Pathogenesis of Intracranial Aneurysms

Brian Vala Nahed

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PATHOGENESIS OF INTRACRANIAL ANEURYSMS

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

Brian Vala Nahed
2005
To My Family:

– Dad, Mom, and Steve –

Your unconditional love, guidance, and friendship are ever constant and without doubt the structure on which I stand. Throughout my life, you have listened without questioning, guided without directing, and heard without my saying…

Thank you and I love you
ACKNOWLEDGEMENTS

Last year, I met with my mentor, Murat Gunel, to discuss the possibility of applying for the Doris Duke Fellowship to study the genetics of aneurysms. As all great mentors do, he cautioned me that trying to take this project from start to its fruition in under a year would be very risky but highly rewarding…

Eager to contribute to the field, I embarked on this project with Dr. Gunel as my mentor and his many accomplishments as the standard against which I would measure myself. During my fellowship, Dr. Gunel guided me as a scientist, geneticist, neurosurgeon and a great friend. The passion and dedication he showed to his patients, his science, and me, have provided the blueprints on which I hope to develop throughout my career.

Our success in identifying a novel chromosomal locus leading to intracranial aneurysms is due to Dr. Gunel as well as the mentorship of Matthew State and Richard Lifton whose guidance and wisdom were driving forces to our success. Thanks to David Hovda and Stefan Lee, for taking me under their wings as a high-school student and fostering my interests in Neuroscience.

In particular, I would like to thank the members of the Gunel laboratory for their friendship and constant support: Nduka Amankulor, Michael Diluna, Grahme Gould, Bulent Guclu, Abigail Hawkins, Angeliki Louvi, Ali Ozturk, Katie Pricola, Askin Seker, and Jennifer Voorhees. Thank you to the Lifton laboratory: Carol-Nelson Williams, Yoav Kohn, Antia Farihi, Arya Mani, Cat Mendenhall, Isabelle Beerman and Kris Kahle; The State laboratory: Danny Baek, and Gulhan Ercan-Sencicek. Special thanks to Andrea Chamberlain, Patti Richitelli, Brandy Howell, Shrikant Mane, Maria Spodick, and Adolfo Cumplido for their ability to make the impossible possible.

Thank you to my original mentor, my father, whose zeal towards medicine and science are constant reminders to chase after that which you love. Thank you to my mother and brother whose love, support, and friendship are unwavering. Thank you to my friends who provided laughter and helped maintain my perspective on things.

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PATHOGENESIS OF INTRACRANIAL ANEURYSMS
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Introduction:
Intracranial aneurysms (IA) are a common neurological problem, the rupture of which frequently constitutes a catastrophic neurological event. While the pathogenesis is largely unknown, it is believed that both genetic and environmental factors work in concert to some degree within patients. Our goal was to take a comprehensive approach to understanding the pathogenesis of IA by identifying factors leading to the formation, growth and rupture of IA.

Methods:
Since 1994, we have recruited patients and families with IA into the Yale Brain Aneurysm Database. Information regarding aneurysm characteristics (size, location, number), patient characteristics (age, medical, and social history), and family history were recorded. We analyzed this database for environmental factors associated with aneurysmal rupture. Within the same database, we identified and analyzed kindreds with a high IA incidence and penetrance using genome-wide linkage analysis. Collaborations with other centers provided additional kindreds to analyze and confirm our results.

Results:
Analysis of our database revealed hypertensive patients with IA \( \leq 7\)mm were 2.6 times more likely to rupture (\( p = .01, 95\% \) CI: 1.21, 5.53) than normotensive patients. Posterior circulation aneurysms were 3.5 times more likely to rupture than anterior circulation aneurysms (\( p = .048, 95\% \) CI: 0.95, 19.4). Further, genome-wide linkage analysis revealed significant linkage to a single locus, with a lod score of 4.2 at 1p34-36.

Conclusions:
We identified hypertension, young age, and posterior circulation as significant risk factors for rupture among patients with small aneurysms (\( \leq 7\)mm). Additionally, we are the first to map the gene responsible for IA to chromosome 1p34-26.
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CLINICAL OVERVIEW

Aneurysmal subarachnoid hemorrhage (SAH) is a serious neurosurgical emergency with poor prognosis; approximately 12% of patients die before reaching medical attention (1), and 40% die in hospital care (2-4). Survivors of SAH frequently leave the hospital severely disabled requiring a lifetime of care (5, 6).

Although the majority of intracranial aneurysms (IA) do not rupture, those that do account for around 85% of SAH (7). The incidence of SAH is 6 in 100,000 per year with approximately 28,000 ruptures per year. Those individuals who survive the initial bleed experience a 40% mortality rate during the first month; 25% of those who live past the first month recover completely (8).

SAH accounts for 3% of all strokes (8), 5% of stroke deaths, and more than one-quarter of potential life years are lost through stroke (9). Although the 20th century has seen great advances in diagnosis, treatment, and prevention of complications of SAH, the overall outcome has only modestly improved (10) leaving formidable challenges ahead for physicians caring for these patients.

Given the devastating sequelae of SAH, surgical or endovascular intervention prior to rupture is considered to be of paramount importance. Guidelines have been established to assist in the decision between treatment and careful monitoring, with the goal of prophylactically treating those aneurysms that are likely to rupture. Attempts to identify risk factors and the pathophysiology leading to aneurysm formation and rupture have been unsuccessful.

By studying populations in which the etiology of IA is relatively homogeneous one can begin to resolve these complexities and reveal the basic mechanisms of pathogenesis.
INTRACRANIAL ANEURYSMS

Intracranial aneurysms (IA) are characterized by abnormal localized dilatations representing cerebral arterial wall compromise. The origin of the word aneurysm stems from the Latin word aneurysma, which means dilatation. IA affect 5 – 10% of the general population (11) and represent a major public health problem. It is estimated that 2.3% of the population have undetected aneurysms (12), the majority of which will not rupture. When they rupture, the morbidity and mortality is devastating with approximately half resulting in immediate death.

Currently there are no reliable screening methods to identify at-risk individuals. Therefore, clinicians resort to imaging at-risk individuals (loosely defined as having a parent or sibling with IA) using magnetic resonance angiography (MRA) or computerized tomography angiography (CTA). Moreover, there are no widely accepted guidelines defining high-risk individuals.

For many years, the decision to operate on an unruptured aneurysm was based solely on the size of the aneurysm determined by imaging studies. One large multi-center trial suggested that IA $\geq 10$ mm had a risk of rupture of 1% per year (13) with smaller aneurysms having a much smaller risk of rupture. However, this initial data conflicted with clinical experience in which a significant number of patients present with SAH due to aneurysms less than 10 mm in size. Furthermore, the data contradicted a previously published series in which aneurysms $<10$ mm were at risk of rupture (14).

Progress in understanding the pathogenesis of IA has been hampered by its multi-factorial nature. Neither the conditions that lead to aneurysm formation nor rupture are well understood. Instead, clinicians are left to speculate on the importance of risk factors such as hypertension, smoking, alcohol, low body mass index, drug use and family
history when deciding the degree of intervention. Given the large number of familial cases and increased incidence with other genetic diseases such as adult polycystic kidney disease (ADPKD), the genetic basis of aneurysms has been alluded to but a gene has yet to be identified.

Recent studies suggest that both environmental and genetic factors contribute to the pathogenesis of IA. The degree to which each contributes to an individual’s aneurysm is likely patient specific. We aim to identify both the genetic mutations and environmental factors that work independently and synergistically to form IA.

**ENVIRONMENT**

The literature is rich with association studies linking risk factors with the formation and rupture of aneurysms. Guidelines have been established to identify which aneurysms should be treated and which need to be watched. Until now, these guidelines have been largely based around aneurysm size and location.

**Aneurysm Size**

The notion that aneurysm size correlates with rupture risk is as old as aneurysm surgery itself. Blood vessel walls are exposed to a combination of hydrostatic pressure and shearing stress. It is thought that aneurysm wall stress and eventual rupture is directly correlated with aneurysm size. Prior to the 1990s, aneurysms greater than 10 mm were considered at high risk for rupture and treated surgically. However, contrary to the guidelines, aneurysms < 10 mm did rupture and led to SAH in significant numbers, prompting a reevaluation of the guidelines.
Recent studies, including the prospective arm of the International Study of Unruptured Intracranial Aneurysms (ISUIA), suggest aneurysms < 10 mm in size have higher than previously predicted rates of rupture (15). These studies led to the adjustment of clinical guidelines that treatment with either microsurgical or endovascular techniques should be considered with aneurysms > 7 mm in size (15). Despite this recommendation a significant number of patients present with SAH due to aneurysms \( \leq 7 \) mm in daily clinical practice. In addition, several studies have shown the decision to treat unruptured aneurysms should not be based on aneurysm size alone (16-20). However, the results of these studies are not yet incorporated into the treatment guidelines.

