NEXT GENERATION SEQUENCING TO DETERMINE A GENETIC CAUSE OF FAMILIAL INTRACRANIAL ANEURYSMS

by

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Abstract

Intracranial aneurysms (IA), a common disease that occurs when cerebral arteries weaken and expand, can lead to subarachnoid hemorrhage upon rupture. The prevalence of IA is estimated to be around 3% and is known to increase with age. A small subset of the patient population has a familial form IA, where two or more first- to third- degree relatives have IA. At this time, one gene, *THSD1*, has been associated with familial IA (FIA). Here we present the preliminary findings from whole exome sequencing on five families diagnosed with FIA. Each family appears to have Mendelian segregation of disease (autosomal dominant, autosomal recessive, or X-linked) and has had their aneurysms clinically confirmed through brain imaging. Sequencing data from the proband of each family was used to identify family-specific candidate genes and was overlapped between families to identify genes that contain rare, possibly pathogenic variants in three or more families. Four genes -- *DST*, *CRIPAK*, *DNAH1*, and *TTN* -- were found to contain rare variants in four out of the five families. Three top candidate genes were selected based on gene function or previous association to cerebral vascular disease from 38 genes that contain rare variants in three out of the five families.

Preface

Identifying patient data are not reported. Ethics approval was required for Chapters 2 and 3, and was obtained from the University of British Columbia Children's and Women's Research Ethics Board (certificate numbers H09-01228 and H08-00784). Written, informed consent was obtained from all participants.

A version of Chapter 1 and the pedigrees for Families 1, 2, and 3 in Sections 3.2.1, 3.3.1, and 3.4.1, respectively, have previously been published: Hitchcock, E., and Gibson WT. **A Review of the Genetics of Intracranial Berry Aneurysms and Implications for Genetic Counseling**. *Journal of Genetic Counseling* 2017, Volume 26, Issue 1, pp 21–31, **First Online:** 14 October 2016. My review article summarizes previous genetic research on intracranial aneurysms, and on pleiotropic syndromes that confer increased risk for intracranial aneurysms. I researched and wrote the entire manuscript. Dr. W.T. Gibson contributed revisions and intellectually to the manuscript's content.

Dr. W. T. Gibson and I designed a strategy for family recruitment and DNA analysis, in order to seek genetic causes of familial intracranial aneurysms. Families 1 and 2 were first identified and enrolled by Dr. W.T. Gibson. I enrolled all subsequent families in collaboration with Dr. G. Redekop, Head of Surgery at Vancouver General Hospital, and in collaboration with the Brain Aneurysm Foundation.

I collected DNA samples for this research. Jillian Diamond, a former summer student in the Gibson Laboratory, collected samples from five members of Family 1. I extracted genomic DNA from samples and performed all quality control and validation measures.

Dr. P. Eydoux, director of the Cytogenetic Laboratory at the Women's and Children's Health Centre of BC, performed genome-wide microarray on the third generation of Family 1, in

order to identify regions of identity-by-descent among the affected sibs that were not shared with the unaffected sibs. I analyzed the results to select candidate copy number variants, and used quantitative PCR (qPCR) to perform validation of candidate variants.

Drs. S. Jones and Yaoqing Shen, both at the Michael Smith Genome Sciences Centre (GSC) carried out exome or genome sequencing on participant DNA, as well as the bioinformatic alignment of sequencing results. I then analyzed the variants identified by the GSC to create a list of candidate variants. Additionally, I compared candidate variants between families.

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List of Abbreviations

AAA	Abdominal aortic aneurysms
Acom	Anterior communicating artery
ADPKD	Autosomal dominant polycystic kidney disease
AVM	Arteriovenous malformation
BBB	Blood brain barrier
CADD	Combined annotation dependent depletion
CCM	Cerebral cavernous malformation
ChAS	Chromosome Analysis Suite
CNP	C-type natriuretic peptide
CNV	Copy number variant
CTA	Computerized tomographic angiography
Cx	Connexin
DGV	Database of Genomic Variants
DLL4	Delta-like-4 ligand
DNA	deoxyribonucleic acid
ECM	Extracellular matrix
EDS	Ehlers-Danlos syndrome
EPC	Endothelial precursor cells
ExAC	Exome Aggregation Consortium
FATHMM	Functional Analysis Through Hidden Markov Models
FIA	Familial intracranial aneurysms
FMD	Fibromuscular dysplasia

FORGE	Finding of Rare Disease Genes in Canada
FTAAD	Familial thoracic aortic aneurysms and dissections
GSC	Genome Sciences Centre
GTEx	Genotype-Tissue Expression
GWAS	Genome wide association study
HHT	Hereditary hemorrhagic telangiectasia
HUVEC	Human umbilical vein endothelial cells
IBA	Intracranial berry aneurysm(s)
ICA	internal carotid artery
JAM	junctional adhesion molecule
Indel	Insertion/deletion
LDS	Loeys-Dietz syndrome
MAF	Minor allele frequency
MCA	Middle cerebral artery
MMP	Matrix metalloproteinases
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NF1	Neurofibromatosis type I
NHLBI	National Heart, Lung, and Blood Institute
NO	Nitric oxide
OMIM	Online Mendelian Inheritance in Man
Pcom	Posterior communicating artery

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PET	Paired-end tag
PGI_2	Prostaglandin I2
Polyphen	Polymorphism phenotyping
qPCR	Quantitative PCR
RNA	Ribonucleic acid
ROH	Regions of homozygosity
SAH	Subarachnoid hemorrhage
SIFT	Sorting Intolerant From Tolerant
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
TGF-β	Transforming growth factor beta
TJ	Tight junction
UBC	University of British Columbia
USCS	University of California, Santa Cruz
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WES	Whole exome sequencing
WGS	Whole genome sequencing

Acknowledgements

I would like to thank my supervisor, Dr. William Gibson, for his continuing support and encouragement throughout my graduate studies, as well as for his mentorship and investment in me as a geneticist. I would also like to acknowledge my committee members, Drs. Steven Jones and Jan Friedman, for their practical and extremely helpful advice on my research.

Thank you to my lab, Ana Cohen, Katey Townsend, and CK Wong, for their patience and kindness, and constant willingness to teach, discuss, and laugh that made it easy to come to lab each day.

I cannot express how grateful I am to my family for their unwavering love and support. Thank you Mom, Dad, Will, Sue and Ian. I would like to say a special thank you to Grams for listening to me talk about my research over many cups of tea and cookies.

Finally, thank you to the Brain Aneurysm Foundation (www.bafound.org), not only for granting my research the Cynthia Lynn Sherwin Chair of Research, but also for being willing collaborators who are truly invested in seeing this project succeed.

Chapter 1: Introduction [A]

1.1 Overview

Intracranial berry aneurysms (IBA) develop in the walls of cerebral arteries, where the endothelial layer has weakened and formed a sac-like abnormality. IBA are also referred to as saccular aneurysms and differ from other intracranial aneurysms in shape, forming an out-pocketing off of a cerebral artery, reminiscent of a berry on a vine (Figure 1.1). Fusiform aneurysms involve dilatation of the entire vessel wall for some component of its length, and in the cerebral circulation they are much rarer than berry aneurysms.[1] IBA can vary in size, location, and shape, with some aneurysms being multi-lobed. IBA greater than 2.5 cm in diameter are considered "giant aneurysms." IBA predominantly form along the internal carotid artery or the other major arteries of the circle of Willis in the anterior circulation, often at branching points or other areas under high hemodynamic stress.[2,3] It is estimated that 3% of the general population have at least one IBA,[4,5] a prevalence that increases to 3.6-6.5% in people over 30 years of age.[6] Aneurysmal rupture accounts for a significant proportion (80-85%) of subarachnoid hemorrhage (SAH), a type of hemorrhagic stroke.[7] SAH is fatal in 35-50% of patients with IBA, and leads to permanent brain damage in 25-50% of survivors.[8-11] From a genetic epidemiology perspective, IBA are considered to be a common, complex condition with multiple risk factors including advanced age, ancestry, sex (women affected more often than men), smoking, longstanding hypertension, and family history.[12-15] A meta-analysis examining 33 studies on aneurysmal SAH found that the mean age of hemorrhage was 62 years, and that

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[A] A version of this chapter has been published as: Hitchcock, E., and Gibson WT. A Review of the Genetics of Intracranial Berry Aneurysms and Implications for Genetic Counseling. *Journal of Genetic Counseling* 2017, Volume 26, Issue 1, pp 21–31, **First Online:** 14 October 2016.

63% of patients who experience rupture were women.[16] However, population-wide screening is not currently recommended, as most cerebral aneurysms are asymptomatic and will never rupture.[11]

The introduction of this thesis will discuss the structure of cerebral vasculature, familial intracranial aneurysms (FIA), as well as the genetics of both IBA and FIA, including associated syndromes.

Figure 1.1 Intracranial berry aneurysm

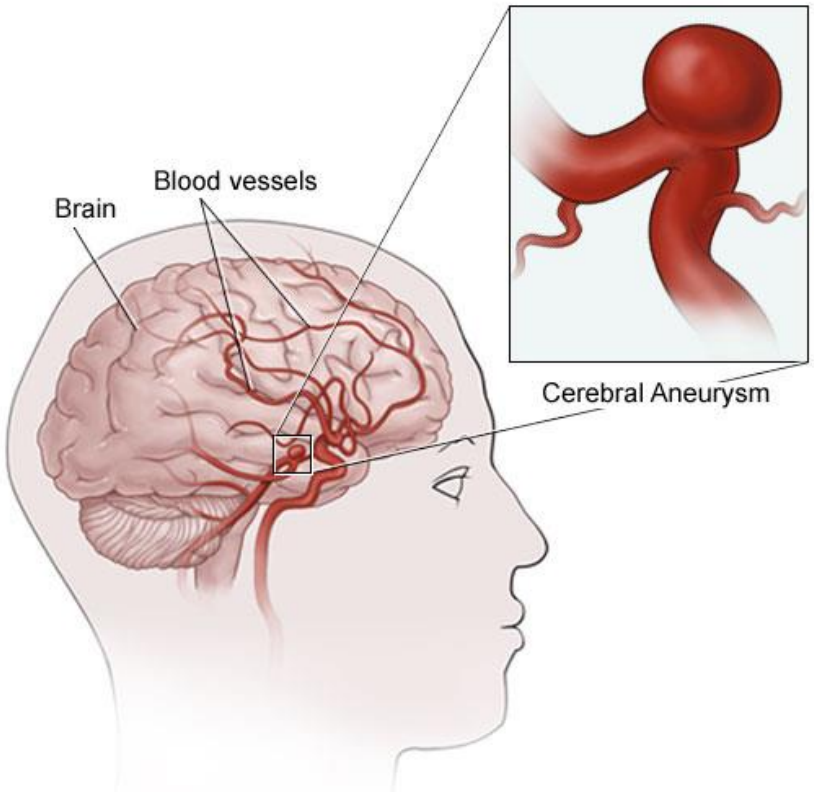


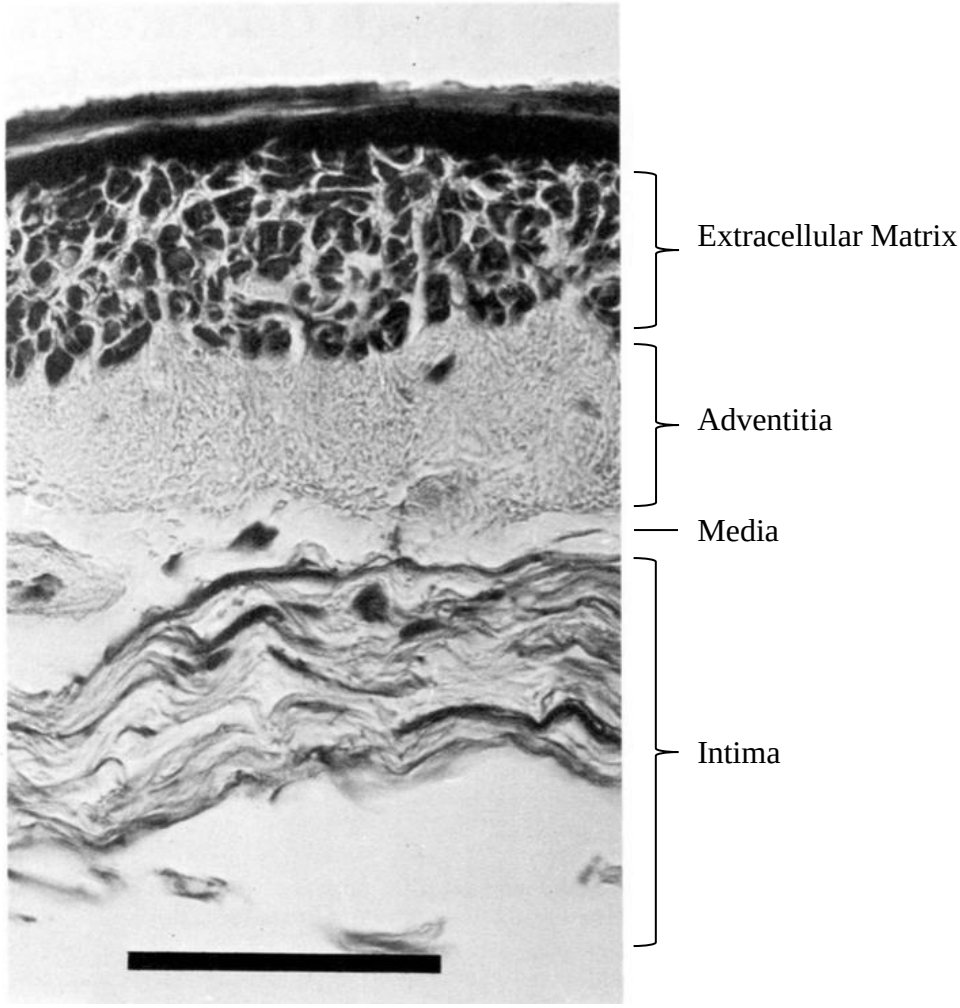
Figure of an intracranial berry aneurysm, also called a cerebral aneurysm, on cerebral arterial tissue. (Adapted from Columbia University Medical Centre accessed at: http://www.columbianeurosurgery.org/conditions/cerebral-aneurysm/)[17]

1.2 Vascular Structure, Function, and Development

Arteries are comprised of three layers of tissue: closest to circulating blood is a layer of squamous endothelial cells with a basal membrane and the elastic lamina called the intima;

second is media, which consists of vascular smooth muscle cells (VSMC) as well as some collagen and elastin fibrils; this is covered by the adventitia, consisting of diffuse connective tissues (Figure 1.2).[18,19] Case reports and reviews have documented aneurysms at most major arteries throughout the body.[20,21] Defects in a variety of connective tissue genes lead to vascular fragility and aneurysm formation in the aorta (see Section 1.3.1.2), but the pathogenesis of aneurysms in the brain has not been solved at the molecular level. Nevertheless, defects in the endothelial and smooth muscle linings of arterial walls are a known cause of IBA at the cellular level. Several molecular mechanisms have been proposed, including loss of tight junction proteins, infiltration of macrophages leading to loss of vascular smooth muscle tissue, and remodeling due to hemodynamic stress.[22-24] Since 1980, when Furchgott and Zawadzki demonstrated that arterial smooth muscle requires the presence of endothelial cells to relax when stimulated by acetylcholine,[25] multiple papers have shown signaling occurs between endothelium and VSMC to control functions of the vasculature. Endothelial cells have the ability to regulate vasodilation and vasoconstriction, VSMC growth, angiogenesis, and inflammation,[26] such that numerous types of dysfunction within these cells could lead to aneurysm formation.

