 people like you to study this bleeding disease.

Please read this form to help you decide if you would like to participate in our study. You do not have to decide today, so please take your time. There may be some medical words or concepts that are foreign to you so please let us know if you have any

confusion or would like to ask any questions either now or later. You are allowed to talk about this study with anyone you choose to help you decide.

Purpose of the research

Intracranial aneurysms are a common problem in the blood vessels of the brain. Sometimes these aneurysms rupture, much like a balloon, causing bleeding inside of the head. The problem we have is that only some of these balloons pop, and we are not very good at knowing when or why they do so. Taking pictures of the brain can help us know when they may pop, but we are hoping to learn about a blood test that will also help us know. This research is going to help us see if our blood test will help us keep people with the disease from having this brain bleeding.

Type of Research Intervention

This research will involve asking you a few questions, doing a brief physical exam, and taking a small amount of your blood from your arm once and testing it in a laboratory. One year later we will take a picture of your brain to see how your aneurysm is doing as well as ask you questions to see if anything has changed for you. Your doctor may have told you that taking pictures of your brain over time is a normal part of how they would take care of you anyway. This study will only add a few extra questions and a blood test to the normal way we take care of aneurysms.

Participant selection

We are inviting all adults with an intracranial aneurysm that has been found within the past 3 years in the Yale New Haven Health care system and who have not had surgery to fix it to participate in our study. You have been invited because you have at least one intracranial aneurysm that was found in the last three years and have decided to not have surgery to fix it. You have been identified using your electronic medical record.

Voluntary Participation

Your participation in this study is completely voluntary. We cannot and are not forcing you to participate, it is your choice. You are allowed to change your mind and stop participating later even if you have already agreed. Whether you chose to participate or not will not affect your normal care with your normal doctor. If you choose to participate, you will still be offered the normal treatment for intracranial aneurysms that is routinely offered in the Yale New Haven Health system. We will tell you more about this treatment later.

A. Procedures and Protocol

You will receive the normal treatment for your condition according to the decisions that you and your doctor make. Often these are made by following national or institutional recommendations. Treatment is either to have surgery to fix the aneurysm or to watch and wait to see if it gets worse before having surgery. Often aneurysms will not get so bad as to need surgery, but sometimes they do. This means that if your aneurysm starts to cause you new problems, such as headaches or vision changes, you will likely receive a picture of your brain. If the picture shows that the aneurysm may

begin bleeding, you will be offered the option to have surgery to fix it. If the picture does not show any of the bad warning signs, you will not get surgery, but your other problems will still be treated normally. Sometimes an aneurysm begins to bleed without warning. These can be deadly, and all efforts will be made to help make sure you are alright.

An important part of our research is a small blood test. We will take blood from your arm using a needle and place it in a test tube. We will have to do this once. We will take about two spoonsful of blood for our tests. If there is any leftover blood at the end of our research we will destroy it and not let anyone else use it.

B. Description of the Process

- You will make two visits to the clinic during the research
- A small amount of blood will be taken from your arm during the first visit. This blood will be sent to a laboratory to see how much of a particular substance is in it normally. We will also ask you a few questions about how your health is and take a few measurements such as your blood pressure and your weight.
- One year later we will take a picture of your brain in the same way your doctor has been doing. Then we will compare the new picture to your old pictures to see if anything has changed. We will also ask you some questions and repeat measurements like blood pressure and your weight to see if anything has changed.

Duration

This research takes place over 18 months or one and a half years. The first 6 months will be spent recruiting subjects like yourself to join our study. After this, you will be asked to come to the clinic a total of 2 times. The first visit will include a blood collection and a physical exam. Then a year later we will take a picture of your brain. At the end of that time our research will be finished.

Side Effects

You may have a few side effects during the study. We will need to use a needle to get your blood to test and the pinch from the needle can be uncomfortable. Some people are very afraid of needles and sometimes can pass out. The needle can sometimes cause bleeding in your arm that may hurt, or rarely cause an infection. To help avoid an infection we will always use a new needle and clean your skin. Some people are allergic to our skin cleaner and may have a reaction. If this is the case we will use a different cleaner. We will do everything we can to make sure this does not happen and will watch you closely to keep track of any bad effects. If any of these side effects occur we will discuss it together with you to help decide if certain medications can help fix the problem.

Risks

There is the risk that your aneurysm will get worse during the study. If this happens your doctor will be told, and you and your loved ones will be able to talk with them to help you decide what to do. This study will not keep you from getting any treatment you may need. Also, while we will make every effort to protect your personal information, there is a risk of this occurring.

Benefits

Your participation in our study will help us gain knowledge about aneurysms. In the future, this information can be used to help people with this disease, to keep them from bleeding, and to help doctors know when they should have surgery. Because intracranial aneurysms are common throughout the world, your participation may help save lives in the future. Many aneurysms also run in families and your participation may even help one of your loved ones.

Reimbursements

There will be no costs to you for participation in the study. Your blood tests and imaging will be paid for by our funding. There are no reimbursements for participation.

Confidentiality

The information that we collect from this research project will be kept confidential and only disclosed if required by the law. Information about you that will be collected during the research will be put away and no-one but the researchers and the research supervisors at Yale University institutions. These people are also required to keep all your information confidential. Any information about you will have a number on it instead of your name. Only the researchers will know what your number is, and that information will be locked away. None of your personal information will be included without your permission when the research is published.

We will help protect your information by only accessing it on university approved and secured electronic devices. Any physical papers that we collect will be locked in a cabinet in my locked office.