**Other Risk Factors**

Many studies have attempted to identify risk factors, other than size, that predict the formation, growth, and rupture of IA. Factors such as hypertension, atherosclerosis, diabetes, and vascular anatomical differences have been implicated in pathogenesis (4, 21). In addition, social factors such as smoking and diet have also been suggested to play a role in the disease (4, 22). Although family history and the above-mentioned modifiable risk factors have been suggested to increase the risk of rupture, there is insufficient data to support a definitive clinical recommendation for surgical treatment of small aneurysms. (23-33)

A review by Teunissen et al. revealed only hypertension, cigarette smoking, and alcohol consumption (greater than 150 g / week) were significant risk factors (34). These results were confirmed in a review of five trials (North America, Canada, and Europe) which demonstrated cigarette smoking to be a major risk factor (28).
While there is evidence for environmental factors contributing to the pathogenesis of IAs, they fail to explain the complete picture, especially in young adults. Thus, genetic factors, particularly in the younger population, have been suggested to play a crucial role in the pathogenesis of aneurysm formation.

**GENETICS**

It is now well accepted that genetic risk factors, in addition to environmental risk factors, contribute to the formation and/or rupture of cerebral aneurysms. In this section, we highlight familial intracranial aneurysms and previous linkage analysis studies.

**Familial Aneurysms**

The notion that aneurysms cluster in families was first noticed in identical twins during the 1960s. Ullrich and Sugar reported 4 families, each with at least 2 members with cerebral aneurysms (35). This was followed by several case reports of multiple familial IA (36-41).

In 1980, Fox and Ko reported the largest family reported to date of thirteen siblings, 6 had proven IA and 5 had normal findings on cerebral angiogram; 2 refused angiogram (42). Subsequently, one of the two who refused workup suffered a SAH and was found to have 2 aneurysms by angiogram. Interestingly, there was no disease in their parents and relatives. Since then, we have ascertained and recruited this family in our study (IA 20, figure 3) and found that there are newly affected members in subsequent generations.

In 1993, Ronkainen et al reported a 10% incidence of familial IAs in family members of 1,130 patients with proven aneurysmal SAH from east Finland (26).
Similarly, Kojima et al. reported a 10% prevalence of IAs in families with positive history (43). Two studies, a prospective and retrospective study, found a three to five fold increase in incidence for first degree relatives in comparison to the general population (44).

A study spanning from 1970 to 1989 evaluated the families of patients with aneurysmal SAH reporting that 15 of 76 patients (20%) had a first- or second-degree relative with aneurysmal SAH (45). The number of observed first-degree relatives with aneurysmal SAH was 11, compared to an expected number of 2.66, giving a relative risk of 4.14.

Nakagawa et al. found a significantly higher incidence of asymptomatic cerebral aneurysms among Japanese patients with family history of SAH within the second degree of consanguinity versus healthy volunteers (13.9% versus 6%) (46). When combined with other risk factors such as hypertension and habitual smoking, these patients were found to have the highest incidence.

Several studies have reported that familial IA behave differently than sporadic IA. Familial cases are detected at an earlier age and ruptured at a smaller size when compared to sporadic cases (47-51). In fact, 70% of familial IA rupture by the age of 50 versus 43% of non-familial aneurysms (47). Additionally, a study by Leblanc et al. found higher than expected concordance of the age at rupture in a prospective study of 30 individuals in 13 families with multiple affected individuals (52). In the Saguenay-Lac Saint Jean region of the Province of Quebec, Canada, Mathieu et al. found that siblings of patients with ruptured IA had a greater risk of ruptured IA than the general population (53).

IA transmission from generation to generation has been difficult to determine. A review of the literature covering 238 affected families, by Schievink et al., did not find
one pattern of inheritance nor Mendelian model that uniformly applied (44). The most commonly affected relatives were siblings. Twenty-two percent of siblings of male probands had an IA compared with 9% of sibs of female probands. Interestingly, angiographic screening in 12 families detected IAs in 29% of 51 asymptomatic relatives. Although genetic heterogeneity might be present, screening of asymptomatic relatives could provide information as to the mode of inheritance. There is significant debate over the mode of transmission (two-hit phenomenon, haploinsufficiency, or defective protein (dominant negative)).

**Candidate Genes**

While several candidate genes have been implicated in the pathogenesis of aneurysms, none have led to any significant findings. These genes range from those associated with vascular wall formation to those that are mutated in connective tissue disorders. By studying known genetic diseases with high concordance of IA, researchers have identified proteins associated with the genetic disease as potentially related to the pathogenesis of aneurysms. Diseases such as Adult Polycystic Kidney Disease (MIM #173900) (54), Marfan syndrome (MIM #154700) (55), Glucocorticoid Remediable Aldosteronism (MIM #103900) (56), and Ehlers-Danlos syndrome type IV (MIM #130050) (57), appear to increase the risk of IA formation. This phenomenon prompted researchers to study the gene and gene products responsible for the disease with the hopes of explaining the increased IA formation.

Recent advances in genetic disorders and vascular abnormalities have revealed several alterations in gene and gene products involved in the remodeling of the extracellular matrix (ECM). The dynamic nature of the ECM has been theorized to go
awry leading to a weakening of the vasculature ultimately resulting in an aneurysm. Supporting this notion, the content and structure of collagen and elastin, the predominant elements in aneurysmal walls, is significantly altered. The below mentioned ECM-related proteins have been identified in genetic disorders and could be related to IA formation.

**Elastin**

In 2001, a genome-wide linkage study of 104 Japanese affected sib-pairs identified an area near the elastin gene (ELN) as the best evidence of linkage (58). Linkage was found at a total of three sites: 5q22-q31 (maximum lod score (MLS), 2.24), 7q11 (MLS, 3.22), and 14q22 (MLS, 2.31). None of the fourteen SNPs identified within ELN were associated with aneurysms. However, the haplotype between intron-20 / intron-23 polymorphism of ELN was strongly associated with intracranial aneurysms \([P = 3.81 \times 10^{-6}]\). Further, patients who were homozygous for the mutation were at highest risk \((P = 0.002)\), with an odds ratio of 4.39. These findings strongly support the body of literature implicating the ELN locus in aneurysm genesis, more specifically the locus on chromosome 7q11.2. However, no frank mutation was identified.

**Elastase / α1-Antitrypsin**

Elastase is a proteolytic enzyme that degrades elastin, collagen, and other proteins within the ECM (59-61). Secreted by polymorphonuclear leukocytes, it is inactivated once it binds to α1-antitrypsin forming a serum complex with protease (62, 63). The balance between active and inactive elastase has been implicated in aneurysmal formation. Tartara et al have suggested that α1-antitrypsin activity is decreased in the walls of intracranial aneurysms (64). Accordingly, elevated levels of elastase are found at
the site of intracranial aneurysms. (65) Although controversial, these complimentary studies suggest that α1-antitrypsin levels are decreased at the site of aneurysms leading to increases in elastase levels. However, two studies contradict these findings demonstrating elevated elastase levels in healthy individuals without aneurysms. (66, 67) Debate over the importance of this ratio will require further work delineating aneurysmal causes from those of normal physiology.

**Collagen (I and III)**

Studies have uniformly demonstrated a decrease and alteration in the collagen within aneurysmal walls. In particular, a deficiency in type III collagen occurs within aneurysm walls. (68-73). Studies have proposed a defect in type III collagen (COL3A1) (57, 72). EDS type IV is a connective tissue disorder caused by mutations in the COL3A1 gene on chromosome 2q31. EDS type IV is characterized by vascular abnormalities consisting of increased ruptures, thin transparent skin, and ligament weakness. Ostergaard and Oxlund demonstrated 6 of the 14 patients who died from ruptured intracranial aneurysms had type III collagen deficiency in the middle cerebral and brachial artery postmortem. (71) However, others have suggested that mutations in the COL3A1 gene are not a common cause of intracranial aneurysms nor are cervical artery dissections. (74) Although collagen constitutes the majority of the ECM, its role in aneurysm formation, and it’s ratio to collagen III, remains unclear.

**Endoglin**

Endoglin, a component of the transforming growth factor-beta receptor complex is highly expressed on endothelial cell surfaces. One study demonstrated an association
between intracranial aneurysms and a 6-base insertion polymorphism in intron 7 of the endoglin gene in a Japanese population. (75) This region codes for a component of the transforming growth factor-β receptor complex and is also mutated in Hereditary Hemorrhagic Telangiectasia 1 (HHT1). However, two other studies, one on a Caucasian population (63) and another on a separate Japanese population (58) failed to replicate this result.

The discrepancy in results from the above-mentioned studies suggests that polymorphism sequences are likely influenced by genetic and environmental factors which differ according to ethnicity. To prove this point, ethnic related differences are reported for polymorphisms and are considered within the norm. While the Kres and Onda studies refute the findings of Takenaka’s study, it is possible that endoglin encodes multiple proteins, one of which could lead to aneurysm formation. The polymorphisms expressed in endoglin represent one of the many reasons genetic testing is complex.