Figure 1.2 Cross section of middle cerebral artery



Cross-section of the middle cerebral artery showing the intima, media, adventitia, and extracellular matrix. The bar represents 50 um. Adapted from Walmsley et al. (1983). *Stroke* 14;5 781-790

1.2.1 Vasculogenesis and Angiogenesis

Vasculogenesis describes the process of forming rudimentary blood vessels during embryogenesis, whereas angiogenesis describes the expansion and repair of vasculature both pre- and postnatally. Endothelial precursor cells (EPC) are critical for both vasculogenesis and angiogenesis. Embryonic EPCs stem from the periphery of blood islands, and respond to vascular endothelial growth factor (VEGF) by migration, proliferation, and differentiation into endothelial cells. Exposure to platelet-derived growth factor (PDGF) also induces their

development into VSMC.[27] Angiogenic sprouting of vascular endothelial cells is regulated by Notch receptors and their Delta-like-4 ligand (DLL4) though lateral inhibition by neighbouring endothelial cells.[28-30] The growth of the sprouting vasculature is guided by a gradient of VEGFA.[31,32] Endothelial cells can promote VSMC growth through growth factors and signaling molecules, such as nitric oxide (NO) and Prostaglandin I2 (PGI_2),[33-35] and can also suppresses VSMC growth through signaling of C-type natriuretic peptide (CNP), [36] which is initiated by sheer stress. Matrix metalloproteinases (MMP) can contribute positively to angiogenesis by degrading collagen and other structural proteins between endothelial cells, thereby facilitating sprouting and release of pro-ECM factors.[37-39] This process enables organization of endothelial cells on the scaffold of the extracellular matrix (ECM), which provide the necessary support for the sprouting blood vessels. Jagged-1 signaling from endothelial cells received by Notch receptors on VSMC promotes adhesion between the endothelium and VSMC and leads to maturation of vasculature.[40]

1.2.2 Blood-Brain Barrier

Cerebral vasculature differs from other blood vessels by the presence of the blood-brain barrier (BBB), which restricts the transport of certain molecules from circulation into the cerebral tissue. Junctional complexes, including tight junctions (TJ), adherens junctions, and gap junctions, form an interdependent network of proteins that restrict the permeability around the cells that comprise the BBB. Reese and Karnovsky first documented the BBB in 1967 when they identified rows of "belt-like" TJ between endothelial cells that blocked the transport of peroxidase, and a scarcity of transport vesicles within the endothelial cells.[41] While the BBB has most frequently been studied at the level of capillaries, a recent paper by Hanske and colleges

(2015) used electron microscopy and fluorescent staining to determine that the most common TJ proteins associated with the BBB are distributed evenly along all types of cerebral blood vessels, including along arteries.[42] Claudin-5, claudin-3, zonula occludens-1 (ZO-1), occludin, and junctional adhesion molecules (JAM) are critical to the integrity of tight junctions.[43-47] Similarly, the cadherin family of proteins make up an important part of the adherens junctions. These transmembrane proteins primarily form adhesions between cells, and between cells and scaffolding proteins. The most commonly expressed cadherin in vascular endothelial cells is VE-cadherin, with N- and E-cadherins found in much lower abundance.[48,49] On cerebral endothelial cells, gap junctions are composed of the connexin (Cx) family of proteins, specifically Cx37, Cx40, and Cx43.[50-52] Notch signaling is also important in BBB function; *Notch3*$^{-/-}$ mice show a disruption of the BBB that is characterized by "patchy" VSMC on blood vessels in the central nervous system and blood vessel leakage.[53]

1.3 Familial Intracranial Aneurysms

Familial intracranial aneurysms (FIA), a hereditary subtype of IBA, is suspected when two or more affected first- to third- degree relatives are present in a family.[54] Differences in study populations and methodology have contributed to variability in the reported prevalence of FIA. For example, the rate of detection of intracranial aneurysms when screening first-degree relatives with at least two affected family members has been reported to be 9.2-9.8% in patients older than 30 years, which is approximately 2-3 times higher than the risk within the general population.[11,55] A more recent study screened for aneurysms among asymptomatic first-degree relatives of families with two affected first-degree relatives *or* three affected second- to third-degree relatives, and found detection rates as high as 20.6% among patients older than 30

years.[56] Individuals with FIA seem to have a more severe phenotype: they are more likely to develop more than one brain aneurysm,[4,7] and have 17 times greater risk of rupture compared to those with sporadic IBA.[56] Furthermore, aneurysms that do rupture tend to rupture at a younger age and at a smaller aneurysmal diameter among patients with FIA.[56-60] Along with the increased risk for aneurysm formation and rupture, patients with FIA appear to have a poorer outcome after rupture.[61,62]

Families with FIA do not seem to experience anticipation in subsequent generations, so the underlying genetic cause (if present) is more likely attributable to single nucleotide variants (SNVs) or copy number variants (CNVs) than expansion of a trinucleotide repeat. Anticipation might also appear to be present in FIA families because of ascertainment bias; as the family history grows, each subsequent affected generation is more likely to be brought to the attention of a health care provider and diagnosed via imaging at an earlier age.[63] Generally, screening with magnetic resonance angiography (MRA) or computerized tomographic angiography (CTA) is recommended for individuals with two or more first-degree relatives diagnosed with intracranial aneurysms.[11,14,64-66] In the absence of prospective longitudinal studies, expert consensus appears to be that relatives at sufficient risk to merit screening should begin such screening 10 years prior to the earliest age-at-diagnosis in their pedigree.

After diagnosis of IBA or FIA, patients either remain under observation, receiving periodic brain imaging to assess the size of their intracranial aneurysm(s), or receive invasive treatment. The two primary means of treating IBA are surgical clipping, which requires a craniotomy, or endovascular coiling, which requires the insertion of a microcatheter in the femoral artery.[67] Surgical clipping removes circulation to the aneurysms, whereas endovascular coiling within the aneurysm helps to stabilize the vascular tissue. Endovascular coiling has lower

risk of mortality and long-term morbidity when treating ruptured or unruptured IBA, but a slightly higher risk of aneurysm recurrence or re-bleeding compared to surgical clipping.[66-71]

1.3.1 Genetics of IBA and FIA

1.3.1.1 Previous Research

Previous genetic research on IBA and FIA has consisted of familial mapping studies, genome-wide association studies (GWAS), and whole-exome sequencing (WES) studies. The first gene-disease association for FIA was made by Santiago-Sim *et al.* in 2016.[72] From a cohort of over 100 families with intracranial aneurysms (saccular, fusiform, or both), a causative gene was found in a single extended pedigree. WES of two affected first-cousins from this pedigree produced 53 variants that were shared between the cousins. A truncating variant (p.R450X) in *THSD1* was found to segregate with disease in their five-generation family, being present in all affected members, obligate carriers, and also in three members whose disease status is unknown. The variant was not found in the 11 clinically unaffected family members. The researchers also validated their findings in a cohort of 507 cases of sporadic intracranial aneurysms against a control cohort consisting of individuals on the ExAC database and 305 locally-collected individuals. Rare *THSD1* variants were found in 8 of the 507 sporadic cases, which are enriched compared to controls.

A knock-in fluorescence reporter of the mouse orthologue Thsd1 in mouse brain co-localized with endothelial cell markers. Knock-out mouse models and knock-down morpholino zebrafish models of *THSD1* led to intracranial hemorrhage in the animals. *In vitro* knock-down of *THSD1* in human umbilical venous endothelial cell (HUVEC) lines with small interfering RNA (siRNA) showed that *THSD1* interacts with a key member of focal adhesion complexes,

talin, and that HUVEC adhesion to collegen I in the basement membrane was reduced significantly. Co-transcription of siRNA-resistant wildtype *THSD1* mRNA rescued the impaired adhesion phenotype in HUVECs. However co-transcription of siRNA resistant *THSD1* mRNA containing patient-derived THSD1 mutations did not rescue the cellular phenotype.[72]

1.3.1.1.1 Familial Mapping Studies

Familial mapping studies have been done in large pedigrees that appear to have a single causal mutation transmitted as a Mendelian trait for intracranial aneurysm development. Mapping follows the hypothesis that such a variant will be found in a chromosomal region that has been inherited by all affected family members. Markers of known genomic location, such as single nucleotide polymorphisms (SNPs), are used to derive haplotypes that can in turn be used to identify loci that co-segregate with FIA. Investigations of families and affected sibling-pairs have suggested numerous loci associated with FIA (Table 1.1). From these studies the loci with the strongest association are 7q11, 19q13, and Xp22.[73-87] A recent meta-analysis of five familial mapping studies revealed an additional two loci in linkage disequilibrium with FIA, 3q27.3-3qter and 17p12-q21.33.[88] A SNP association study aimed at replicating loci previously flagged by linkage analysis confirmed the association at 14q23 (found by Ozturk et al., 2006) in a cohort of 266 affected and 288 unaffected Japanese individuals.[89] The number of loci identified though linkage analysis indicates there is genetic heterogeneity in FIA.

1.3.1.1.2 Genome-Wide Association Studies

Genome-Wide Association Studies (GWAS) interrogate the genome for statistically significant associations between SNPs and disease at a population level. Several loci have been

associated with sporadic IBA by GWAS, primarily using large discovery and replication cohorts from the Dutch, Finnish, and Japanese populations (Table 1.2).[90-98] The most frequently replicated locus is 9p21.3, which contains the long non-coding RNA, *CDKN2B-AS1*, and is adjacent to the cyclin-dependent kinase inhibitor genes, *CDKN2A* and *CDKN2B*. The same linkage block in 9p21.3 associated with IBA has also been associated with other vascular diseases, including coronary artery disease, myocardial infarction, and abdominal aortic aneurysms: this suggests that there may be a single locus that predisposes to all of these conditions via a common pathology.[99,100] Alg et al. (2013) conducted a meta-analysis of 61 GWAS studies that replicated the association between three loci (9p21.3, 8q11, and 4q31.23) and IBA.[101] In contrast to the postulated high-penetrance Mendelian loci in FIA, loci discerned through GWAS each have a relatively small effect size on the risk of developing IBA. For example, SNPs at the most strongly associated locus, 9p21.3, have reported odds ratios between 1.29-1.34 in the major GWAS studies.[91,93,97]

Table 1.1 Loci associated with familial intracranial aneurysms by linkage analysis.

Associated Loci	LOD Score	Study Population(s)	OMIM Locus Name	OMIM Number	Studied in >1 Population	Study Cohort [affected (unaffected)]	References
1p36.13-p34.3	4.2 -	North American, Dutch	ANIB3	609122	Yes	12(8) from 1 family 7(10) from 1 family	Nahed et al. (2005) Ruigrok et al. (2008)
2p13	3.55	Dutch	-	-	No	7(9) from 1 family[a]	Roos et al. (2004)
4q32.2	2.5 2.6	FIA Study	-	-	Yes	192 families[b,c] 333 families[b,c]	Foroud et al (2008) Foroud et al (2009)
5p15.2-p14.3	3.57	French-Canadian	ANIB4	610213	No	9(3) from 1 family	Verlaan et al. (2006)
5q22-31	2.24	Japanese	-	-	No	104 ASP from 85 families	Onda et al. (2001)
7q11.2	3.22 3.22	Japanese, North American	ANIB1	105800	Yes	104 ASP from 85 families 39(0) from 13 families	Onda et al. (2001) Farnham et al. (2004)
8p22	3.61	South Korean	ANIB11	614252	No	9(22) from 5 families	Kim et al. (2011)
11q24-q25	4.3	Colombian, North American	ANIB7	612161	Yes	2 families[b]	Ozturk et al. (2006)
12p12.3	3.1	FIA Study	-	-	Yes	333 families	Foroud et al. (2009)
13q14.12-q21.1	4.56	French-Canadian	-	-	No	10(25) from 1 family	Santiago-Sim et al. (2009)
14q22	2.31	Japanese	-	-	No	104 ASP from 85 families	Onda et al. (2001)
14q23	3.0	Colombian, North American	ANIB8	612162	Yes	2 families[b]	Ozturk et al. (2006)
17cen	3.0	Japanese	-	-	No	93(27) from 29 families	Yamada et al. (2004)
19q13	2.58 2.58 2.6 2.15	Finnish, Japanese	ANIB2	608542	Yes	48 ASP from 22 families 222 ARP from 121 families 93(27) from 29 families 41(0) from 9 families	Olson et al. (2002) van der Voet et al. (2004) Yamada et al. (2004) Mineharu et al. (2007)
Xp22	2.16 2.08 4.54	North American, Japanese, Dutch	ANIB5	330870	Yes	48 ASP from 22 families 93(27) from 29 families 7(10) from 1 family	Olson et al. (2002) Yamada et al. (2004) Ruigrok et al. (2008)

Study cohort data corresponds with the reference in each horizontal row. ASP: affected sib-pair; ARP: affected relative pair

a. Family is consanguineous. **b.** The number of affected and unaffected individuals included in the linkage analysis was not available. **c.** A total of 1155 affected and 1895 unaffected family members were genotyped in Foroud et al. (2008) and Foroud et al. (2009). Families were enrolled at recruitment sites located in North America, New Zealand, and Australia

Table 1.2 Loci associated with intracranial berry aneurysms through genome-wide association studies.