Sharing the Results

The knowledge that we get from this study will be shared with you after the study is complete and before the results are published. If we have any reason to think that you are at any increased risk of brain bleeding we will tell you immediately.

Right to Refuse or Withdraw

It is your choice to participate in this study. It is also your choice to change your mind if you wish to not complete the study at any time. If you choose to not complete the study you will not be allowed to rejoin again.

Alternatives to Participating

If you do not want to take part in this study you will be provided with the care that you have agreed upon with your doctor.

Who to Contact

If you have any questions you may ask them now or later. If you wish to ask questions later you may contact the Principal Investigator at his office: Peter Stampfel, 555-555-5555 or peterstampfel@fakeemail.com

If you have any questions about your privacy rights, please contact the Yale Privacy Officer at 444-444-4444.

This proposal has been reviewed and approved by the Yale Institutional Review Board, which is a committee whose task it is to make sure that research participants are protected from harm. If you have any complaints about this research or questions about your rights as a participant, please contact the Yale Institutional Review Boards at 333-333-3333 or email hrpp@yale.edu.

PART II: Certificate of Consent

I have read, or someone has read to me, the information in this document. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. By signing this form, I consent voluntarily to participate as a participant in this research. By refusing to sign I understand that I will not be able to participate in this research in the future. My signature also indicates that I have received a copy of this document.

Print Name of Participant_____

Signature of Participant _____

Date _____
Day/month/year

If illiterate:

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness_____ **AND** **Thumb print of participant**

Signature of witness _____

Date _____
Day/month/year

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. Health Information will be gathered
2. Blood will be collected

3. Repeat imaging will be completed at a later date
4. Their information will be protected in accordance with standard practice
5. They can withdraw from the study at any point and are choosing to participate by their own choosing

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Print Name of Researcher/person taking the consent_____

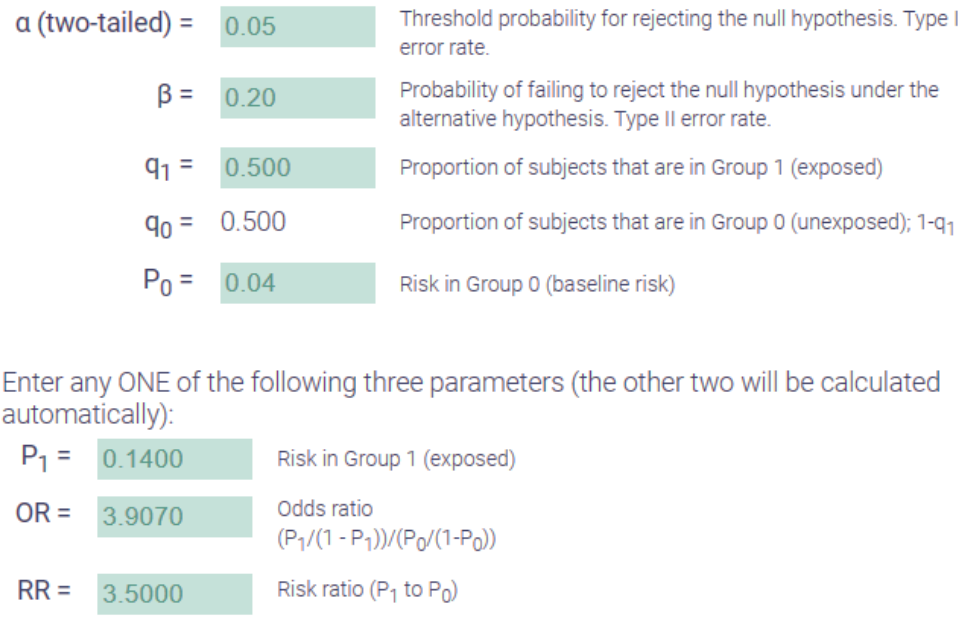
Signature of Researcher /person taking the consent_____

Date _____
Day/month/year

Appendix C: Sample Size Calculation

Our Sample size was based on a calculation program assuming a normal distribution for MMP levels and a two tailed hypothesis. This program was cross referenced using the UCSF Sample Size calculator to compare dichotomous outcomes using the Chi-square statistic. Our calculated sample size of 256 subjects was expanded by 10% (26 subjects) to a total of 282 subjects to account for expected loss to follow up. Specific parameters of the calculation are listed below:

Alpha = 0.05
Beta = 0.20
Power = 0.80
Effect size = 10%



The standard normal deviate for $\alpha = Z_\alpha = 1.9600$

The standard normal deviate for $\beta = Z_\beta = 0.8416$

Pooled proportion = $P = (q_1 * P_1) + (q_0 * P_0) = 0.0900$

$A = Z_\alpha \sqrt{P(1-P)(1/\bar{q_1} + 1/\bar{q_0})} = 1.1218$

$B = Z_\beta \sqrt{\bar{P_1}(1-\bar{P_1})(1/\bar{q_1}) + \bar{P_0}(1-\bar{P_0})(1/\bar{q_0})} = 0.4743$

$C = (P_1 - P_0)^2 = 0.0100$

Total group size = $N = (A+B)^2/C = 255$

Continuity correction (added to N for Group 0) = $CC = 1/(q_1 * |P_1 - P_0|) = 20$

Sample size (without continuity correction)

	N	Outcome+	Outcome-
Group 1:	128	18	110
Group 0:	128	5	123
Total:	256	23	233

Note: This calculator uses the normal distribution (with and without the continuity correction) as an approximation to the binomial distribution.

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