Polycystin

Autosomal dominant polycystic disease (ADPKD) is characterized by renal cysts, renal failure and vascular pathology. Disease results in a mutation in one of two genes, PKD 1 and 2, which encode polycystin 1 and 2, respectively. Polycystin participates in protein-protein (multiprotein membrane-spanning complex) and protein-carbohydrate interactions in the extracellular matrix. As early as 1971, Jankowicz et al. reported an increase in incidence of berry aneurysms in patients with ADPKD. While mutations in both genes have been linked to intracranial aneurysms, work on PKD 1 has revealed a specific mutation in chromosome 16p13.3 exon 15 of PKD 1 in two patients. (76). Further, the position (not the type of PKD 1 mutation) influences a patient’s likelihood of
developing an aneurysm. Rosetti et al. demonstrated that mutations in the 5’ half of the gene was associated with poorer prognosis (77). The prevalence of asymptomatic intracranial aneurysms in patients with ADPKD is five times that of the general population (54, 78). The average age of aneurysmal rupture is 41, a decade earlier than sporadic cases (78-80). One could speculate that the PKD 1 gene either encodes multiple proteins or that a mutation in the 5’ end produce a dominant negative. The relationship with PKD1 and intracranial aneurysms is rather complex and possibly due to one of the multiple proteins encoded by the PKD1 gene.

Fibrillin

Marfan syndrome, an autosomal dominant disorder of the connective tissue, affects the cardiovascular, skeletal and ocular system. It is characterized by arachnodactyly, unusual height, pectus abnormalities, enlargement of the aorta and possibly aortic aneurysms. The disease is due to a mutation in the gene encoding fibrillin (FBN1) located on chromosome 15q21.1. There is debate over the association between FBN1 mutation and intracranial aneurysms. A recent study of 25 autopsy cases with Marfan syndrome revealed no statistical difference in the prevalence of intracranial aneurysm in patients with and without Marfan syndrome.

There has been no association between FBN1 mutations and IA, however that is not the case for thoracic aortic aneurysms (TAA). Two influential studies have linked chromosomes 5q13-q14 and 11q23.2-q24 with aortic and thoracic aneurysms (81, 82). The pathogenesis of aortic and thoracic aneurysms is likely similar to that of intracranial aneurysms and identification of the gene and gene products of one may divulge a wealth of knowledge about the other. Currently, our laboratory is involved in a project studying
families with both intracranial and thoracic aortic aneurysms in an effort to identify common gene mutations.

**Genome-Wide Linkage Analysis**

The discrepancy in results from the above-mentioned studies reveals the limitations of candidate gene analyses. While approaches driven by hypotheses regarding disease mechanisms are intuitively attractive, experience has shown repeatedly that positional cloning is the most productive method for isolating causative genes when the pathophysiology and molecular biology of a disorder is not well elaborated. For example, in other complex disorders such as hypertension, positional cloning approaches by our laboratory revealed 19 genes that contribute to human blood pressure homeostasis, some defining novel physiological pathways that would have been extremely difficult to predict prior to our studies being completed (83). Importantly, the results of these types of analyses serve as the basis for a wide array of hypothesis driven investigations that can elaborate the mechanisms by which a particular gene or genes, once identified, contribute to a disorder of interest.

To date, a number of studies have used linkage approaches to attempt to identify loci contributing to IA risk (57, 58, 72, 74, 75, 84-86). Sib-pair studies in the Japanese and Finnish populations and a recent report of a consanguineous Dutch family have identified additional candidate intervals (58, 85, 87). However, only two intervals have significant genome-wide linkage and have not been disproven; 19q13.3 in the Finnish population (88) and 2p13 in the Dutch family (87).
Genetics of Complex Disease

Although many diseases have genetic components, relatively few segregate in Mendelian fashion with an identifiable single gene. A number of explanations may account for this observation. First, the disease may be attributable to inheritance of a single gene of incomplete penetrance, in which case only a fraction of recipients of the mutant gene may develop the disease. Second, the disease may be caused by the combined effects of multiple factors in individual subjects—these factors may be a combination of genetic and environmental exposures, obscuring the effects of inheritance of each susceptibility gene. Until recently, it seemed that the identification of genes for these multi-factorial traits was an insurmountable barrier. However, the ongoing revolution in genetic analysis now permits us to identify genes for complex disorders such as IA in which there is a likely genetic component in some patients.

Importantly, the advent of imaging studies have revealed that IA is much more common in the general population than had been previously recognized, with estimates of the population prevalence ranging up to 10% (11). With a high prevalence such as this, it becomes increasingly likely that, as a general rule, this trait will prove to be of multi-factorial determination rather than due to inherited variation in a single gene. The upside to this observation, however, is that it is highly likely that one will be able to identify a sizable cohort of multiplex families relatively rapidly by ascertaining multiplex families through affected index cases. Moreover, the availability of large pedigrees still provides the opportunity to identify the unusual circumstance in which IA is transmitted as a consequence of a mutation in a single with major effect. Consistent with these observations, since 1994 we have collected nearly 142 families including both large and small families in order to give us the power to positionally map the IA gene(s).
Large kindreds with high incidence and prevalence of IA are likely due to a single gene responsible for the large effect. Therefore, genome-wide linkage analysis of these kindreds will allow one to find the chromosomal position of the disease gene by using genetic markers. Confining analysis to a single or a few large families minimizes the chance of obscuring linkage due to genetic heterogeneity in which the disease is caused by mutation in different genes in different families. The disadvantage is that it is unclear whether genes identified in such families will prove to play a role in the general population or whether they will be of significance only in rare families. Further complicating things, the ability to collect large extended kindreds may prove to be exceptionally difficult due to the lethality of the trait, such that samples from known affected subjects are unavailable. Such analysis is confounded by the uncertain genotype of unaffected subjects.

We plan to use the large extended families we have already collected to identify a single gene of major effect using parametric linkage analysis. Once a locus is identified, we propose to confirm the loci by analyzing additional families.

**Specific Aims**

The goal of my thesis was to identify the genetic and environmental causes of IA. The primary project was to discover the genetic causes of intracranial aneurysms by studying the largest-yet-reported kindred with IA. The other large IA families in our database were used to help confirm and develop a more thorough understanding of the molecular genetics of IA.

The second stage of the project was to define the environmental risk factors related to IA formation. We conducted a retrospective case-control study, to identify the
risk factors associated with the rupture of aneurysms \( \leq 7 \text{ mm in size} \) in a consecutive series of patients. We hope to identify patient-specific risk factors that should be considered along with IA size in determining rupture risk and treatment decisions.

**MATERIAL AND METHODS**

*Patient Recruitment*

Through collaborations with vascular neurosurgeons at a number of domestic and international centers, we have established the Yale Brain Aneurysm Database (table 1). Study investigators from Yale and/or physicians caring for the patient discussed participation in the study with the index patient. Similarly, relatives were contacted and recruited to the study either by the Yale group or the physician of the index case (after appropriate HIC / HIPAA consent has been obtained).

Both patients with sporadic (<2 affected members within kindred) and familial disease (>2 affected members within kindred) were enrolled in this database. Since 1994, we have screened over 3000 patients with IA and identified 142 multiplex families with a total of 345 affected patients. Twenty-one of these families have more than 4 affected members and 4 of them have more than 7 affected members. These families are large enough to support linkage independently. A representation of a number of the large families is shown in Figure 1. While some families fit an autosomal pattern, analysis of IA pedigrees show that inheritance of IA is complex. We believe these patients and families constitute one of the largest IA databases in the world.
Table 1: List of Collaborators and their respective institutions

<table>
<thead>
<tr>
<th>Medical Center</th>
<th>Collaborator</th>
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<tr>
<td>Beth Israel Medical Center</td>
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Patient Questionnaire

The first approach to identification of multiplex families was via questionnaire administered to IA patients (see appendix). This approach was simple, rapid, and selected for more severely affected subjects by virtue of the severity of disease presenting to medical attention. This questionnaire includes questions regarding the patient’s current health, past medical and surgical history, medications, allergies, and family history of IA, stroke, and cerebral hemorrhage. In addition, participants were specifically asked about histories of known causes of aneurysm or cerebral hemorrhage, i.e., diagnoses such as Ehlers-Danlos, Polycystic Kidney Disease, and GRA. Data regarding the patient’s history was obtained either directly from the patient or through participating physicians.