Associated Loci	O.R. (95% C.I)	p-value	Study Population(s)	OMIM Locus Name	OMIM Number	Replicated in >1 Population	Size(s) of Study Cohort	References
2q33.1	1.22 (1.13-1.32)	5.8×10^{-7}	Dutch, Finnish, Japanese	ANIB9	612586	Yes	2,196 cases; 8,085 controls	Bilguvar et al. (2008)
4q31.22	1.25 (1.16-1.34)	9.58×10^{-9}	Japanese	-	-	No	2,431 cases; 12,696 controls	Low et al. (2012)
4q31.23	1.22 (1.14-1.31)	1.1×10^{-5}	Dutch, Finnish, Japanese	-	-	Yes	5,891 cases; 14,181 controls	Yasuno et al (2011)
5q31.3	1.92 (1.53-2.40)	3.17×10^{-8}	Finnish, Dutch	-	-	Yes	2,335 cases; 9,565 controls	Kurki et al. (2014)
8q11.12 –12.1	1.36 (1.24-1.49) 1.17 (1.10-1.25) 1.86 (1.40-2.47) 1.25 (1.11-1.40)	1.4×10^{-10} 9.0×10^{-7} 9.2×10^{-5} < 0.001	Dutch, Finnish Japanese	ANIB10	612587	Yes	2,196 cases; 8,085 controls 5,891 cases; 14,181 controls 406 cases; 392 controls 1,483 cases; 1,683 controls	Bilguvar et al. (2008) Yasuno et al. (2010) Deka et al. (2010) Foroud et al. (2012)
9p21.3	1.29 (1.19-1.40) 1.32 (1.19-1.45) 1.24 (1.01-1.52) 1.36 (1.22-1.52) 1.34 (1.23-1.45) 1.21 (1.13-1.30) 1.41 (1.05-1.89)	1.4×10^{-10} 1.5×10^{-22} 0.017 < 0.001 4.07×10^{-12} 1.55×10^{-7}	Dutch, Finnish, Japanese, Portuguese	ANIB6	611892	Yes	2,196 cases; 8,085 controls 5,891 cases; 14,181 controls 406 cases; 392 controls 1,483 cases; 1,683 controls 4,133 cases; 7,869 controls 2,431 cases; 12,696 controls 200 cases; 499 controls	Bilguvar et al. (2008) Yasuno et al. (2010) Deka et al. (2010) Foroud et al. (2012) Foroud et al. (2014) Low et al. (2012) Abrantes et al. (2015)
10q24.32	1.29 (1.19-1.40)	1.2×10^{-9}	Dutch, Finnish, Japanese	-	-	Yes	5,891 cases; 14,181 controls	Yasuno et al. (2010)
12q22	1.16 (1.10-1.23)	1.2×10^{-5}	Dutch, Finnish Japanese	-	-	Yes	5,891 cases; 14,181 controls	Yasuno et al. (2011)
13q13.1	1.20 (1.13-1.28)	2.5×10^{-9}	Dutch, Finnish Japanese	-	-	Yes	5,891 cases; 14,181 controls	Yasuno et al. (2010)
18q11.2	1.22 (1.15-1.28)	1.1×10^{-12}	Dutch, Finnish, Japanese	-	-	Yes	5,891 cases; 14,181 controls	Yasuno et al. (2010)
20p12.1	1.20 (1.11-1.28)	1.5×10^{-5}	Dutch, Finnish, Japanese	-	-	Yes	5,891 cases; 14,181 controls	Yasuno et al. (2011)

The size of each study cohort corresponds with the reference in the same horizontal row. Yasuno et al. (2010) and Yasuno et al. (2011) analyzed the same discovery and replication cohorts, which were expanded on from the cohort studied in Bilguvar et al. (2008).

1.3.1.1.3 Whole-Exome Sequencing Studies

Unlike GWAS, but similar to linkage analysis, whole-exome sequencing (WES) aims to identify rare variants that have a large effect size and impart a high risk of developing intracranial aneurysms. Recently, three WES studies of multiplex families diagnosed with FIA have revealed new candidate genes in the development of intracranial aneurysms. All three studies excluded individuals who were diagnosed with a syndromic form of IBA.

The Familial Intracranial Aneurysm (FIA) Study, a separate initiative from those who discovered *THSD1*-related FIA, published two exome sequencing studies on seven multiplex families of European-American ancestry. These families were selected for having high numbers of affected individuals, and for having pedigrees consistent with either autosomal dominant or autosomal recessive inheritance. Initially, WES was carried out on 50 affected and unaffected individuals from these families, and analysis identified 96 candidate genes (Foroud for the FIA Study Investigators, 2013).

In their second publication the FIA Study researchers analyzed WES data from 36 affected and 9 unaffected family members.[102] Unaffected family members were only included if they were above 45 years of age and had received a negative screen by MRA – it is of course possible that some of the family members who were scored as unaffected may yet develop an IBA in their lifetime. For this analysis, Farlow et al. (2015) employed six biological filters on their WES data to generate a list of 68 candidate variants in 68 genes. These filters examined the variant type (non-synonymous SNVs or exonic and splice site indels), variants with a minor allele frequency (MAF) of <0.01, the segregation within families, and the predicted effect on protein (via combined annotation dependent depletion (CADD) score ≥10, and predicted damaging by Polyphen2 or SIFT). Five variants, found in *GSTCD, DUSP16, LMBR1L, HAL* and

TSC2, segregated fully with disease in one family and were present in the affected individuals of a second FIA family. None of the variants found in this study overlapped with any GWAS flagged loci (Farlow et al., 2015). This is presumably because rare, highly-penetrant pathogenic variants that result in a Mendelian inheritance pattern in a multiplex family tend not to spread through to the general population (unless some sort of balancing selection occurs).

Yan et al. (2013) had broader inclusion criteria and sequenced families with three or more affected first- to third- degree relatives.[103] They sequenced 42 affected people from 12 families of Japanese ancestry. After filtering for their presence in all affected family members, minor allele frequency (MAF) <0.05, and a predicted damaging effect to the protein (Polyphen2 and SIFT), WES analysis resulted in 78 candidate variants. Two variants, p.Y193F in *GPR63* and p.R142H in *C10orf122* (*TEX36*), segregated with affected individuals in more than one family. While both of these protein changes are predicted to be deleterious, relatively little is known about the gene function, and there was not sufficient evidence to classify either mutation as pathogenic. Yan et al. (2015) then selected ten variants from nine genes, based on functions that were plausibly associated to the pathogenesis of intracranial aneurysms, for Sanger sequencing and replication in two additional Japanese cohorts. The first replication cohort consisted of probands from 24 independent FIA families, and the second replication cohort included 426 individuals diagnosed with sporadic IBA. A variant in *ADAMTS15* was significantly associated with the familial cases in the first replication cohort, while variants in *THBD, IL11RA, PAFAH2,* and *ZNF222* had a slightly increased MAF among the sporadic IBA cases in the second replication cohort compared to the Japanese population. None of these variants had sufficient evidence for the authors to determine them as causative for FIA.

For rare diseases with characteristic phenotypes, a gene-disease association is established when rare variants are present in the same gene in three independent families. For more common diseases such as IBA, issues such as non-penetrance and phenocopies make additional evidence (such as functional studies) highly desirable, in order to lower the false discovery rate. Whereas none of the candidate genes yet flagged through the three WES studies mentioned above have sufficient genetic evidence to prove a causal association, the recent publication of *THSD1* is an important milestone in the field, as it included multiple lines of functional inquiry and effectively "sets the bar" for acceptance this type of gene-disease association when a potentially causal variant is segregating in a single extended pedigree (at least until other pedigrees are identified with different functional variants in the same gene).

1.3.1.2 Associated Syndromes

There are several Mendelian syndromes that confer susceptibility to IBA. These syndromes are predominately connective tissue disorders that lead to decreased integrity of the extracellular matrix of vascular tissue, but also include conditions such as autosomal dominant polycystic kidney disease (ADPKD) and Neurofibromatosis Type I (NF1).

1.3.1.2.1 Autosomal Dominant Polycystic Kidney Disease

Apart from family history, diagnosis of autosomal dominant polycystic kidney disease (ADPKD) imparts the highest risk for developing IBA (Table 1.3). Between 4% and 17% of patients with ADPKD will develop IBA, with an equal risk distribution between sexes.[104-108]As seen with non-syndromic IBA, the prevalence of IBA increases with age in these syndromes.[106,108] The combined risk for aneurysmal formation in patients diagnosed with

ADPKD and a family history of IBA or SAH increases to approximately 22-25%.[105,107] Screening for IBA in ADPKD is recommended in patients above 30 years of age or in patients with a family history of IBA.[108] Hypertension, found commonly in ADPKD patients, is not considered to be an obligatory risk factor for aneurysm formation in this context. Individuals with ADPKD who have well-controlled hypertension, or blood pressure within the normal range, have still been seen to develop intracranial aneurysms.[106,108-110]

Pathogenic variants in the *PKD1* and *PKD2* genes are causative for ADPKD and account for 85% and 15% of diagnoses, respectively.[111] Patients with pathogenic variants in PKD1 or PKD2 appear to have an equivalent risk of IBA.[112] *PKD1* and *PKD2* mutations cause defects in the mechanosensory cilia found on renal and vascular endothelium, which leads to cyst and aneurysm formation, respectively.[113-116] Patients with tuberous sclerosis complex can also have a concurrent diagnosis of ADPKD when their disease is caused by a contiguous gene deletion affecting *TSC2* and *PKD1* at chromosome 16p13.3. These patients not only have more severe kidney disease, but are also at risk for intracranial aneurysms.[117-119]

1.3.1.2.2 Ehlers-Danlos Syndrome

Ehlers-Danlos syndrome (EDS) is an autosomal dominant connective tissue disorder. In classical EDS, the majority of patients have pathogenic variants in *COL5A1* or *COL5A2*, while in vascular EDS, previously referred to as EDS Type IV, variants in *COL3A1* are responsible for disease.[120] Vascular EDS patients have a high risk of mortality, due to vascular fragility that often leads to hemorrhage.[120] Intracranial aneurysms have been reported in both classical and non-classical forms of EDS, but relatively speaking it is patients with the vascular subtype of EDS who are at the highest risk for IBA formation.[121-128] Kim et al. (2016) found intracranial

aneurysms in 12 individuals, seven of whom had vascular EDS, three of whom had classical EDS, and one who had the hypermobility form, from a chart review of 99 EDS patients (mean age 41.7 years) who underwent brain imaging.[128] There is currently no consensus on the clinical utility of screening for intracranial aneurysms among otherwise asymptomatic vascular EDS patients. Results of a positive test on such screening may not be easily actionable, because of the high risks associated with surgical intervention. If screening is desired, a non-invasive approach (such as MRA) should be strongly considered in these patients, in order to avoid further weakening of the vasculature.[125,129] Certain tertiary care centers with specialized expertise have reported low rates of complications from endovascular procedures in EDS,[130,131] such that treatment may be safe for EDS patients receiving care at experienced centers.

1.3.1.2.3 Loeys-Dietz Syndrome

Loeys-Dietz syndrome (LDS) is a connective tissue disorder characterized by severe vascular defects, primarily arterial aneurysms, that can hemorrhage or dissect very early in life.[132,133] Autosomal dominant mutations in TGF-β pathway genes, most frequently *TGFBR1* and *TGFBR2*, cause LDS. Although LDS is not commonly listed as having an association with IBA, a number of patient cases have reported IBA as a feature.[132-137] Cerebrovascular bleeding is the third leading cause of death in LDS patients, and intracranial aneurysms have been seen at a frequency ranging between 10-28%.[128,134,137] Although further studies are needed to assess the clinical utility of screening LDS patients specifically for intracranial aneurysms, surveillance of each part of the vascular tree is currently recommended every two years.[138]

1.3.1.2.4 Marfan Syndrome

Marfan syndrome is an autosomal dominant connective tissue disorder caused by pathogenic variants in *FBN1*. It is characterized by skeletal, ocular, and cardiovascular anomalies with wide phenotypic variability. Aortic aneurysm, dissection, and root enlargement are the most commonly reported vascular defects.[139,140] IBA have been associated with Marfan syndrome through multiple case reports.[141-147] Conway et al. (1999) did not find evidence for an association between intracranial aneurysms and Marfan syndrome upon autopsy of 25 patients.[148] Only one patient autopsied was found to have an intracranial aneurysm, a number that would agree with the general population frequency. However, a recent retrospective chart review of 59 Marfan syndrome patients estimated that 14% have one or more intracranial aneurysms.[128] Specific screening for intracranial aneurysms is not routinely recommended in Marfan syndrome patients, although it could be considered in family members of affected individuals who have had a diagnosed IBA.

1.3.1.2.5 Neurofibromatosis Type I

Neurofibromatosis Type I (NF1) is an autosomal dominant condition caused by pathogenic variants in *NF1*. The primary characteristics of NF1 are café-au-lait spots, iris Lisch nodules, and benign neurofibromas.[149] Vascular abnormalities are also a recognized characteristic of NF1, and patients under 29 years of age have an increased prevalence of intracranial aneurysms relative to the general population risk.[150] Though Conway and coauthors (2001) did not detect any intracranial aneurysms in their autopsy study of 25 NF1 patients between 3 and 69 years of age,[149] intracranial aneurysms have been documented as a cerebrovascular feature of NF1 by other investigators.[151,152] Retrospective reviews of NF1 patient

data have reported the prevalence of intracranial aneurysms to be between 9-11%, and the youngest NF1 patient reported to have an intracranial aneurysm was 1 year of age. However, routine screening for intracranial aneurysms is not currently recommended for NF1 patients.[153-159] Moyamoya angiopathy, which is also associated with NF1,[160] appears to have a pathogenesis that is distinct from that of intracranial berry aneurysms; it is not clear whether the occasional association of intracranial aneurysms with moyamoya[161,162] reflects a common pathophysiology at the molecular level, or whether it reflects pathology at one vascular site that perturbs downstream hemodynamics.

1.3.1.2.6 Other Syndromes

Multiple Endocrine Neoplasia Type I,[163] Pseudoxanthoma Elasticum,[164-166] and Hereditary Hemorrhagic Telangiectasia (HHT)[167] are often mentioned as being associated with IBA. While all have had individual patient case reports which document the presence of intracranial aneurysms, these data are not as robust as are the data for the syndromes listed above and in Table 1.3. Specific recommendations for IBA screening in these disorders must await larger cohort studies, although in the case of HHT, the currently recommended screening protocols to detect cerebral arteriovenous malformations (AVM) would be expected to detect IBA as well.[168] Most frequently in HHT patients intracranial aneurysms form along arteries leading into AVM.[167]

Patients with fibromuscular dysplasia (FMD) are also believed to be at risk for aneurysms in the brain and elsewhere, so current recommendations are that all FMD patients have cross-sectional imaging from head to pelvis with a sensitive method like CTA or MRA.[169] Though

autosomal dominant inheritance has been suggested for fibromuscular dysplasia, definitive genetic candidates have yet to emerge.[170]

1.3.1.2.7 Concluding Remarks

Among the syndromes discussed above that confer increased risk of IBA, ADPKD has the strongest association. Published estimates of IBA prevalence in Marfan syndrome, NF1, EDS, and LDS are likely biased toward reporting a higher percentage of affected patients. In the studies discussed, patients found to be at risk for vascular complications received brain imaging, whereas patients lacking these risk factors were not screened and were not included in the prevalence calculation. Additionally, many of these studies also included fusiform aneurysms when documenting the presence of intracranial aneurysms.

Table 1.3 Prevalence of intracranial aneurysms in selected syndromes

Relative Prevalence	Syndrome	OMIM Number(s)	Associated Genes	Prevalence of patients with IBA
Frequent	Autosomal Dominant Polycystic Kidney Disease	173900 613095	*PKD1*, *PKD2*	4-17%
Infrequent	Vascular Ehlers-Danlos Syndrome	130050	*COL3A1*	12%[a]
	Loeys-Dietz Syndrome	609192 610168	*TGFBR1*, *TFGBR2*, *SMAD3*[b]	11-28%[a]
	Marfan Syndrome	154700	*FBN1*	14%[a]
	Neurofibromatosis Type I	162200	*NF1*	9-11%[a]
Rare [c]	Pseudoxanthoma Elasticum	264800	*ABCC6*	-
	Hereditary Hemorrhagic Telangiectasia	187300	*ENG*	~10%[d]
	Multiple Endocrine Neoplasia Type I	131100	*MEN1*	-

a. Prevalence estimates may be influenced by selection bias and the inclusion of both fusiform and berry aneurysms.
b. Pathogenic variants in *TGFB2* and *TGFB3* also account for < 5% of LDS cases. **c.** Individual case reports only. **d.** As the prevalence of IBA in HHT patients is unknown, the prevalence of arteriovenous malformations, which can also lead to cerebral hemorrhage, has been given.

These two methodological limitations have prevented an accurate measurement of IBA prevalence in the syndromes listed, which may or may not be significantly higher than the background population risk. LDS prevalence measurements may not be as affected by ascertainment bias, if patients are receiving screening every two years as recommended. As more data are collected on the natural history of LDS, this syndrome may move to a higher risk category for IBA.

1.3.1.3 Genetic Counselling

Certified genetic counsellors are familiar with family trees that suggest Mendelian inheritance that follows autosomal dominant, autosomal recessive or X-linked patterns of inheritance. Even if a DNA-level diagnosis is not available in those situations, counsellors will

be able to estimate recurrence risks and suggest appropriate follow-up. Where feasible, risk for apparently-unaffected family members may be refined by imaging studies in living parents or grandparents and other relatives (sibs, aunts and uncles, etc.).

Unusual situations may arise that demand consideration of screening protocols that have not been validated by prospective studies. For example, pediatric IBA is extremely rare, with cases often only being described as case reports. A retrospective study found only 2% of patients diagnosed with intracranial aneurysms were children, and many of those aneurysms were either fusiform or associated with an underlying syndromic diagnosis.[164] Family 3 in this study has a child under age 15 who suffered a subarachnoid haemorrhage attributed to a berry aneurysm of the cerebral circulation. Although this child is not known to have another affected first-degree relative, when a child develops a disease that is rare in children but common among elderly adults, an underlying high-penetrance risk allele may well be a major contributing risk factor. Challenging situations such as these may demand a multidisciplinary approach that includes engagement of the genetic counsellor with other medical and surgical practitioners involved in the family's care. Counsellors may also need to provide an opinion to insurance providers as to the utility of screening in one family member for the purposes of estimating risk in other family members. In the examples given above, screening of clinically-unaffected sibs may be worthwhile, but screening of clinically-unaffected parents may also provide useful information. Where one or more aneurysms are detected in a parent, the unaffected sibs would definitely be considered to be at risk and to merit screening under current guidelines.

As there is not yet a genetic test, or a conclusive familial recurrence rate, genetic counsellors will need to estimate patient risk for developing non-syndromic IBA on a case-by-case basis, through analyzing inheritance within a pedigree. Counsellors should also investigate

the possibility of an associated syndrome by taking a targeted family history; if warranted by specific findings, they could then refer the patient to a medical geneticist or other suitable specialist. The heterogeneity of findings on IBA within Marfan syndrome, NF1, EDS, and LDS does not give clear direction to genetic counsellors. However, genetic counsellors should be aware that patients with these syndromes could be at increased risk for IBA development, and may want to recommend screening for patients who present with additional risk factors such as a specific history of IBA in their family members.

1.4 Objectives and Hypotheses

My objective was to identify one or more disease-causing genes for intracranial berry aneurysms. While several loci and candidate genes have been identified, only a single conclusive gene-disease association has been made for non-syndromic families. Further next-generation sequencing studies were needed to identify causative genes in families with intracranial aneurysms as the only clinical finding. Patients with FIA are at increased risk for aneurysm formation and rupture compared to the general population, and screening of all first-degree relatives using MRA or CTA is currently recommended. Definitive identification of a single-gene cause of FIA in a particular family or group of families would allow genetic confirmation of which family members are at risk. Family members who did not inherit the pathogenic variant would not need to undergo regular screening, and could obtain peace of mind regarding aneurysm formation later in life.

I have hypothesized that major Mendelian genes for intracranial aneurysms exist. A recent example of this is the pathogenic mutation in *THSD1* was reported to account for a small proportion (~1%) of nonsyndromic brain aneurysm families.

My hypotheses are that:

1. In multiplex families with FIA segregating in a Mendelian manner (autosomal dominant, autosome recessive, or X-linked) there will be a single pathogenic variant per family that leads to the formation of intracranial aneurysms.
2. Among multiple independent families diagnosed with FIA, different rare pathogenic variants will be found to affect the same gene in a subset of these families.

Chapter 2: Methods and Materials

2.1 Family and Clinical History

After informed consent, a detailed family history was taken from each study participant and/or family through an in-person interview, or via an over-the-phone interview for individuals who do not live locally. Additionally, a clinical history was taken from each participant to gather information on the screening and treatment of their intracranial aneurysms. Participants were also offered the option of signing a release of information form permitting access to their medical records regarding the care of their aneurysms. This provided further resolution of the oral clinical history given by the patient and allowed for collection of more granular data such as size and location of IBA.

2.1.1 Data Collection Form

A data collection form was created for this study to ensure a standardized set of information was collected from each participant (Appendix A). This form included collection of basic demographic information, such as age and ethnicity, clinical information such as age of diagnosis, number of aneurysms and type of surgical intervention, as well as research information, such as ADPKD status. The data collection form was updated in October 2016 to include questions about smoking, hypertension, and connective tissue disorders.

2.1.2 Family Selection

Participants were required to fulfill a minimum of one of the following three criteria in order to be enrolled in this study:

1. Had ≥1 family member also diagnosed with intracranial aneurysms.

2. Were diagnosed with ≥2 intracranial aneurysms.
3. Were <40 years of age when diagnosed with intracranial aneurysms.

Individuals fulfilling criterion 1 were prioritized for sequencing above those fulfilling criteria 2 or 3, as family history is a stronger predictor of genetic risk factors for disease than are age of onset or disease severity. Individuals with a concurrent diagnosis of a disease or syndrome known to confer a genetic predisposition to IBA/FIA (such as ADPKD) were excluded from this study.

2.2 DNA Collection and Extraction

DNA samples were obtained from study participants using the Oragene DNA OG-500 collection kit by DNA Genotek (Kanata, Ontario) to collect a saliva sample. As the majority of study participants reside outside of Vancouver, British Columbia, collection kits with detailed instructions were mailed to each study participant. Participants then provided saliva samples following Oragene DNA protocol, and mailed samples back using the return envelope provided. The collection kits include a preservative that permits the transportation of the saliva without DNA degradation or the need for refrigeration.

Two methods were utilized to extract DNA from the Oragene kits. For Family 1, DNA was extracted manually according to the DNA Genotek prepIT-L2P laboratory protocol for manual purification of DNA. To ensure samples were free of RNA contamination, a subsequent RNase treatment was performed. First, DNA samples were diluted with elution buffer to a total volume of 500 uL, and 0.5 uL of RNase was added so that the final RNase concentration was 10 ug/mL. The DNA/RNase solution was incubated at 37 ˚C for 30 minutes. Next, 50 µL of 3M sodium acetate (pH 5.2) and 1 mL of 100% ethanol were added to each sample to precipitate the

DNA. Samples were then incubated overnight (18.5 hours total) at -20 ˚C to allow for complete precipitation of the DNA. After incubation, samples were centrifuged at 14,000 rpm for 10 minutes at 4 ˚C. The supernatant from each sample was removed, and then 200 µL of ice cold 70% ethanol was added to each sample. Samples were centrifuged at 14,000 rpm for 10 minutes at 4 ˚C for a second time, and the supernatant and all remaining 70% ethanol was pipetted out. The lids of the Eppendorf tubes containing the samples were left open for 5.5 hours to evaporate any residual ethanol. Lastly, DNA was re-suspended in 100 µL of elution buffer (a pH neutral nucleic acid solvent), for a final volume of 100 µL per sample.

For all subsequent families after Family 1, and for one member of Family 1 who required DNA to be re-collected due to poor quality, the Maxwell RSC Blood DNA AS1400 extraction kit from Promega (Madison, Wisconsin) was used following the blood/saliva RSC DNA protocol. The Maxwell extraction kit is an automated process that extracts DNA from blood or saliva samples through the use of magnetic beads. Saliva was heated for one hour at 50 ˚C in a water bath before the extraction process. With clean gloves, extraction kit cartridges and 0.5 mL elution tubes were placed into the tray. Then 50 µL of elution buffer was added to each of the 0.5 mL elution tubes. For each study participant, DNA was extracted from two 300 µL aliquots of saliva sample. First, 300 µL from the collected saliva sample was pipetted into the first well of each extraction cartridge. After the automated DNA extraction process was complete, the Maxwell processor deposited the genomic DNA into the 50 µL of elution buffer in a 0.5 mL PCR tube. Residual magnetic beads were also deposited into the PCR tube; these were concentrated by centrifuging the DNA samples at 10,000 G for two minutes. Duplicate samples were then combined by pipetting 40 µL of each one into a single sterile 1.5 mL Eppendorf tube, taking care to exclude the magnetic beads. This resulted in 80 µL total of extracted DNA from

each study participant. Concentrations varied from 8 – 230 ng/µL, depending on the initial quality of the saliva sample. None of the samples extracted using this automated process required RNase treatment.

2.2.1 Quality Control

Assessment of DNA quality was achieved using three different measures: agarose gel, NanoDrop 2000 spectrometer, and Quantus fluorometer. All DNA samples were quantified using the NanoDrop 2000 and by visualizing the DNA on a gel. Additionally the Quantus fluorometer was used to measure DNA concentration in samples extracted using the Maxwell RSC Blood DNA AS1400 extraction kit.

An agarose gel was made at 1% concentration using 40 mL of 1x TBE buffer, 0.4 g of agarose, and 4 µL of 10,000x CyberSafe DNA gel stain. DNA samples were prepared on parafilm, combining 1 µL of 6x DNA loading dye with a combination of 5 µL sample and elution buffer so that absolute amount of DNA added was ~200 ng, and the final volume was 6 µL. Next, 5 µL of 1 kb ladder was added to the first well of the agarose gel, with samples in neighbouring wells. The agarose gel was run for 30 minutes at 100 V before DNA within the gel was visualized using a UV transilluminator.

NanoDrop-based quality assurance was conducted using the following protocol. The NanoDrop (Thermo Fisher Scientific, Waltham, MA) was set to measure nucleic acid quality. First, 1 µL of elution buffer (from the DNA Genotek or Promega kits, depending on prior extraction method) was placed on the NanoDrop and measured as the blank, setting the background wavelengths that would be present in the DNA samples. Between each

measurement, the NanoDrop was cleaned with a Kimtech wipe. Next, 1 µL of patient sample was placed on the Nanodrop and measured to determine DNA concentration and purity.

To measure DNA concentration 1 µL of sample was added to 199 µL of Quantus fluorometer dye in a 0.5 mL Eppendorf tube, vortexed briefly, and incubated in a dark drawer for 5 minutes. After incubation, each 0.5 mL Eppendorf tube containing the sample was placed in the Quantus fluorometer and the nucleic acid concentration was measured. Human cancer cell K562 genomic DNA was used as the standard for Quantus measurements.

2.3 Microarray

Aliquots of participants' samples from Family 1 were sent to the Cytogenetics Laboratory at Children's and Women's Health Centre of BC for whole-genome SNP microarray on the CytoScan HD Array from Affymetrix (Santa Clara, California). Microarray data were analyzed on Affymetrix Chromosome Analysis Suite (ChAS) software to identify candidate genomic regions of interest. In collaboration with Dr. Patrice Eydoux, regions of homozygosity (ROH) and copy number variants (CNVs) shared between affected and unaffected siblings were eliminated as potential candidate regions. Thresholds for detection of candidate pathogenic CNVs in affected subjects were set to require a minimum of 20 CNV markers for deletions and 30 CNV markers for duplications. The clinical thresholds for minimum length (200kb for deletions and 400kb for duplications) were not used for calling CNVs, as we reasoned that pathogenic CNVs would likely be smaller than these thresholds and did not want to limit our scope of discovery. These thresholds were chosen after consulting with Dr. Patrice Eydoux, Director of the Cytogenetics laboratory at Children's and Women's Health Centre of British Columbia.

2.3.1 Validation of Candidate CNV by qPCR

Candidate CNVs were validated with quantitative polymerase chain reaction (qPCR). Four sets of primers, two sets within the deleted region and two sets in the undisrupted region (approximately 2 Kb in length) 5' of the microdeletion, were designed to carry out the qPCR. Primers were design using Primer BLAST on NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), with an amplicon size between 105 – 115 bp (an optimal length for qPCR), and melting temp below 62°C (Appendix B). To check for self-complementarity of primer pairs, OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) algorithm was used to scan for hair-pin formation and self-annealing. Primer sequences were inputed into *in silico* PCR by the UCSC algorithm (https://genome.ucsc.edu/cgi-bin/hgPcr), to ensure specificity by determining the location(s) and number of predicted PCR products. *G6PD* was used as a gene control to check for amplification of DNA from each sample. The qPCR reaction was optimized for these primers in two steps:

First, the optimal amount of gDNA to be added to each reaction was determined. Stock DNA was diluted eight times by multiples of 10 from its initial concentration of 38.7 mg/µL, and run following Promega's GoTaq qPCR Master Mix protocol using primers already known to work, in this case the *G6PD* primers. Based on the CT value (26 for 100x dilution) it was determined that 5 ng of gDNA per reaction would be appropriate. Second, each designed primer pair was confirmed to produce an amplified product using 5 ng of test DNA per reaction.