The questionnaire was completed either with supervision from study personnel or by direct mailing to study participants for completion at home. Follow-up phone calls helped ensure complete data capture. Previous protocols reveal a greater than 90% response rate, and our protocol had similar response rates. Patients were often aware of relatives with diagnoses or treatment for aneurysm or who have suffered a SAH.
 Relatives identified with history of aneurysm or SAH were directly contacted by study personnel to obtain medical records to either confirm or exclude the diagnosis. Once relatives are identified, we expanded the pedigree to include all distant relatives in order to identify the total number of living affected members and determine at-risk siblings and children. Individuals with unknown phenotype were imaged using MRA or CTA. Finding a family with high prevalence of disease increased the likelihood that a single gene accounts for most or all of the risk of aneurysm development.

**Phenotype Assignment**

Phenotype was ascertained through MRI/A, CTA or cerebral angiography. Families with at least one additional affected individual (total of two affected) were selected for study. Medical records of relatives helped determine affection status. Those with unknown phenotype were screened by using non-invasive tests such as MRA or CTA (which was indicated clinically). In cases where the diagnostic imaging studies were performed at outside institutions, we obtained official radiological reports of diagnostic studies. Relatives were classified as affected if presence of IA during these imaging techniques. Unaffected relatives <30 years old were classified as phenotype unknown. All phenotypes were assigned prospectively by Dr. Gunel.

Screening of family members with more than 2 affected is indicated clinically (89-93) and has been funded by insurance companies. Despite the consensus within the neurosurgical community regarding scanning of these at-risk individuals, funding of the imaging tests is often a potential problem with the insurance companies. In a number of occasions when we encountered this problem, one-to-one discussion with the insurance companies was necessary in order to gain approval to scan the at-risk individuals.
Figure 1:
Representation of several of the large kindreds with intracranial aneurysms in the Yale Brain Aneurysm Database. Affected and unaffected individuals are shown as blackened and nonblackened symbols, respectively. Obligate carriers are shown as partially blackened symbols.
Record Review

In order to identify eligible patients with SAH, we initially conducted a retrospective chart review on 336 patients presenting with any type of intracranial bleed from January 2001 to 2004 to the Yale Brain Aneurysm and AVM Center. Of the 336 charts reviewed, 100 eligible patients with SAH due to IA ≤ 7mm were identified. Subjects with SAH due to aneurysms > 7mm (n = 52) were excluded from the study, as were SAH without documented IA on angiogram (n = 26), intracerebral hemorrhage (n = 97), subdural hemorrhage (n = 40), or bleeding due to trauma (n = 21). This was the only SAH for any of the patients in the ruptured group. Furthermore, no pre-hemorrhage data was available for many of the ruptured patients and this was excluded from the data series. Control subjects were referred due to a variety of reasons ranging from trauma, family history, or workup for headaches. Patients with unruptured aneurysms, but a prior history of subarachnoid hemorrhage were excluded from the control group.

Aneurysm size was obtained from conventional angiography or 3D computerized angiography (CTA). Clinical data, past medical history, and other data were obtained from clinic, hospital charts, and radiological reports. We contacted patients directly in the event of incomplete medical record data. In two cases of deceased patients, we obtained information from the patient’s next of kin and confirmed these results with the patient’s primary care physician. All aneurysms were berry aneurysms of the circle of Willis vessels. Dissecting aneurysms were excluded from the analysis.

Clinical Definitions

Patients were coded with hypertension or hypercholesterolemia if either diagnosis was present in the clinical chart prior to admission or clinic visit. Hypertension and/or
hypercholesterolemia were identified by the patients’ primary care physicians prior to hospital admission or presentation to clinic. Information regarding smoking, alcohol and cocaine use, and family history (intracranial aneurysms hypertension, and abdominal aortic aneurysms) was obtained from charts. Age at presentation was defined as the patient’s age upon admission, clinic visit, or diagnosis of unruptured aneurysm.

Aneurysm location was classified as anterior circulation (anterior cerebral, anterior communicating, internal carotid, middle cerebral, ophthalmic, para-ophthalmic, and posterior communicating artery) or posterior circulation (basilar, posterior inferior cerebellar, and posterior / superior cerebellar artery) according to anatomical convention.

**Statistical Methods**

Using the Fisher exact test for categorical variables and Spearman’s correlation coefficient for ordinal variables, we performed univariate comparisons between putative predictor variables and the outcome of aneurysmal rupture. Age was analyzed as an ordinal and dichotomous variable comparing patients younger than 50 years of age to those older than 50. A two-tailed p value < 0.05 was chosen as the threshold for statistical significance. All variables with a p value of 0.2 or lower were entered into a multivariable logistic regression model. Adjusted odds ratios were reported based on the results of logistic regression analysis. Model fit was assessed by standard methods, including residual diagnostics and Hosmer-Lemeshow goodness-of-fit testing. Model performance was assessed by the Nagelkerke R² estimate and computed prediction errors. All statistical analyses were done using SPSS 12.0 (SPSS, Inc, Chicago, IL).
Meta-Analysis

Under the guidance and expertise of Dr. Tom Morgan, we sought to aggregate all previously published data to improve the precision of our estimates in defining the impact of risk factors leading to rupture of IA ≤ 7 mm. To accomplish this, we used methods previously described for meta-analysis of case-control data (94) We performed a comprehensive literature review by searching PubMed and Medline using various combinations of the following keywords: "subarachnoid hemorrhage", "intracranial aneurysms", "unruptured", "ruptured", and "risk factors". In addition, we manually searched the bibliographies of existing reports to identify citations not included in Medline. Using previously established guidelines we systematically reviewed these articles (95) Our inclusion criteria were: prospective or retrospective study; must contain comparison of ruptured vs. unruptured ICA; risk factor data must be reported by subcategory of aneurysm size (up to 10 mm accepted as cut-off for subgroup analysis); and populations must be comparable in terms of age and co-morbidity.

Preparation of Human Genomic DNA

A 20 ml sample of venous blood was collected in acid citrate-dextrose tubes from each adult subject and shipped to Yale at room temperature via overnight courier. For pediatric subjects, no more than 10 ml/30 kg of body weight was collected. DNA was prepared by isolation of nuclei followed by proteinase K - SDS lysis and subsequent phenol and chloroform extractions, after which the DNA was precipitated with ethanol and resuspended in 10 mM Tris, pH 8.0, 0.1 mM EDTA. For samples that had been previously frozen, the yield of intact nuclei was very low, and we consequently modified the method to perform direct lysis of whole blood using high concentrations of proteinase
K, following which the protocol followed as above. This approach was inexpensive and had a long standing history of producing good yields. We have used this protocol for over 10 years and have had no problems with PCR amplification, restriction endonuclease digestion, cloning, or long-term stability of samples.

DNA isolation was performed in a dedicated room to prevent potential contamination of the laboratory environment with genomic DNA. Yield averages approximately 1 mg DNA, and was less than 200 ug in only 2% of samples. Because we use 50 ng DNA per PCR reaction, we could genotype 400 markers with only 20 ug DNA, leaving large quantities of each sample for subsequent analysis as needed. The optical density at A260 and A280 is read to determine the concentration of each sample, and is ≥1.8 in 98% of samples prepared from fresh blood and 85% of samples prepared from frozen specimens. Primary isolates were stored in eppendorf tubes at –70°C. Access to these samples was restricted to the DNA database manager. When samples were used, a master stock of samples were aliquoted at a concentration of 100 ug/ml in 96-well plates and working stock dilutions were prepared from this master stock, and these stocks were maintained by individual investigators.

SNP Genotyping

GeneChip genotyping was performed by and in collaboration with the Keck Affymetrix GeneChip Center under the expertise of Shrikant Mane Ph.D. We used a two-stage design in linkage analysis (96). We first genotyped all available affected individuals (n=6) using an early access version of the Affymetrix 10K GeneChips containing 10,044 SNP markers (Affymetrix: Santa Clara, CA). The median intermarker distance with this approach was 105 kb, and the mean heterozygosity of markers was
0.39. The SNPs genotyped on these chips provide an estimated information content equivalent to a microsatellite screen density of one marker per 1 - 2.5 cM (97). SNP genotypes are obtained by following the Affymetrix protocol for the GeneChip Mapping 10K Xba Array. Briefly, 250 ng of genomic DNA was digested per sample with the restriction endonuclease XbaI for 2.5 h. Digested DNA was mixed with Xba adapters and ligated using T4 DNA ligase for 2.5 h. Ligated DNA was added to four separate PCRs, cycled, pooled, and purified to remove unincorporated ddNTPs. The purified PCR products are then fragmented and labeled with biotin-ddATP. Biotin-labeled DNA fragments are hybridized to the mapping 10K array 130 chips for 18 h in a standard Affymetrix 640 hybridization oven. After hybridization, arrays were washed, stained, and scanned using an Affymetrix Fluidics Station F400 with images obtained by use of the Affymetrix GeneArray scanner 2500. Affymetrix MicroArray Suite 5.0 software was used to obtain raw microarray feature intensities (raw allele scores (98)). Using Affymetrix Genotyping Tools software package we derived the SNP genotypes.