Patient DNA samples were then diluted to a final concentration of 1.25 ng/µL and following the GoTaq qPCR Master Mix protocol by Promega. Each qPCR reaction contained 10 µL GoTaq qPCR Master Mix (2x), 1 µL 5M forward primer, 1 µL 5M reverse primer, 0.2 µL

CXR reference dye, 4 µL of patient DNA and 3.8 µL ddH_2O for an overall volume of 20 µL per reaction.

2.4 Next-Generation Sequencing

Whole-exome sequencing for Family 1 was carried out by Perkin Elmer Inc. with the SureSelect V4 (51 Mb) All Exon Kit (Agilent) for targeted exome selection and sequenced on an Illumina HiSeq 2000 to 50x mean coverage. Variant calling, filtering, and annotation were carried out using VarSeq software by Golden Helix. WES for Families 3, 4 and 5, was carried out using Agilent Human SureSelect kit for multiplex exome capture and Illumina HiSeq 2500 with four samples per 125 base paired-end tag (PET) to 30X mean coverage lane by the Michael Smith Genome Sciences Centre (GSC) in Vancouver, British Columbia under the supervision of Dr. Steven Jones. Whole-genome sequencing for Family 2 was carried out using an Illumina HiSeq 2500 with two lanes of 125 base PET to 30X mean coverage, also by the GSC under the supervision of Dr. Jones. Read alignment and variant calling, filtering and annotation were done via the GSC in-house pipeline.

2.5 Variant List Annotation

For Family 1, VarSeq used five algorithms (Polyphen 2, MutationTaster, MutationAssessor, FATHMM and SIFT) to predict the effect of each variant on the cognate protein. The GSC pipeline annotated variants using two protein prediction algorithms, Polyphen 2 and SIFT. Both VarSeq and the GSC annotated variants with known Online Mendelian Inheritance in Man (OMIM; https://www.omim.org/) associations to disease.

Additionally, all candidate variant lists were annotated manually using the Gene Ontology Consortium (http://geneontology.org), National Centre for Biotechnology Information Gene database (https://www.ncbi.nlm.nih.gov/gene), International Mouse Phenotyping Consortium (http://www.mousephenotype.org), Genotype-Tissue Expression (GTEx) Portal (http://www.gtexportal.org/home/), and through a literature search of the gene name with a defined set of search terms (Table 2.1).

Table 2.1 Literature search terms

BROAD	NARROW	RELATED
• Angiogenesis • Artery • Blood vessel • Brain • Cerebral • Endothelium/ Endothelial • Smooth muscle • Vascular/Vasculature	• Aneurysm • Intracranial berry aneurysm (IBA) • Saccular aneurysm • Subarachnoid haemorrhage (SAH) • Stroke	• Abdominal aortic aneurysm (AAA) • Inflammation • Thoracic aortic aneurysm (TAA)

2.6 Sanger Sequencing

Sanger sequencing was used to validate or reject candidate variants identified through exome sequencing. Primers were designed for PCR amplification of participant DNA prior to Sanger sequencing using the same tools as were used to design primers for qPCR on CNVs. In Family 1 primers were designed for one candidate variant in each of 6 genes (*RYR1*, *BSN*, *VAT1*, *HSPB6*, *ARHGAP33*, and *FRY*) and two candidate variants in one gene (*SLC7A9*). Primer sequences are shown in Appendix C. All additional primers for Sanger validation were designed following the same methods as in Family 1. Primers for validation of one candidate variant in *CCM2* in Family 2 are shown in Appendix D. Primers for validation and segregation analysis of rare variants in *THSD1* and *PKD1* in Family 5 are shown in Appendix E and Appendix F, respectively.

Primer pairs were tested and optimized using test DNA at six annealing temperatures (52°C, 55°C, 57°C, 59°C, 61°C, and 63°C). Before Sanger sequencing, each DNA sample was amplified using GoTaq Master Mix (2x concentration) by Promega following the associated protocol on the Veriti 96 Well Thermal Cycler by Applied Biosystems. The settings for the Thermal Cycler were as follows: 2 minutes at 94°C; 45 seconds at 94°C; 45 seconds at optimal primer annealing temperature, followed by 1 minute at 72°C, repeated a total of 35 times; 10 minutes at 72°C; hold at 4°C until the Thermal Cycler run stopped. Each reaction contained 10 µL GoTaq Master Mix, 1 µL Primer pair (with each forward and reverse primer at 5 µM concentration), 1 µL 100% DMSO, 3 µl ddH_20, and 5 µl of sample DNA (at a concentration of 8 ng/µL). Amplified DNA was then sequenced using the ABI Prism 3130xl Genetic Analyzer, and ABI BigDye v3.1 Terminator chemistry. Sequenced reads were analyzed using Sequence Scanner 2 by Applied Biosystems to identify variants, and CLC Sequence Viewer 7 by Qiagen to align sequenced reads to the reference genome.

2.7 Overall Analysis Strategy

Most rare disease phenotypes that have been solved to date are caused by rare coding variants in the exome that are predicted to be protein-damaging.[171] Thus, this study used next generation sequencing to identify rare coding variants in families with FIA. A minimum of one affected member from each FIA family received sequencing. Variants in the exome of each sequenced individual were filtered to meet quality thresholds (Read Depth and Genotype Quality > 20) and to have a minor allele frequency (MAF) <0.01. MAF for each variant was initially determined using the National Heart, Lung, and Blood Institute (NHLBI) database, and then confirmed against broader population data found in the Exome Aggregation Consortium (ExAC)

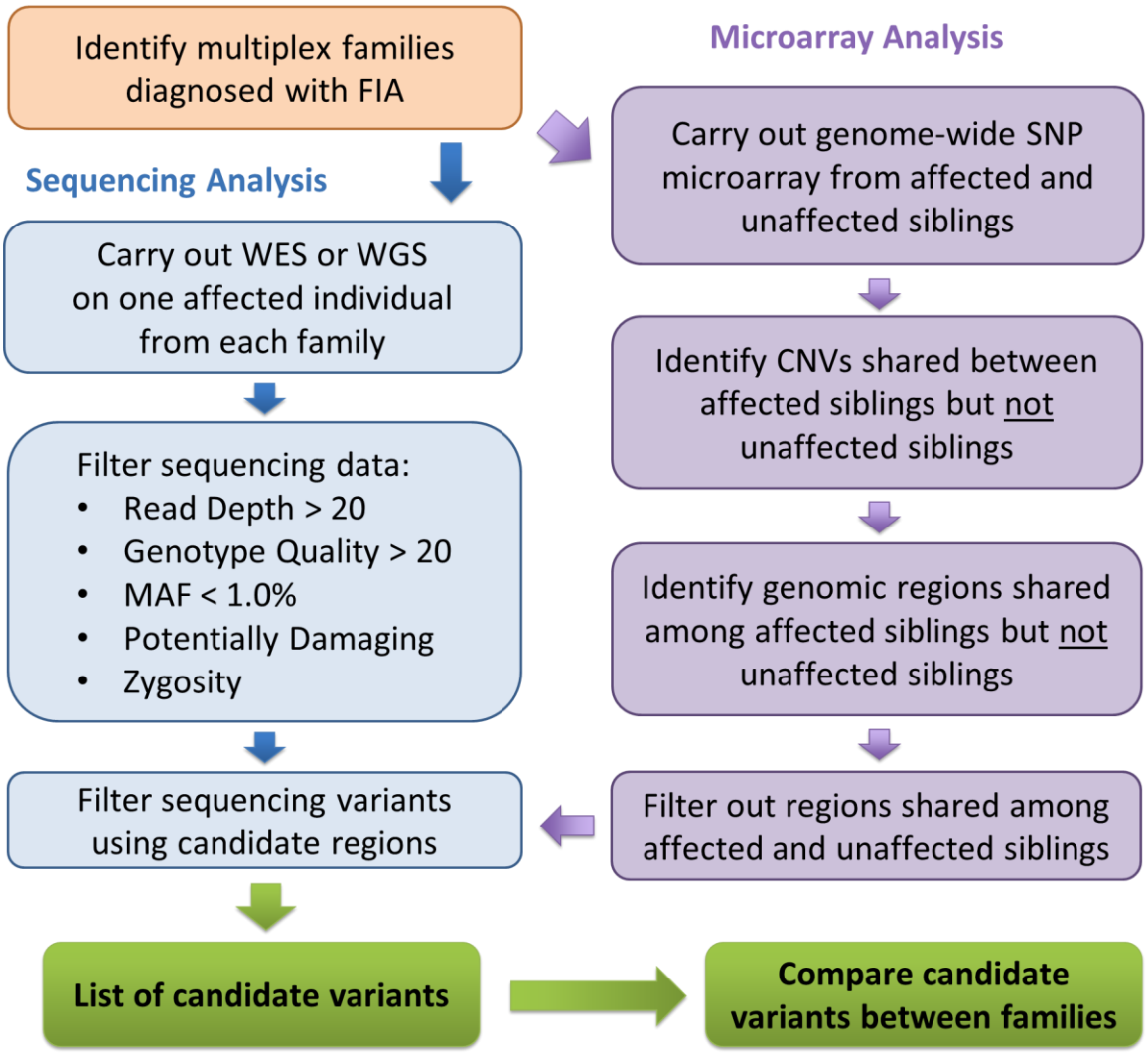
database. Variants were also filtered by zygosity to match the hypothesized inheritance pattern in the family (e.g. for families that appear to have dominant inheritance, heterozygous variants were retained) (Figure 2.1). Through this filtering, both the very rare variants that cause Mendelian disease (MAF <0.001) as well as variants that have an intermediate penetrance (MAF between 0.001 and 0.01) were identified (Figure 2.2). We used a less stringent MAF, because of what we know about the genetics of other common, complex diseases. Diseases that rarely present as a Mendelian disorders, and more commonly present as sporadic disease, typically feature very rare and highly-penetrant alleles in multiplex families, as well as an enrichment for rare but less-penetrant alleles in apparently sporadic late-onset cases. An example of a disease that exhibits this type of allelic architecture is coronary artery disease; both rare loss-of-function variants and uncommon variants in *ANGPTL4* significantly lower triglyceride levels, and are protective against this common, complex disease in the individuals and families who carry them.[172,173]

The list of rare, potentially damaging candidate variants identified in each affected family member was then overlapped to identify candidate genes that contained rare variants in three or more families. Upon the recent discovery of the role of *THSD1* in intracranial aneurysm formation, sequencing data were used to seek rare variants in this specific gene in all families in our cohort. All 5 exons of *THSD1* had mean 30x coverage from the exome or genome sequencing.

Among large multiplex families, such as Family 1, whole-genome SNP microarray was used to find genomic regions shared among affected family members that were not shared by unaffected family members. Due to the variability in family size, only families for which we had large numbers of both affected and unaffected members received microarray for haplotype mapping. The genomic coordinates of the candidate regions shared by affected family members

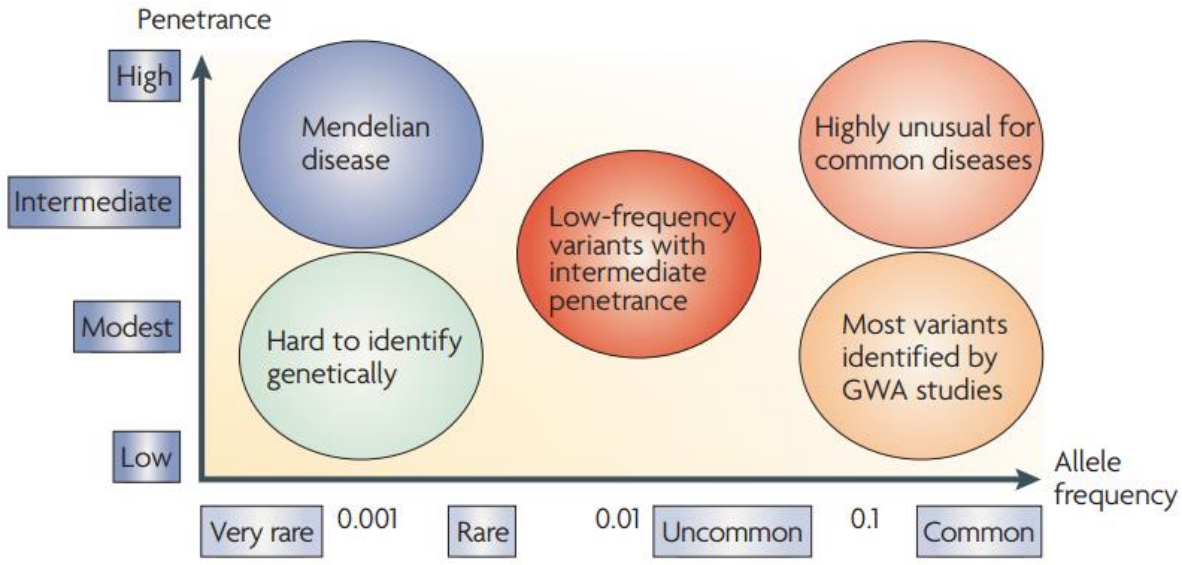
were then used to filter the rare variants found via WES even further (Figure 2.1). In families that received microarray, CNVs were also identified and investigated as the potential cause of IBA formation.

Figure 2.1 Outline of analysis of FIA families



CNV: Copy number variant; MAF: Minor allele frequency; SNP: single nucleotide polymorphism

Figure 2.2 Disease penetrance versus allele frequency



This graph shows the genetic architecture of common, complex disease. Both rare and common disease variants contribute to disease risk for conditions such as IBA.[174]

2.7.1 *De Novo* Variant Analysis

In families such as Family 3, wherein the parents appear to be unaffected, the presence of severe and/or early onset of disease in the proband is suggestive of a *de novo* mutation leading to aneurysm formation. Since *de novo* variants would be considered top candidates for pathogenic mutations causing IBA formation in families with this structure (followed by compound heterozygous and homozygous variants), trio-based exome sequencing can be a useful strategy to find these variants. However, without diagnostic brain imaging in the parents, it is not possible to rule out conclusively that one of them may be harbouring an unruptured IBA. Furthermore, it is impossible to be truly certain that a parent is unaffected, since he or she might develop an IBA later on in life. Lastly, a parent may carry the risk allele and be non-penetrant. Thus, in families

with pediatric-onset IBA, rare variants in genes that overlap with candidates from other FIA families should also be considered, even when these variant(s) are inherited from unaffected parents.