**Genechip Data Analysis**

Using the Genome Analysis Programs provided by Affymetrix, we analyzed Genechip data. We created a UNIX based program (Chunky) that parses the data sheet into individual files per chromosome in linkage format by generating a data sheet with the following information: chromosome number, SNP markers, Decode Map Distances, Genotype Calls, and Allele Frequencies (provided by NetAffymetrix).

We used multipoint linkage analysis assuming autosomal dominant inheritance and assign either a 70, 90, or 99% penetrance. Analysis will be done using the Allegro program (DeCode Genetics, Iceland). Allele frequencies for the GeneChips SNPs will be

However, since a number of our pedigrees had a mixture of different ethnic groups, the allele frequencies provided by Affymetrix which was based on 54 individuals, may not have been suitable for the study population. Therefore, we also examined the robustness of linkage analysis using alternative approaches to specifying allele frequencies.

The 10K Genechips provided enough power to identify broad structures in the sample using statistical methods (e.g. the STRUCTURE program developed by Dr. Pritchard and colleagues). In this analysis, unrelated individuals from each family were used to infer population structure. This allowed us to group families into more homogeneous groups. Then based on the inferred groups, we considered three approaches for allele frequency specification. First, for groups with a large number of families, we collected data to infer allele frequencies based on unrelated individuals in these families. Second, as these large groups likely corresponded to populations with known allele frequencies, e.g. European Americans, and allele frequencies from such populations may be available in the public domain (e.g. Affymetrix and the international HapMap project), we used population data to investigate the robustness of linkage analysis results. These different approaches ensured that our report of linkage had enough confidence that they were not due to allele frequency misspecifications. The map order and distances between SNP markers are based on the latest build of the UCSC Genome Browser (May 2004, genome.ucsc.edu).

We confirmed the results of the Allegro program by using GENEHUNTER. Since GENEHUNTER can only handle 50 markers at most, we created a Unix based perl script that finds 50 markers within the maximum lod score interval. This program also
generates pedigree and data file to be fed into the GENEHUNTER program. Previous runs have shown consistent results between Allegro and GENEHUNTER programs.

Establishment of proper threshold for statistical significance is crucial for linkage association studies. In general, LOD scores of 3.3 are accepted as significant for parametric linkage studies, while for non-parametric tests threshold values \( p = 2 \times 10^{-5} \) or LOD >3.7 are necessary to declare significance (99, 100). Loci were also examined under models of locus heterogeneity, using 3.3 as threshold for significance (101). For Mendelian traits segregating in many independent families, if linkage under models of locus homogeneity were not apparent after exhausting potentially linked intervals, we then performed analysis allowing for locus heterogeneity (101). If a locus was identified, multilocus analysis was performed to search for additional loci that explained disease in remaining kindreds (102, 103). We used the suggested threshold point-wise p-value of 0.01 which corresponded approximately to a LOD score of 1.5 (99). These guidelines are generally accepted for genome-wide significance levels for linkage studies. (99, 100).

**Confirmation of Linkage Using Microsatellite Short Tandem Repeat (STR) Markers:**

The above-mentioned approach provides suggestive genomic regions with lod scores close to the theoretical maximum lod score. Microsatellite short tandem repeat (STR) markers were identified within these regions using the physical map data from the UCSC Genome Browser (May 2004, genome.ucsc.edu). These were genotyped for further mapping using all available members of a particular family, affected and unaffected. This strategy has been often referred as a two-stage design in linkage analysis (104). The theoretical properties of this strategy have been explored by researchers and some software provided guidance on the application of this strategy (e.g.
DESPAIR in SAGE). We followed these established principles in the selection of promising regions to follow up using microsatellite markers. All genotyping for microsatellite analysis were performed by polymerase chain reaction, with detection of fluorescent products on an ABI 3700 sequencer from Applied Biosystems equipped with Genescan and Genotyper software (ABI, Norwalk, CT).

PCR reactions were performed in 96-well plates using MJ Research DNA Engine Tetrad thermal cyclers, using 50 ng DNA as template in a 10 uL reaction (MJ Research: Waltham, MA). The reaction mixture contains 1 uL 10X buffer, 1.25 nmole of each dNTP, 50 pmole of each primer. PCR conditions involve denaturing for 30 sec at 95°C, annealing at specified temperature for 30 sec, elongation for 45 sec at 72°C, for 35 cycles.

Electrophoresis

Genotyping and electrophoresis were performed in collaboration with the Keck Laboratory at Yale. Keck DNA Sequencing Resource provided high volume DNA 96- or 384-well plate genotyping under its DNA Sequencing Resource. During 2002, the DNA Sequencing Resource completed 164,910 services for 286 Yale and 102 non-Yale investigators at 52 different institutions. Detailed information regarding this service is available at: http://info.med.yale.edu/wmkeck/dnaseq. Sequence turnaround time is typically two to three days for all services offered.

Advantages of the ABI 3700 include automated sample loading, shortened run times, and elimination of gel pouring and lane tracking. One uL of pooled sample were mixed with 2.0 uL formamide, 1 uL ROX labeled size standard and 1 uL of loading buffer and denatured for 3 minutes. In brief, 96–well plates containing these denatured
PCR mixtures were loaded on the sequencer which automatically transfers and injects individual samples into 96 glass capillaries filled with a stationary polymer.

After electrophoresis through the capillary tubes, the samples were ejected into a transparent cuvette, which were scanned by a laser beam that detects fluorescence. The ABI 3700 capillary sequencer cannot distinguish between TET and 6-FAM labeled PCR products. To avert this problem, a novel dye, NED, has been developed by Perkin-Elmer to replace TET in ABI 3700 runs. We converted the TET labeled primers (N=139) used for the ABI 377 to the NED label. In addition, ROX-labeled size standards were used instead of TAMRA labels. Genotypes are obtained with the ABI 3700 (Perkin Elmer, CA) DNA sequencers using the compatible Genescan and Genotyper software programs.

Once the data was read, it was processed and stored as a sample file in the instrument database and displayed as an electropherogram. The sample files were imported into the Genescan analysis software (Version 3.5 NT) and run on a PC compatible computer with the Windows NT operating system. Sample sizes were calibrated using the Genescan 500 ROX standard. After analysis with the size standard, the samples files were imported into the Genotyper software (Version 3.5) for determination of genotypes. Allele sizes were determined by an investigator blinded to patient characteristics. The allele size data were exported into a data sheet and converted to allele numbers and associated with patient identification numbers and phenotypic status for analysis of linkage.

Data Management and Storage

All GeneChip data was stored in Sun Sparc workstations and all microsatellite STR genotyping data was stored in the Cyrillic program. These files were backed up on
CD-ROM and UNIX-based linkage input and output were backed up on high capacity tape drives. ABI files containing sequence runs which were typically 25-28 MB in size were achieved by backup to CD-ROM discs that were stored in the laboratory.

RESULTS

Hypertension

SAH secondary to IA ≤ 7 mm occurred in 76.5% of patients with pre-existing hypertension, whereas only 55.9% of people with normal blood pressure experienced IA rupture. In the univariate analysis, hypertension was a significant risk factor for IA ≤ 7 mm rupture with odds for rupture of 2.58 (p value = 0.01).

Aneurysm Location

Although the majority of aneurysms were in the anterior circulation, rupture rates differed greatly according to aneurysm location. Within the unruptured group, carotid (n=15), MCA (n = 13) and ACoA (n = 8) IAs were the most common. Among the SAH group, ACoA (n = 26), PCoA (n = 26), and MCA (n = 19) were the most common locations of IAs. Eighteen of the 21 (85.7%) aneurysms located in the posterior circulation ruptured, whereas 82 of the 130 (63.1%) anterior circulation IAs ruptured. Posterior circulation conferred increased risk for IA rupture, OR = 3.51 (p < 0.05).

Age of Presentation

The median age of patients in our sample was 52. We chose to dichotomize at 50 years old as this is a clinically useful cutoff point where surgical morbidity and mortality increases (morbidity 5.6% at 1 year for patients < 50 years old). (15) There was no
significant correlation between age and rupture in the univariate analysis (Spearman’s rho = -0.11; p = 0.17). However, the adjusted multivariate analysis showed a risk increase of 2.6% (p = 0.07) with each one-year decrease in age.