Chapter 3: Results

3.1 Study Cohort

The families presented in this thesis are the first to be sequenced and analyzed in an ongoing research study on the genetics of familial intracranial aneurysms. The size of the entire cohort is 49 affected and 28 unaffected individuals from 18 families, and 5 isolated affected cases, for a total of 82 individuals (Table 3.1 and Table 3.2). At the time of writing, DNA from 17 affected and 2 unaffected individuals from this cohort has undergone next-generation sequencing. Sections 3.2, 3.3, 3.4, 3.5, and 3.6, describe genome or exome sequencing results specific to each family. Section 3.7 describes the results from the analysis done by overlapping the genes containing rare variants between all of the families.

Table 3.1

Demographics of Cohort	
n	82
No. of singletons	5
No. of familes	18
by ethnicity (%)	
African	1 (5.6)
European	14 (77.8)
First Nations	3 (16.7)
Age, mean*	49.4
Range	12-78
No. Women, (%)	56 (68.3)
No. Affected (%)	49 (59.8)
No. DNA samples collected (%)	34 (41.5)
No. Families with ≥1 affected sequenced	9 (50.0)
No. Affected sequenced (%)	9 (11.0)
No. Unaffected sequenced (%)	2 (2.4)
No. Rare variants	2147

*This refers to the mean age at diagnosis or screening.

Table 3.2

Demographics of Affecteds	
n	49
No. Women (%)	36 (73.5)
Age, mean*	47.9
Range	12-78
No. Ruptured	16 (32.7)

*This refers to the mean age at diagnosis or screening.

3.2 Family 1

3.2.1 Pedigree and Phenotype

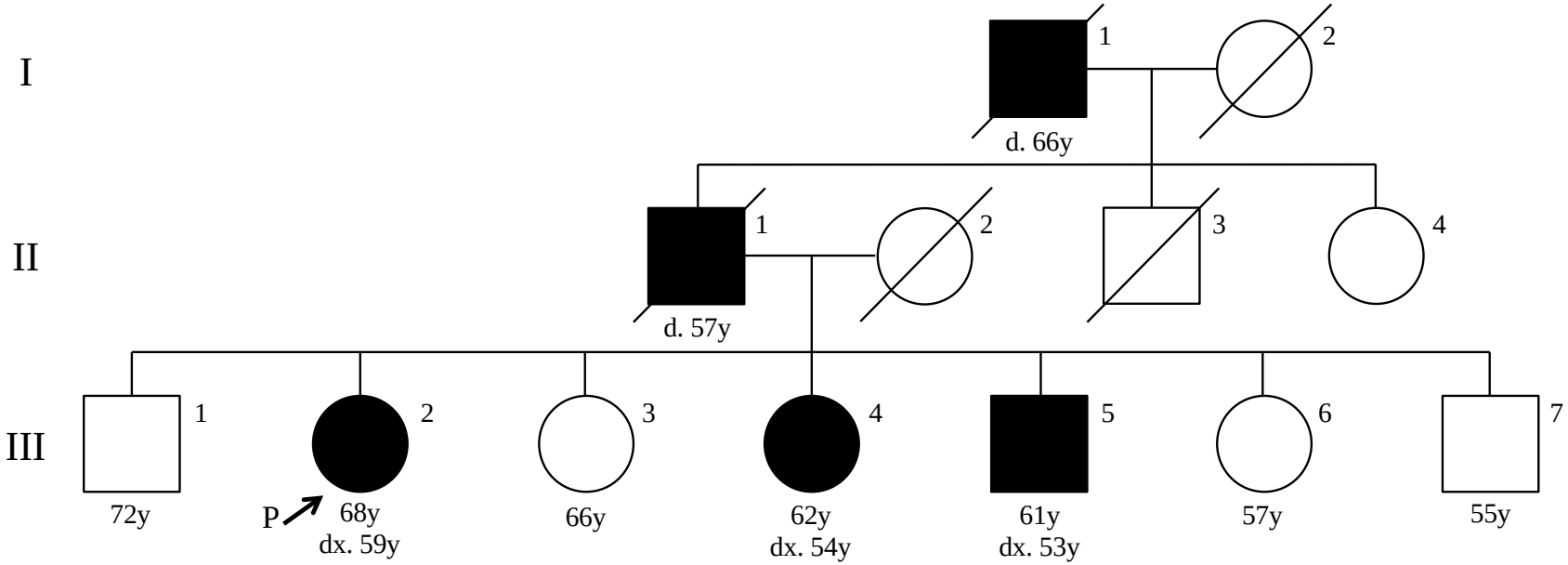
The first family has a history of IBA spanning three generations and is of European ancestry. Family 1 contains three affected siblings and four clinically unaffected siblings in the third generation, all of whom have had clinical brain imaging (Figure 3.1). Family members III-2, III-4, and III-5 were all screen-positive by age 60 years, and have since had surgical intervention (either by clipping or coiling) (Table 3.3).

The proband, III-2, was diagnosed by CT scan at 59 years of age with a single aneurysm on her anterior communicating artery. She chose not to pursue treatment at that time, but subsequently had two coiling procedures done at the age of 63. The first coiling procedure was done on an emergent basis because of aneurysmal hemorrhaging, and a second was done 6 months later in the same location because her aneurysm had increased in size to 4 mm in diameter. III-2 smokes approximately half a pack of cigarettes per day and has controlled hypertension, both of which are additional risk factors for aneurysm formation and rupture.

III-4 has been diagnosed with four IBA: bilateral lesions on the middle carotid artery (MCA), one on the left internal carotid artery (ICA), and the last on the posterior communicating (Pcom) artery. At age 54 she had the bilateral MCA aneurysms clipped surgically and the left ICA treated with endovascular coiling. The Pcom aneurysm was 3 mm in size when last scanned and remains untreated.

III-5 had two aneurysms treated through surgical clipping at the age of 53. III-1, III-3, III-6, and III-7 have all been screened using MRI, MRA, or CT and do not show any indications of intracranial aneurysms by ages 72, 66, 57, and 55, respectively. I-1 and II-1 both died from SAH at age 56 years and 66 years, respectively.

Figure 3.1 Pedigree of Family 1 [B]



"P↗" indicates the proband; "dx." denotes the age at diagnosis; "d." denotes the age at death

[B] A version of this figure has been published as: Hitchcock, E., and Gibson WT. A Review of the Genetics of Intracranial Berry Aneurysms and Implications for Genetic Counseling. *Journal of Genetic Counseling* 2017, Volume 26, Issue 1, pp 21–31, **First Online:** 14 October 2016. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, with attribution.

Table 3.3 Phenotypes of the third generation in Family 1

Family Member	III-1	**III-2**	III-3	**III-4**	**III-5**	III-6	III-7
Affected (Y/N)	N	**Y**	N	**Y**	**Y**	N	N
Sex (M/F)	M	**F**	F	**F**	**M**	F	M
Age (years)	72	**68**	66	**62**	**61**	57	55
At diagnosis or screening	64	**59**	60	**54**	**53**	50	48
Number of IBA	-	**1**	-	**4**	**2**	-	-
Location and size of IBA	-	**Acom (5 mm) recurred (4mm)**	-	**L. ICA (6 mm) L. MCA (7.4 mm) R. MCA (6.4 mm) R. Pcom (3 mm)**	**unknown**	-	-
Symptomatic (Y/N)	-	**Y**	-	**N**	**N**	-	-
Headache		**Severe headache and nausea at time of SAH**					
Dizziness		**N**					
Other		**N**					
SAH (Y/N)	-	**Y**	-	**N**	**N**	-	-
Age (years), Location		**63, Acom**					
Treatment (Y/N)	-	**Y**	-	**Y**	**Y**	-	-
Age (years), Location					**53, unknown**		
Surgical clipping		**-**		**53, bilateral MCA**			
Endovascular coiling		**Y**		**54, Pcom**			
Other		**-**					
Hypertension (Y/N)	Y	**N**	-	**N**	**Y - post-treatment, controlled by medication**	-	-
Smoking (Y/N)	N	**Y**	-	**-**	**-**	-	-
Additional Phenotype(s)	-	**-**	-	**-**	**-**	-	-

Affected family members are indicated by bold text. Information was not collected, or not applicable, for fields marked by a dash.

Acom: Anterior communicating artery; IBA: Intracranial berry aneurysm; L. ICA: Left interior carotid artery; L. MCA: Left middle communicating artery; R. MCA: Right middle communicating artery; R. Pcom: Right posterior communicating artery; SAH: Subarachnoid hemorrhage

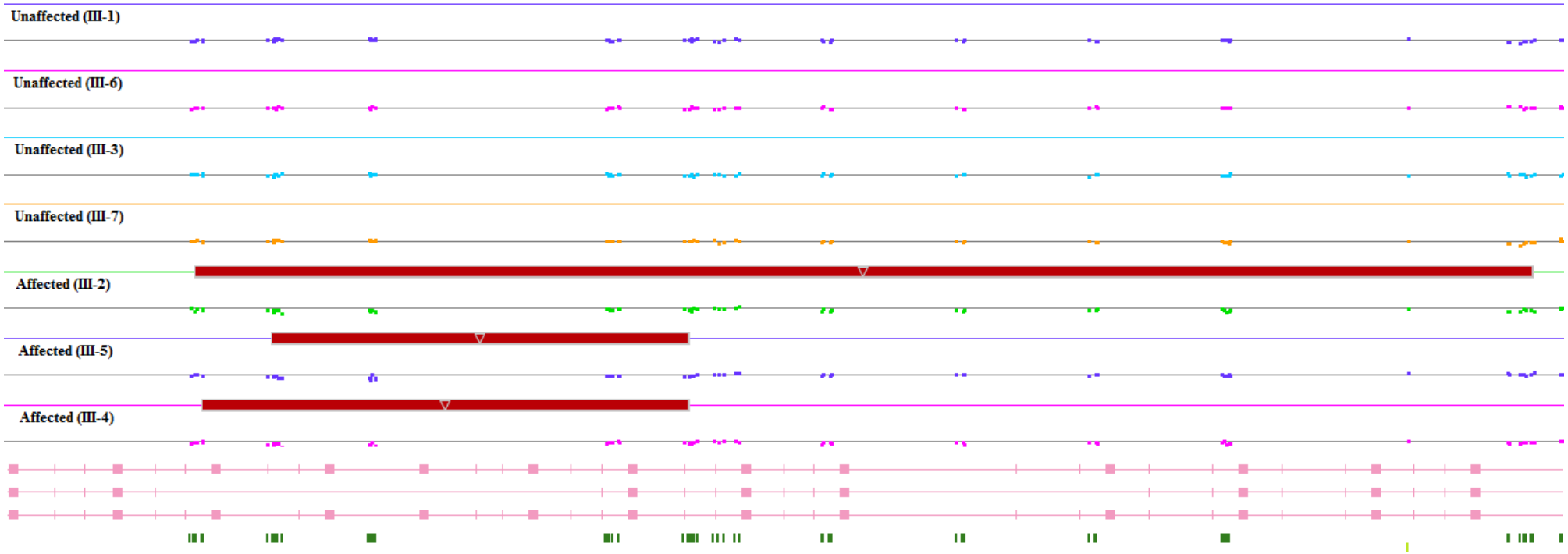
3.2.2 Microarray

A single candidate CNV, a microdeletion, was identified using ChAS. This CNV was found to be common only between the three affected siblings and absent from the DNA of the four unaffected siblings. This copy number loss disrupted one gene, deleted in malignant brain tumors 1 (*DMBT1*), on chromosome 10 and deleted a minimum of 12 exons at the 5'end of the gene (Figure 3.2). The length of this microdeletion varied between affected siblings, and is almost three times as long in III-2. The chromosomal section of the microdeletion that is absent in all three siblings is approximately 12.7 kb in length from chr:124,343,937-124,356,641 (Figure 3.4). Its presence in the three siblings with brain aneurysms and absence in the four siblings without brain aneurysm was confirmed by qPCR of genomic DNA (Figure 3.5 and Figure 3.6).

DMBT1 is expressed by endothelial cells and secreted into the extracellular matrix, and is also found to bind with known components of angiogenic pathways, including VEGF. In *DMBT1* knock-out mouse models vascular repair after hind limb ischemia was impaired compared to wild-type mice.[175] Although this microdeletion was found to segregate with disease, it was ultimately discarded as a candidate due to the apparent size discrepancy between affected siblings and the highly polymorphic nature of the region in which the CNV was found. *DMBT1* also transcribes salivary agglutinin protein (SAG). This protein is thought to interact with oral bacteria and certain isoforms are associated with a decrease in dental carries. *DMBT1* is known to have two highly polymorphic regions within the gene, which are characterized by both copy number gain and copy number loss formed through non-allelic homologous recombination. These CNVs have a 5% *de novo* mutation rate per gamete and change the number of tandem scavenger-receptor cysteine-rich domains (SRCR) within the protein.[176] The first highly variable