Table 2: Clinical variables and risk of cerebral aneurysmal rupture

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
<th>Proportion with IA rupture (%)</th>
<th>Unadjusted OR (95% CI)</th>
<th>Fisher exact p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&lt; 50 years</td>
<td>49/69 (71.0)</td>
<td>1.49 (0.71, 3.13)</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>≥ 50 years</td>
<td>51/82 (62.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>81/123 (65.9)</td>
<td>0.91 (0.35, 2.37)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>19/28 (67.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA size</td>
<td>5-7 mm</td>
<td>50/73 (68.5)</td>
<td>1.22 (0.59, 2.53)</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>1-4 mm</td>
<td>50/78 (64.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA number</td>
<td>2+</td>
<td>23/37 (62.2)</td>
<td>0.79 (0.34, 1.83)</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>77/114 (67.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA location</td>
<td>Posterior</td>
<td>18/21 (85.7)</td>
<td>3.51 (0.95, 19.4)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Anterior</td>
<td>82/130 (63.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension*</td>
<td>Yes</td>
<td>62/81 (76.5)</td>
<td>2.58 (1.21, 5.53)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>38/68 (55.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia*</td>
<td>Yes</td>
<td>27/40 (67.5)</td>
<td>0.74 (0.30, 1.86)</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>56/76 (73.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking*</td>
<td>Yes</td>
<td>59/83 (71.1)</td>
<td>1.26 (0.56, 2.83)</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>35/53 (66.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cocaine use*</td>
<td>Yes</td>
<td>5/7 (71.4)</td>
<td>1.08 (0.16, 12.3)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>44/63 (69.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history IA*</td>
<td>No</td>
<td>77/111 (69.4)</td>
<td>1.51 (0.60, 3.75)</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>18/30 (60.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history HTN*</td>
<td>Yes</td>
<td>23/31 (74.2)</td>
<td>1.52 (0.58, 4.10)</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>72/110 (65.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history AAA*</td>
<td>Yes</td>
<td>3/3 (100)</td>
<td>Undefined</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>92/138 (66.7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Differences in denominators reflect incomplete or missing data in some chart reviews.
**Aneurysm Size**

Of the 336 patients with intracranial hemorrhage in this study, 152 had SAH due to an aneurysm; 100 (65.7 %) of these were found to have an aneurysm < 7 mm in size. As shown in Table 2, aneurysm size ranged from 1 to 7 mm. The median size in the rupture group was 5 mm; 74 patients had aneurysms of 5 mm and greater, while 78 patients had aneurysms between 1 and 5 mm. Size was not an independent risk factor for IA rupture under 7 mm (unadjusted OR = 1.22; p value = 0.61). The correlation between aneurysm size and rupture was not statistically significant (Spearman’s rho = 0.10; p = 0.22). Figure 2 presents the respective distributions of ruptured and unruptured aneurysms by size in millimeters, showing no impact of size on risk of rupture.

**Figure 2:**
Frequency of subarachnoid hemorrhage (SAH) in patients with intracranial aneurysms (IA) by IA size.
Sex, Hypercholesterolemia, Smoking, and Cocaine Use

Similar to previous studies, more females (n = 123) than males (n = 28) presented with IA. However, there was no statistically significant relationship between sex and risk of rupture (p = 1.00). Additionally, neither hypercholesterolemia (p = 0.52) nor smoking (p = 0.57) were associated with rupture. Cocaine use was also not statistically significant (p = 1.00), although lack of data in many charts limits the interpretation of this finding.

Logistic Regression Analysis

There was a statistically significant association between hypertension and the risk of IA rupture (p = 0.01). In addition, there was an increased risk of IA rupture with posterior location (p = 0.048). There was an insignificant inverse correlation between age and risk of rupture (Spearman’s rho = -0.11; p = 0.17). No other variable was associated with IA rupture at the p ≤ 0.2 level. Thus, we entered age, hypertension, and aneurysm location into a logistic regression model. Inclusion of these three variables explained 14% of the variance in outcome (Nagelkerke $R^2 = 0.14$). The multivariable model performed better for predicting rupture (89% correct) than non-rupture (24.5% correct). Overall, the model's predictive accuracy was 67.8%.

Hypertension was an independent risk factor for IA rupture, with an adjusted odds ratio (95% CI) of 3.05 (1.33, 6.25), and p value = 0.004. Likewise, posterior aneurysmal location conferred increased risk for IA rupture, OR = 5.35 (1.15, 25.0), p = 0.03. With each one-year decrease in age, the risk of IA rupture in this sample increased by 2.6% (OR = 1.03, 95% CI 1.00, 1.07; p value 0.07).

Meta-Analysis and Literature Review

A thorough review of literature identified 25 studies that reported risk factors for SAH. However, none met our inclusion criteria nor were suitable to add or compare to
our own data (Table 3). The identified studies were excluded due to violation of one of two criteria; (1) study did not contain comparison of rupture vs. non-rupture of IA; or (2) study did not report risk factor data by subcategory of aneurysm size (up to 10 mm accepted as cut-off for subgroup analysis) in both cases and controls.

### Table 3: Literature review results of 25 studies comparing SAH and risk factors

<table>
<thead>
<tr>
<th>Author</th>
<th>Citation</th>
<th>Type of Study</th>
<th>Exclusion criteria*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qureshi et al.</td>
<td><em>Neurosurgery</em> <strong>43</strong>, 22-6</td>
<td>Retrospective</td>
<td>1</td>
</tr>
<tr>
<td>Menghini et al.</td>
<td><em>Neurology</em> <strong>51</strong>, 405-11</td>
<td>Prospective / Retrospective</td>
<td>2</td>
</tr>
<tr>
<td>Nanda et al.</td>
<td><em>Neurosurgery</em> <strong>46</strong>, 1063-7</td>
<td>Retrospective</td>
<td>1</td>
</tr>
<tr>
<td>Forget et al.</td>
<td><em>Neurosurgery</em> <strong>49</strong>, 1322-5</td>
<td>Retrospective</td>
<td>1</td>
</tr>
<tr>
<td>Matsumoto et al.</td>
<td><em>Surg Neurol</em> <strong>60</strong>, 516-22</td>
<td>Prospective</td>
<td>1</td>
</tr>
<tr>
<td>Ogilvy et al.</td>
<td><em>Neurosurgery</em> <strong>52</strong>, 82-7</td>
<td>Prospective</td>
<td>1</td>
</tr>
</tbody>
</table>

Exclusion criteria: (1) Study did not compare rupture vs. no rupture of IA  
(2) Study did not report risk factor data as a subcategory of aneurysm size.
Figure 3:

IA 20 kindred. Affected and unaffected individuals are shown as filled and unfilled symbols, respectively. Individuals I-2, II-5, and IV-1 were assigned affection status unknown prior to linkage analysis and as such are depicted with grey symbols. The genotypes of STR marker loci spanning 14 cM at 1p35-36 are shown and segments of the haplotype linked to the disease phenotype are enclosed in a box.

IA 20 Phenotype

We focused our efforts on the IA 20 family (fig. 3), because it has the largest number of affecteds within our database and is also has the largest number of affecteds within a family reported in the literature (42). When first described, the IA family had six members with proven IA, all in generation II. The pedigree has since been extended and further characterized. In total, there are now 10 documented IAs, one subject with distinctive multiple intracranial vessel occlusions and extensive collateral vessel formation of unknown etiology (subject III-3), and one subject with abdominal aortic aneurysm (AAA) at a young age (age 32; individual II-5); this latter trait is sometimes associated with IA (105). For the purpose of linkage analysis, the documented IAs and
the patient with multiple intracranial vascular occlusions were classified as affected and the one with AAA was prospectively classified as phenotype unknown.

There are also 12 unaffected descendents of subject I-2. Of these, eight were asymptomatic over age 30 and had negative screening MRI or angiography (individuals II-3, II-6, II-11, II-14, III-1, III-2, III-8, IV-2); three offspring of unaffected subjects were asymptomatic over age 30 and did not have screening studies (individuals III-4, III-9, III-10); one was asymptomatic under age 30 without screening studies (individual IV-1). For linkage studies, this latter subject was classified as phenotype unknown, and the others were classified as unaffected.

**Table 4: Clinical data of IA 20**

<table>
<thead>
<tr>
<th>AFFECTED</th>
<th>UNAFFECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>ID</td>
</tr>
<tr>
<td>II-2</td>
<td>II-3</td>
</tr>
<tr>
<td>II-7</td>
<td>II-6</td>
</tr>
<tr>
<td>II-9</td>
<td>II-11</td>
</tr>
<tr>
<td>II-15</td>
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<td>II-16</td>
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<td>III-9</td>
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<tr>
<td>III-7</td>
<td>III-10</td>
</tr>
<tr>
<td>IV-3</td>
<td>IV-2</td>
</tr>
</tbody>
</table>


Clinical features of affected members are presented in Table 4. Age of diagnosis of IA ranged from 21 to 53 years by MRA or angiography prior to SAH (n = 7), and from ages 29 to 57 for patients presenting with SAH (n = 4). There are scant risk factors for IA among kindred members; specifically, there is a history of hypertension in only one
individual and while smoking was prevalent among both affected and unaffected family members, there was no significant difference between the two groups (8 of 10 affected and 10 of 12 unaffected). Specifically, there is no history of polycystic kidney disease (no history of end stage renal disease, and no serum creatinine level > 1.5 mg/dl); no history of Marfan syndrome (no history of aortic dissection, ectopia lentis, etc.); no history of Ehlers Danlos (no history of hypermobile joints, hyperextensible skin, or easy scarring). Finally, in neither the affected only nor the affected plus unaffected genome-wide linkage analysis (see below) was there evidence of linkage to known loci for any of these syndromes. Members of both genders are affected, the trait is present in consecutive generations, all affected members are the offspring of either known or suspected IA cases, and approximately half the offspring of such subjects have IA (fig. 2). These findings are consistent with autosomal dominant transmission of IA with high penetrance.