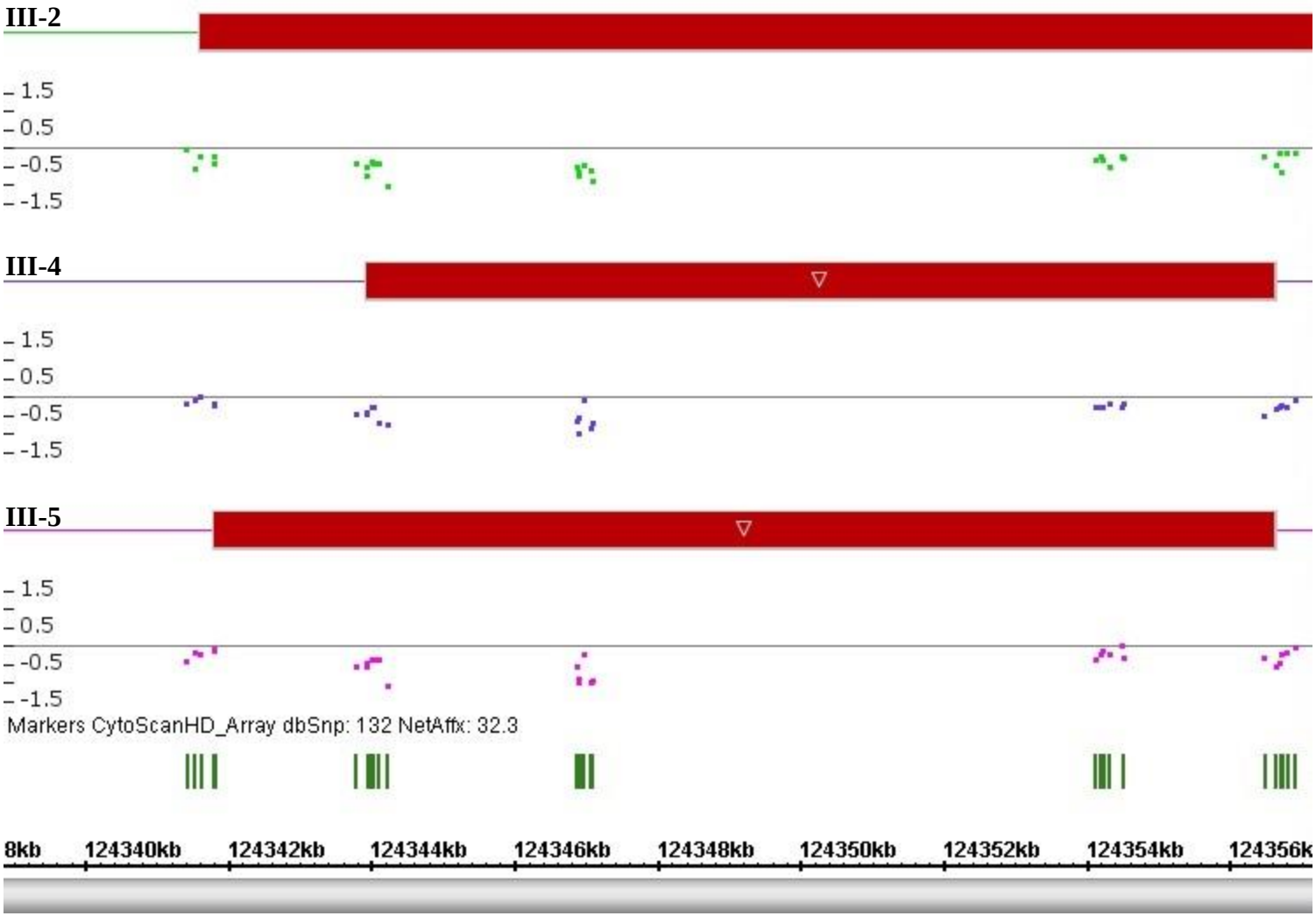
region consisting of many tandem repeat sequencing and involves SRCR 2-6, overlaps with the region that contains all three microdeletions in the affected siblings from Family 1. Although these tandem repeats account for the varying lengths of the microdeletions, the presence of CNVs at this region within *DMBT1* do not appear to be a rare occurrence. Both deletions and duplications have been reported in the Database of Genomic Variants (DGV; Build GRCh37: Feb. 2009, hg19) (http://dgv.tcag.ca/dgv/app/home) numerous times, further indicating that these CNVs are common changes within the general population. Despite the suggestive evidence from animal studies, we considered the combined evidence from the literature and DGV to be stronger predictors of lack of pathogenicity, and discarded this variant as a candidate.

Figure 3.2 Candidate microdeletion in *DMBT1* in Family 1



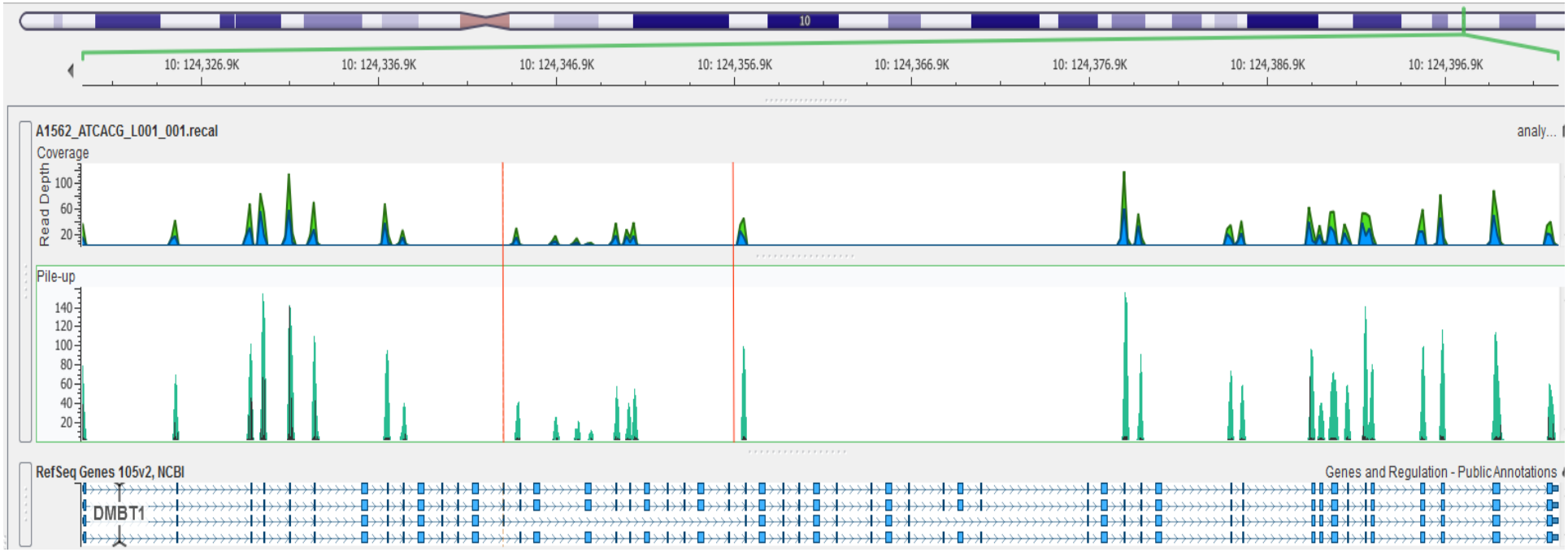
A microdeletion in *DMBT1* found in affected siblings III-2, III-4, and III-5 shown in red on Chromosome Analysis Suite. The boxes and lines in pink show the exons transcribed in three different transcripts of *DMBT1*. The coloured lines (different for each sibling) and dots along each line display the log2 ratio for each CNV probe. Dots that lie on the line represent a normal copy number of two, while dots that lie approximately 0.45 units below the line indicate a single copy number loss, as is seen in the deleted regions. The dark green and light green boxes denote the location CNV markers and SNP markers along this genomic region, respectively.

Figure 3.3 Candidate microdeletion in DMBT1 in affected siblings of Family 1



A microdeletion within *DMBT1* found in affected siblings III-2, III-4, and III-5 shown in red on Chromosome Analysis Suite. The full length of the CNV in III-2 has been truncated in this figure in order to show the log2 ratios. The coloured lines (different for each sibling) and dots along each line display the log2 ratio for each CNV probe. Dots that lie on the line represent a normal copy number of two, while dots that lie approximately 0.45 units below the line indicate a single copy number loss, as is seen in the deleted regions. The dark green boxes denote the location CNV markers along this genomic region.

Figure 3.4 Region of *DMBT1* microdeletion common between affected siblings



Exome sequencing coverage from affected brother III-5 shown on Genome Browse software by Golden Helix. The red bars mark the chromosomal region between chr10:124,343,937 - 124,356,641, approximately 12.7kb in length, which is deleted in all three affected siblings.

Figure 3.5 Relative gDNA in region 5' of DMBT1 between unaffected and affected family members

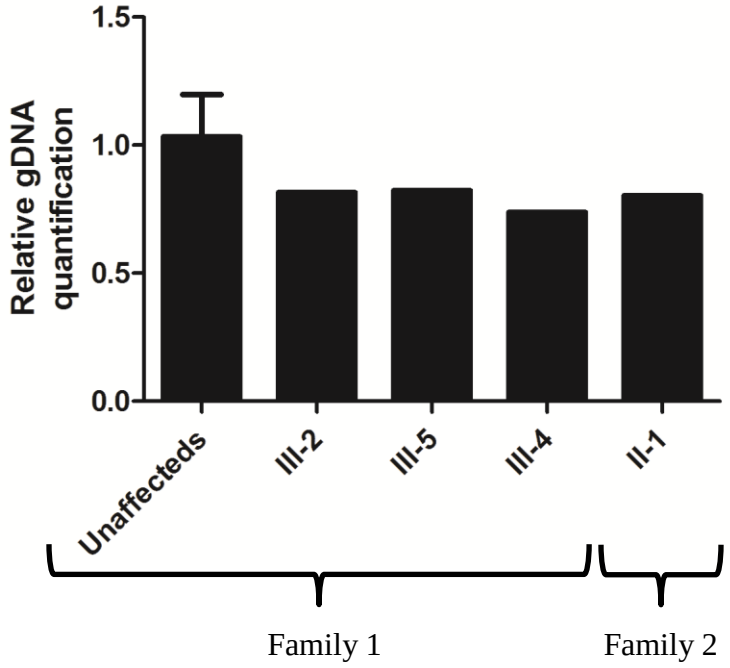
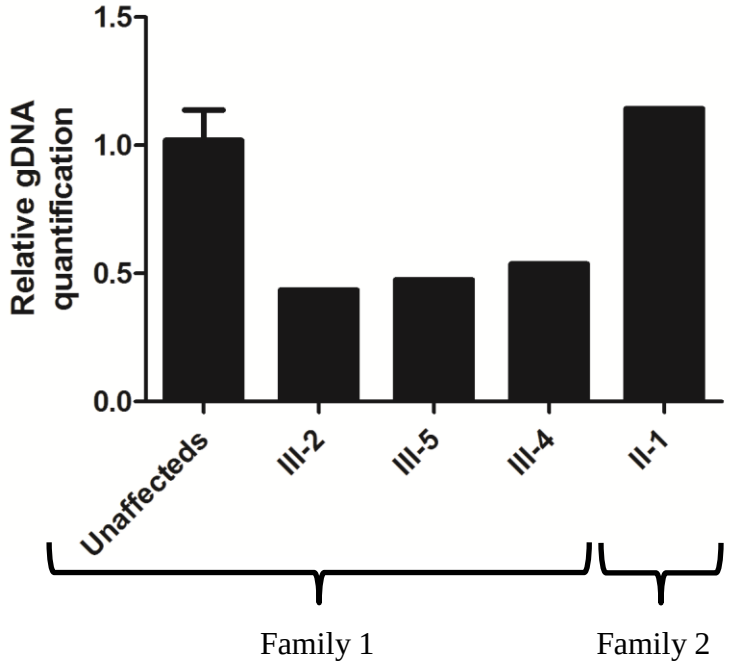


Figure 3.6 Relative gDNA in region of microdeletion between unaffected and affected family members



3.2.3 Exome Sequencing

WES of III-5, filtered following the methods in Chapter 2, produced 1,042 rare heterozygous variants. Further filtering using the coordinates of genomic regions shared by affected siblings resulted in 76 variants in 68 genes. Detailed annotation and literature search of all 68 genes resulted in six variants in *NOTCH4*, *CFB*, *PIK3C2G*, *HLA-DRB1*, and *DDR1* being selected as top candidates (Table 3.4). Eight additional variants in seven genes (*RYR1*, *BSN*, *VAT1*, *HSPB6*, *ARHGAP33*, *SLC7A9* and *FRY*) were not confirmed upon Sanger sequencing and were eliminated as candidates.

NOTCH4 encodes a protein belonging to the evolutionarily conserved NOTCH family of membrane bound receptors, which are known to be important in early embryogenesis for vascular development. The *Notch* genes are expressed in many tissues types, however Notch4 is thought to be expressed only in vascular endothelium and arteries.[177,178] In mouse models, endothelium-specific expression of constitutively activated Notch4 during embryogenesis resulted in embryonic lethality due to abnormal vasculature around 10 days postcoitum. Blood vessels failed to form in the brain and around the periphery blood vessels either did not form or were dilated, indicating a loss of structural integrity.[179] Notch4-deficient mice (through homozygous knock-out) develop normally into adulthood. However, mice with a double homozygous knock-out of Notch1 and Notch4 show a more severe phenotype of abnormal angiogenic remodeling and morphogenesis than do mice with a single knock-out of Notch1. This finding indicates that although Notch4 is not essential for vascular development during embryogenesis, it does have a role in vascular maintenance in adult mice.[180] Mice with constitutively active Notch4, induced by tetracycline at birth, developed arteriovenous

malformations (AVM) by 3 weeks and died of cerebral hemorrhage by 5 weeks of age. Abnormal connections between arteries and veins were seen in these mice, as were enlarged and tangled blood vessels, all of which are hallmarks of AVM in humans.[181]

In Family 1 a six base pair insertion in *NOTCH4*, was predicted to result in two leucine amino acids being added between residues 16 and 17. The two leucine amino acids are inserted into a string of 11 leucine residues near the 5' end of the protein. It is unclear whether or not this variant leads to disease.

CFB encodes complement factor B, a component of the alternative complement pathway, which has been associated with aneurysm formation in mouse models of abdominal aortic aneurysms.[182,183] *CFB* is also expressed in the endothelium of kidney cysts, but not in other types of kidney disease.[184]

Phosphatidylinositol-4-Phosphate 3-Kinase Catalytic Subunit Type 2 Gamma (*PIK3C2G*) remains largely unstudied and its function is not known. PIK3C2A, a protein in the same family, is critical for clathrin-coated vesicle endocytosis-related angiogenesis and the normal formation of vasculature. However, PIK3C2A is structurally distinct from PIK3C2G and other PIK2C2 family members and is limited in its expression to smooth muscle and vascular endothelium. Thus, PIK3C2G is thought to have a different expression profile and function compared to its better-studied family members.[185,186]

As a HLA Class II beta chain molecule, *HLA-DRB1* has been associated with a number of auto-inflammatory conditions such as multiple sclerosis and rheumatoid arthritis. In a cohort of 37 abdominal aortic aneurysm (AAA) patients compared to 90 controls, *HLA-DRB1* alleles *0404 and *15 have a higher frequency in AAA patients compared to controls: 14% versus 3% and 47% versus 27%, respectively.[187] In Marfan syndrome patients, increased HLA-DRB1

expression is associated with aortic root dilation compared to patients with low aortic root dilation.[188]

DDRI encodes a discoidin domain receptor (DDR), a type of tyrosine kinase. DDRs are autophosphorylated upon binding to Type 1 and Type VIII Collagen. *Ddr1* mRNA and protein expression is increased in rat aorta after balloon-catheter injury. Cultured smooth muscle cells from mouse aortas were used for an attachment assay that compared wild type cells with those bearing targeted deletion of *Ddr1*. The smooth muscle cells containing the *Ddr1* deletion had reduced adherence to collagen and reduced proliferation on the collagen matrix. Mice with the targeted deletion in *Ddr1* also showed reduced intimal growth of the carotid arteries in response to wire-induced injury.[189] Another experiment on smooth muscle cells from mouse aorta with knock-out of Ddr1 showed impaired attachment to and migration toward Type I Collagen. Transfection of these cells with *Ddr1b* cDNA lacking the catalytic domain partially rescued the phenotype, restoring attachment but not migration of the smooth muscle cells.[190]

Although each of the genes discussed above have a role in vasculature, none are a compelling candidate and will require further functional work to name them as causative within this family.