Linkage Analysis

An average of 9468 genotypes was scored per subject (SNP call rate range: 91% – 97%). To analyze the Genechip data for linkage we created a UNIX based program (Chunky) that parses the data sheet into individual files per chromosome in linkage format. Information captured includes chromosome number, SNP markers, map distances, genotype calls, and allele frequencies.

We used multipoint analysis of linkage, we specified the disease locus as autosomal dominant, with penetrance that varied from 70% to 99%, a mutant disease-gene frequency of 0.001, and a phenocopy rate of 0.001. SNP allele-frequency data for the white population, as supplied by Affymetrix, were used for the analysis of linkage, which was performed using the Allegro program (de-CODE). This analysis identified
Figure 4:
Analysis of linkage in IA 20 from GeneChip data of affected individuals only. Linkage graphs for all chromosomes are shown: x-axis corresponds to genetic distance (cM) and y-axis shows lod score.
Figure 4 continued:
Analysis of linkage in IA 20 from GeneChip data of affected individuals only. Linkage graphs for all chromosomes are shown: x-axis corresponds to genetic distance (cM) and y-axis shows lod score.
three intervals with LOD scores near the theoretical maximum of 1.8 (1p34.3-p36.13, 1q31-q41, and 2p11-p14), with LOD scores of approximately 0 for nearly all of the remainder of the genome (fig. 4).

The LOD scores were confirmed using the GENEHUNTER program. In an additional analysis, we specified the trait locus as X-linked dominant, with otherwise similar estimates of the trait locus; no interval on the X chromosome achieved a LOD score 1.0.2. Additional genotyping with GeneChip of four unaffected individuals yielded only these same three intervals with LOD scores of 1.0. Changing the phenocopy rate had small effects on the LOD scores and did not identify additional candidate intervals.

Table 5: Maximum lod scores for linkage of STRs and IA with varying penetrances

<table>
<thead>
<tr>
<th>Interval</th>
<th>Penetrance</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>70%</td>
</tr>
<tr>
<td>1p35 – 1p36</td>
<td>3.4</td>
</tr>
<tr>
<td>1q31 – 1q41</td>
<td>1.3</td>
</tr>
<tr>
<td>2p11 – 2p14</td>
<td>-0.3</td>
</tr>
</tbody>
</table>

Maximum lod scores are reported for 1p35 – 1p36, 1q31 – 1q41, and 2p11 – 2p14 using STR markers in all family members with varying estimates of penetrance.

Using data from the University of California–Santa Cruz (UCSC) Genome Browser (May 2004) (UCSC Genome Bioinformatics Web site), we identified and genotyped from five to nine highly polymorphic di- and tetranucleotide microsatellite markers across each of the three candidate intervals in all available kindred members. Genotyping for microsatellite analysis was performed by PCR, with detection of fluorescent products on an ABI 3700 sequencer (Applied Biosystems) equipped with GeneScan and Genotyper software (Applied Biosystems). The results were analyzed using the Simwalk program (we specified marker heterozygosities of 75% and the same
autosomal dominant model of the trait locus used above, with penetrance of 70%–99%). Our analysis diminished the evidence of linkage to 1q31-q41 and 2p11-p14 (table 5). In contrast, it demonstrated that all affected members inherit the same haplotype at 1p34.3-p36.13; this haplotype was transmitted to none of the unaffected members (fig. 3).

Parametric linkage analysis (with 99% penetrance specified) yielded a maximum LOD score of 4.2 at 1p34.3-p36.13 (table 5 and fig. 5); changing estimates of marker allele frequencies had negligible effects on the LOD score. The likelihood of linkage to 1p34.3-p36.13 was nearly 1,000-fold more likely than the next-most-likely interval at 1q31-q41 (table 5). The LOD score peak occurs at UT646; the LOD-1 interval is flanked by loci D1S199 and D1S496 (fig. 5), which define a 12.5-cM interval that corresponds to a 15-Mb segment (from 19.3 million bp to 34.9 million bp). This is the same interval defined by the GeneChip analysis, which indicated a LOD-1 interval flanked by rs950922 and rs514262 that corresponded to a 15.4 million–bp interval (from 21.3 million bp to 36.7 million bp on 1p34.3-p36.13).

Analysis of critical recombinants supports localization of the IA locus within the specified interval. Affected subject II-9 is recombinant at the distal border, and subject III-7 is recombinant at the proximal border (fig. 3). Nearly identical borders define the linked interval by SNP analysis. Examination of the LOD-1 interval identified 240 genes. Among these, a number of genes have been identified as plausible candidate genes, including polycystic kidney disease–like 1, brain-specific angiogenesis inhibitor 2, fibronectin type III domain–containing gene, and collagen type XVI a1.
Figure 5:
Analysis of linkage with microsatellite markers on 1p35-36 localizes the gene causing IA to a 12.5 cM region between markers D1S199 and UT5144 with a maximum lod score of 4.2. Multipoint analysis of linkage comparing segregation of IA and marker loci was performed. The location of marker loci used is indicated at the top of the diagram. The horizontal bar indicates the extent of the lod-1 interval.

DISCUSSION

The pathogenesis of intracranial aneurysms has been associated with genetic and environmental factors; however, a definitive mechanism has yet to be worked out. In this thesis, we took a comprehensive approach to studying the pathogenesis of intracranial
aneurysms. Through efforts both domestic and internationally, we have amassed one of the largest databases of IA patients – a resource which has enabled us to study the environmental and genetic factors associated with formation, growth and rupture of IA.

Previous genome-wide linkage studies have used affected sibling and/or relative pairs to identify various loci throughout the human genome that link to IA (58, 84, 85, 88). However, the results of these studies have been inconsistent, inconclusive, and have not identified a gene leading to IA. Candidate gene studies have been equally unsuccessful (58, 84, 88, 106).

Given the substantial locus heterogeneity, the power of affected sibling pair studies or affected relative pair studies is severely limited (107). Alternatively, using rare Mendelian forms of IA, we focused on individual families to identify genes and pathways that play a key role in the pathogenesis of both the rare and common form of IA (108).

Genetics

In the present study, we have investigated what we believe is the largest-yet-reported kindred with IA; genome-wide analysis of linkage provides significant evidence that the disease in this family is attributable to inheritance of a single locus at 1p34.3-p36.13. Our analysis diminished evidence for linkage to 1q32.1 and 2p12 (table 5). In contrast, it demonstrated that all affected members inherit the same haplotype at 1p34-36, while this haplotype was transmitted to none of the unaffected members (fig.3). Parametric linkage analysis specifying 99% penetrance yielded a maximum lod score of 4.2 at 1p34-36 (table 5 and fig. 3); changing estimates of marker allele frequencies had negligible effects on the lod score. The likelihood of linkage to 1p34-36 was nearly 1000 fold more likely than the next most likely interval at 1q32.1 (table 5).
The lod score peak occurs at UT646; the lod-1 interval is flanked by loci D1S199 and D1S496 (fig. 3), defining a 12.5 cM interval which corresponds to a 15 Mb segment extending from 19.3 to 34.9 million base pairs. This is the same interval defined by GeneChip analysis which indicated a lod-1 interval flanked by rs950922 and rs514262, corresponding to a 15.4 million base pair interval (from 21.3 to 36.7 million base pairs on 1p34-36).

Analysis of critical recombinants supports localization of the IA locus within the specified interval. Affected subject II-9 is recombinant at the distal border, and subject III-7 is recombinant at the proximal border (fig. 3). Nearly identical borders define the linked interval by SNP analysis.

Examination of the lod-1 interval identified approximately 240 genes. Among these, a number of genes have been identified as plausible candidate genes including Polycystic Kidney Disease Like-1 gene, Brain Specific Angiogenesis Inhibitor 2, Fibronectin type III domain containing gene, and Collagen type XVI α1 gene.

To our knowledge, the present kindred is the largest yet reported with IA, with 10 definitively affected subjects and one likely affected subject. Genome-wide analysis of linkage in this kindred demonstrates complete linkage of IA to a 12.5 cM segment of chromosome 1, with evidence for linkage that substantially exceeds thresholds for significance. The phenotyping in the kindred was clear-cut; reclassifying the patient with multi-vessel occlusions and extensive collateral growth as phenotype unknown would reduce the maximum lod score to 3.9. Moreover, the lod score was substantially increased by inclusion of unaffected family members, supporting high penetrance of the trait locus. It is also of note that the subject with the early AAA inherited a segment of the linked haplotype, suggesting that this vascular aneurysm might be attributable to
inheritance at this same locus. It would be of interest to obtain abdominal ultrasounds in kindred members in order to determine whether this phenotype commonly co-segregates with intracranial aneurysms and/or linked haplotypes. The pattern of segregation and the linkage data indicate that this family defines a new Mendelian form of IA that is transmitted as an autosomal dominant trait with high penetrance. Similar to reported cases of familial IAs, members of IA 20 presented with SAH or symptomatic findings at an earlier age than typically found in sporadic cases (47).

These findings represent a first step in identifying a susceptibility gene for intracranial aneurysm. Other than young age, there are no obvious clinical features that would separate IA in members of this family from typical cases in the general population. It is presently unknown whether the locus implicated in this study might play a role in other common forms of IA. In principle, it is possible that this might be a one-of-a-kind family with a rare mutation resulting in a highly penetrant form of IA. It is also possible that other less penetrant mutations in the same gene or pathway play a role in more common forms of IA. To date, a number of studies have used linkage approaches to attempt to identify loci contributing to IA risk (57, 58, 72, 74, 75, 84-86). Sib pair studies from Japanese and Finnish populations and a recent report of a consanguineous Dutch family have identified candidate intervals (58, 85, 87). The only intervals from such studies that meet genome-wide evidence of significant linkage is 19q13.3 in the Finnish population (88) and 2p13 in the Dutch family (87).

The identification of the causative gene in IA 20 will shed light on the pathways leading to disease. Whether this locus or pathway will play a role in more common forms of disease remains to be determined. However, once genes leading to IAs are identified, they may better define the pathophysiology and natural history of aneurysm
formation and rupture. Finally, these findings may contribute to improved diagnostic and therapeutic approaches to this disease.

**Environment**

The management of cerebral aneurysms ≤ 7 mm remains a controversial issue in neurosurgery. Numerous studies have outlined guidelines for treating unruptured aneurysms that range from > 7 to 10 mm. However, despite the clinical observation that a substantial number of IA ≤ 7 mm also rupture, relatively little is known about the risk factors associated with the rupture of these smaller aneurysms. In this study, we show for the first time that among patients with aneurysms ≤ 7 mm, hypertension, younger age, and posterior circulation are significant risk factors for rupture.

We retrospectively analyzed aneurysm characteristics and social history demographics of patients with both unruptured and ruptured aneurysms ≤ 7 mm. In our study population, the average size of ruptured and unruptured aneurysms was 4.65 mm and 4.33 mm, respectively. Consistent with published reports, the majority of ruptured aneurysms ≤ 7 mm in our study were of the anterior circulation, mainly the anterior communicating (ACoA) and middle cerebral arteries (MCA). In our unruptured population, the majority of aneurysms were also of the anterior circulation (carotid and middle cerebral arteries). Interestingly, however, posterior aneurysmal location conferred substantially increased risk for IA rupture. Those whose aneurysms ruptured were more likely to have poorly controlled hypertension.

Our findings extend those of previous retrospective reviews of aneurysmal rupture risk. In particular, aneurysms ≤ 7 mm rupture and the majority of these aneurysms are of the anterior circulation (16, 18-20, 33, 93). Interestingly, other studies, including the
prospective arm of the ISUIA study, showed that aneurysms of the posterior circulation were more likely to rupture at smaller sizes (≤ 7 mm) (15) However, based on a comprehensive systematic review of the medical literature, we found no published data meeting our liberal inclusion criteria for combination with our own data. Accordingly, to the best of our knowledge, this study is the first to report on risk factors specifically associated with the rupture of aneurysms less or equal to 7 mm in diameter.

A recent study analyzing 280 ruptured aneurysms showed that 74% were smaller than 10mm, with a mean size of 7.6 mm (33) The mean size of ruptured aneurysms in patients who were normotensive, had medically controlled hypertension, and had poorly controlled hypertension were 8.3, 7.4, and 6.5mm, respectively. Furthermore, patients with a family history of subarachnoid hemorrhage or who had poorly controlled hypertension were more likely to have ruptured aneurysms less than 5mm. With respect to location, ruptured aneurysms of the anterior communicating artery were smaller on average (6.6 mm) than aneurysms in other locations. These results confirmed the results of a previous single-center retrospective analysis that showed that 50% of ruptured aneurysms were 6-10 mm in size and 35% were < 5 mm with the majority of small aneurysms being of the anterior communicating artery (19)

The prospective arm of the ISUIA trial outlines the clearest guidelines indicating treatment based on size and location of a small, unruptured aneurysm. This study stated that the greatest benefit might be seen when aneurysms > 7 mm of the posterior communicating artery are surgically treated in young patients (<50 years of age) (15) The recommendation that aneurysms ≤ 7 mm of the anterior circulation in patients with no family history of SAH should be left untreated was reinforced by others (32) However, the limitation of these studies is the necessary reliance on 5-year or other
relatively short-term durations of follow-up. Patients will be interested to know near-
term rupture risks, but SAH can occur following any time interval, making retrospective 
study data essential for surgical decision-making.

Because the rupture of small aneurysms is a relatively rare event, it is difficult to 
achieve a sample size that is large enough to detect modest risks for rupture. In this study, 
the absence of a statistically significant relationship between such variables as number of 
aneurysms, smoking, and family history of IA should be interpreted with caution. Type 
II error (false negative) is a possible explanation, and further studies involving multiple 
centers are warranted for the assembly of large, prospective cohorts with the power to 
provide more precise estimates of the risk associated with these clinically important 
variables. Additionally, as a chart review, we were limited in the direct quantification of 
continuous variables. Data on pre-hemorrhage aneurysm size was inconsistent and often 
available in our patients presenting with SAH and therefore this data was excluded 
from our analysis. This is another significant limitation of this study.

Based on our results, we suggest the need for new guidelines incorporating 
relatively young age, hypertension, and posterior aneurysm location as possible factors 
relevant to surgical management of IA ≤ 7 mm. These patients are at higher risk for 
rupture, and have lower age-related surgical mortality and morbidity risks. These results 
are consistent with those presented in the ISUIA prospective study, but add additional 
clinically relevant information regarding the subset of patients with IA ≤ 7 mm.

Furthermore, more rigorous prospective studies with the specific aim of following 
aneurysms less than or equal to 7mm are needed to justify these new sets of guidelines.
CONCLUSION

Although the pathogenesis of intracranial aneurysm remains poorly understood, there is significant evidence associating environmental and genetic factors with disease. Size has long been considered the main risk factor for rupture; however, we demonstrate that hypertension, posterior circulation, and relatively young age should also be considered. In addition we identify 1p34.3-36.13 as a chromosomal region related to the pathogenesis of IA. Although the literature is rich with linkage studies pointing to regions throughout the genome, it appears that more than one locus will be involved in the IA pathogenesis.

In short, it is likely that both locus and allelic heterogeneity along with environmental factors play a role in IA pathogenesis complicating efforts at disease gene identification. Certainly the identification of new genes important in IA pathogenesis will provide insight into the primary determinants of this disease and will result in new opportunities for early diagnosis in the preclinical setting. Identification of risk factors related to formation and rupture of aneurysms will also assist in the understanding of disease, while allowing clinicians the opportunity to modify treatment based upon risk. Ultimately, it is anticipated that novel therapeutic strategies will be developed which will target these newly elucidated genetic susceptibilities.
APPENDIX

PATIENT INFORMATION SHEET

Name ______________________ Telephone # ______________________

Address ______________________________________________________

Date of birth __________ Race _________  Gender __________________

1. How old were you when your aneurysm / stroke was first diagnosed? ________

2. How many intracranial aneurysms did / do you have? _____________________

3. Did your aneurysm rupture and bleed? _________________________________

4. Did you have surgery to treat the aneurysm?   Yes    /     No
   - If yes, what was the date of surgery: _____________________________
     Name of your neurosurgeon: _______________________________
     Hospital where operation was performed: _____________________

5. Do you have high blood pressure: Yes /  No    When was it diagnosed? _______
   - Please list the names of family members with high blood pressure?: _________
   __________________________________________________________________
   __________________________________________________________________

6. Have you ever smoked?   Yes  /  No
   - If yes, for how many years _______  How many packs a day? _______

7. Do you drink alcohol / beer / wine / liquor? Yes / No
   - If yes, what do you drink? _____   How many drinks per day? _______

8. Please list any family members that have had an aneurysm or stroke?
   (For example: cousins, nieces, nephews, grandchildren, grandparents, great aunts / uncles)
   __________________________________________________________________
   __________________________________________________________________

9. Please list any diseases that run in your family:
   (Example stroke, high blood pressure, kidney disease, abdominal aneurysm AAA)
   __________________________________________________________________
   __________________________________________________________________

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