Table 3.4 Top candidate variants from Family 1

Chr:Pos	Ref/Alt Allele	Identifier	MAF	Gene Names	Protein Change	OMIM Disease Association	CADD Score
6:31917898	C/A	-	0.000008493	*CFB*	p.P996T	Complement factor B deficiency	29.8
12:18719887	C/T	rs146312199	0.003642	*PIK3C2G*	p.P1262S	-	28.8
6:32549582	G/T	rs200516145	-	*HLA-DRB1*	p.T135N	Autoimmune susceptibility	23.4
19:35842992	G/A	rs146654047	0.003077	*FFAR1*	p.G180S	-	17.84
6:32549583	T/C	rs17433947	-	*HLA-DRB1*	p.T135A	Autoimmune susceptibility	10.07
6:30862440	A/G	rs55787895	0.009611	*DDR1*	p.N502S	-	0.166
6:32191659	-/AGCAGC	-	-	*NOTCH4*	p.L16_L17insLL	-	0.005
6:24456812	G/T	-	-	*GLPD1*	p.F354L	-	0.003

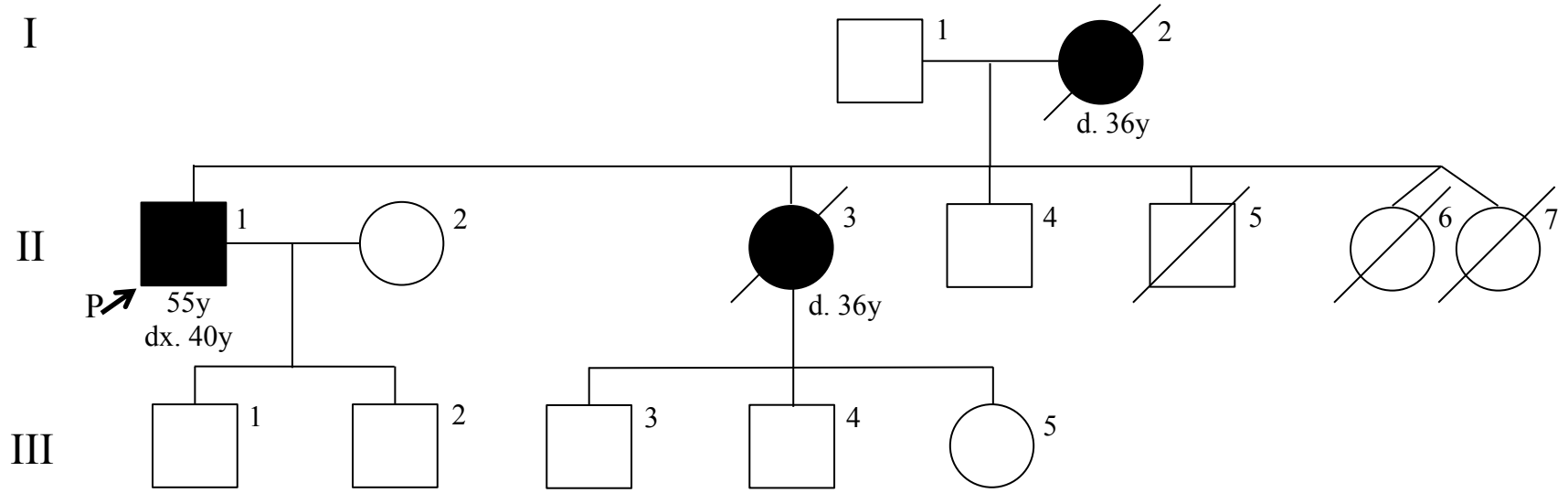
Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, MAF: minor allele frequency (for European ancestry), OMIM: Online Mendelian Inheritance in Man, Ref: reference.

3.3 Family 2

3.3.1 Pedigree and Phenotype

Family 2 has a history FIA spanning two generations with onset 15 to 20 years earlier than the mean age-of-onset in the general population with sporadic aneurysms (Figure 3.7 and Table 3.5). Family 2 is of Canadian First Nations ancestry and has three affected individuals. Affected family members I-2 and II-3 died by SAH, both at age 36 years; additional clinical and phenotypic information is unavailable. There have also been four deaths from SAH in extended family members on the paternal side (I-1) of the family (not shown in pedigree). There is no known consanguinity in this family. The proband, II-1, was first diagnosed with FIA, upon rupture of an IBA on the left middle MCA at age 40 years, for which he was subsequently treated with surgical clipping. The proband has since been diagnosed two additional IBA. The second IBA is located on the left Pcom, which was treated with endovascular coiling at 47 years of age after SAH. The third IBA, located on the right MCA, is currently stable and being monitored. There is also an abnormality on the anterior communicating (Acom) artery, which may turn out to be a fourth IBA. The two sons of the proband, III-1 and III-2, appear unaffected, but are not yet at the age to require screening, meaning their true affected status is currently unknown.

Figure 3.7 Pedigree of Family 2 [C]



"P↗" indicates the proband; "dx." denotes the age at diagnosis; "d." denotes the age at death

[C] A version of this figure has been published as: Hitchcock, E., and Gibson WT. A Review of the Genetics of Intracranial Berry Aneurysms and Implications for Genetic Counseling. *Journal of Genetic Counseling* 2017, Volume 26, Issue 1, pp 21–31, **First Online:** 14 October 2016. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, with attribution.

Table 3.5 Phenotype of Family 2

Family Member	I-2	II-1	II-3
Affected (Y/N)	**Y**	**Y**	**Y**
Sex (M/F)	**F**	**M**	**F**
Age (years)	**-**	**55**	**-**
At diagnosis or screening	**36**	**40**	**36**
Number of IBA	**1**	**3**	**1**
Location and size of IBA	**unknown**	**L. MCA** **R. MCA** **L. Pcom**	**unknown**
Symptomatic (Y/N)	**-**	**N**	**-**
Headache			
Dizziness			
Other			
SAH (Y/N)	**Y**	**Y**	**Y**
Age (years), Location	**36, unknown**	**40, L. MCA** **47, L. Pcom**	**36, unknown**
Treatment (Y/N)	**-**	**Y**	**-**
Age (years), Location			
Surgical clipping		**40, L. MCA**	
Endovascular coiling		**47, L. Pcom**	
Other		**-**	
Hypertension (Y/N)	**-**	**N**	**-**
Smoking (Y/N)	**-**	**-**	**-**
Additional Phenotype(s)	**-**	**Long-QT Syndrome**	**-**

Affected family members are indicated by bold text. Information was not collected, or not applicable, for fields marked by a dash.
Acom: Anterior communicating artery; IBA: Intracranial berry aneurysm; L. MCA: Left middle communicating artery;
R. MCA: Right middle communicating artery; L. Pcom: Left posterior communicating artery; SAH: Subarachnoid hemorrhage

3.3.2 Genome Sequencing

Previous to this study, II-1 tested negative for pathogenic variants in *TGFBR1* and *TGFBR2* at University of Washington.WGS, filtered following the methods in Chapter 2:, produced 1,106 rare heterozygous variants in the exome. Analysis of unique variants in known OMIM disease-causing genes produced one candidate variant in cerebral cavernous malformations 2 (*CCM2*), shown in Table 3.6. Heterozygous mutations in *CCM2* lead to cerebral cavernous malformations (CCM), which are characterized by chronic headaches, seizures, and subarachnoid hemorrhage. While missense variants have been reported in CCM2 to cause CCM, most often the pathogenic mutations are multi-exon deletions or truncating mutations.[191,192] CCM2 is part of a scaffolding protein complex that maintains endothelial cell-cell binding and vascular integrity.[193,194]

Homozygous knock-out and morpholino knock-down of the zebrafish CCM2 orthologue valentine, leads to embryonic lethality and causes enlargement of the heart chambers.[195] In mice, constitutive deletion of *Ccm2* results in a very similar phenotype to that of the zebrafish, with lethality by embryonic day 10.5 due to a grossly enlarged heart and cardiac failure. Endothelial-specific knock-out of *Ccm2* in mice is usually embryonic lethal, and also leads to an enlarged heart and dilated major blood vessels.[196] We hypothesize that if the p.Q11R variant in *CCM2* in this family is pathogenic for FIA, the mechanism of aneurysm formation would be through a weakening of endothelial cell-cell adhesion allowing the blood vessel wall to expand over time.

Table 3.6 Candidate variant in *CCM2* in Family 2

Chr:Pos	Ref/Alt Allele	Zygosity	MAF	Gene Names	Protein Change	OMIM Disease Association	CADD Score
7:45067335	A/G	het	0.000008242	*CCM2*	p.Q11R	Cerebral Cavernous Malformations	11.53

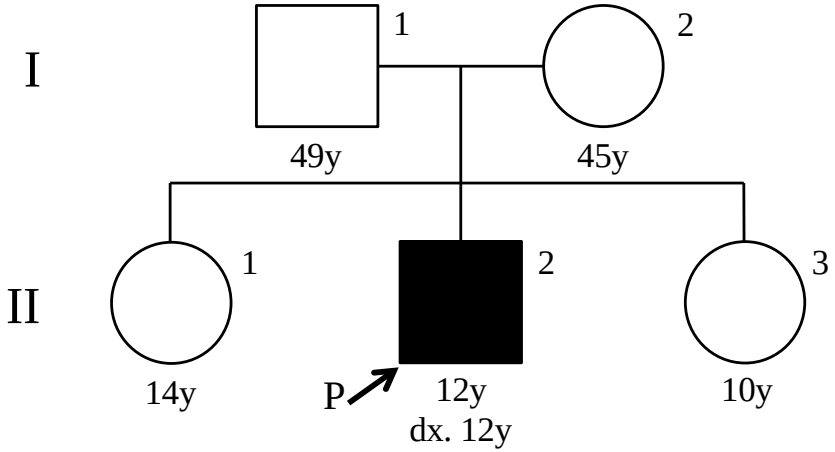
Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, het: heterozygous, MAF: minor allele frequency (for global ancestries), OMIM: Online Mendelian Inheritance in Man, Ref: reference.

3.4 Family 3

3.4.1 Pedigree and Phenotype

Family 3 presents a single pediatric case of IBA and SAH in a 12-year-old boy (Figure 3.8 and Table 3.7). This family is of European ancestry and does not have a family history of intracranial aneurysms. The proband, II-2, was diagnosed with IBA upon hemorrhage of a 4 mm aneurysm that had formed off the anterior communicating artery (Acom). The proband did not experience any symptoms before SAH and is an otherwise healthy, athletic child with no known risk factors. Upon rupture of the Acom aneurysm the proband experienced severe head pain and was taken to his local hospital where he was screened with a CT scan that showed the ruptured aneurysm with a small daughter lobule also present. He was subsequently treated with surgical clipping. None of the other family members have been screened to date (on the advice of their neurosurgeon), so their affected status is not known.

Figure 3.8 Pedigree of Family 3 [D]



"P↗" indicates the proband; "dx." denotes the age at diagnosis; "d." denotes the age at death

[D] A version of this figure has been published as: Hitchcock, E., and Gibson WT. A Review of the Genetics of Intracranial Berry Aneurysms and Implications for Genetic Counseling. *Journal of Genetic Counseling* 2017, Volume 26, Issue 1, pp 21–31, **First Online:** 14 October 2016. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, with attribution.

Table 3.7 Phenotype of Family 3

Family Member	I-1	I-2	II-1	**II-2**	II-3
Affected (Y/N)	N	N	N	**Y**	N
Sex (M/F)	M	F	F	**M**	F
Age (years)	49	45	14	**12**	10
At diagnosis or screening	-	-	-	**12**	-
Number of IBA	-	-	-	**1**	-
Location and size of IBA	-		-	**Acom (4 mm)**	-
Symptomatic (Y/N)	-	-	-	**-**	-
Headache					
Dizziness					
Other					
SAH (Y/N)	-	-	-	**Y**	N
Age (years), Location				**12, Acom**	
Treatment (Y/N)	-	-	-	**Y**	-
Age (years), Location					
Surgical clipping				**Acom (4 mm)**	
Endovascular coiling					
Other					
Hypertension (Y/N)	-	N	N	**N**	N
Smoking (Y/N)	-	-	N	**N**	N
Additional Phenotype(s)	N	N	N	**N**	N

Affected family members are indicated by bold text. Information was not collected, or not applicable, for fields marked by a dash.
Acom: Anterior communicating artery; IBA: Intracranial berry aneurysm; SAH: Subarachnoid hemorrhage

3.4.2 Exome Sequencing

WES of II-2, filtered following the methods in Chapter 2:, produced 770 rare heterozygous or homozygous variants. Trio analysis of sequencing data from the proband and his two parents identified 2 *de novo* variants in *CA5A* and *C2orf16* (Table 3.8). Compound heterozygous variants were found in 5 genes in the proband: *VPS18, AHCTF1, FAM198B, RPF2,* and *ARFGEF1* (Table 3.9). There were no candidate homozygous variants.

Homozygous variants in carbonic anhydrase 5A (*CA5A*) that disrupt enzyme function lead to an autosomal recessive form of hyperammonemia caused by carbonic anhydrase deficiency. Patients with these pathogenic mutations have an early-onset metabolic phenotype.[193] Information on the function of *C2orf16* is not yet available from the scientific literature. *VPS18, AHCTF1, FAM198B, RPF2,* and *ARFGEF1* have not been associated with intracranial aneurysms previously, and do not appear to have functions related to the vasculature.

At this time there is no evidence to support the variants discussed above as being causative for the IBA in II-2. However, as research discovers more information on each genes function, one or more of these variants may become a compelling candidate.

Table 3.8 *De novo* variants in II-2 in Family 3

Chr:Pos	Ref/Alt Allele	Identifier	MAF	Gene Names	Protein Change	OMIM Disease Association	CADD Score
16:87935534	G/A	-	0	*CA5A*	p.P201L	Hyperammonemia due to carbonic anhydrase VA deficiency	24.7
2:27804355	G/A	rs199903910	0.00001499	*C2orf16*	p.R1639H	-	13.22

Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, MAF: minor allele frequency (for European ancestry), OMIM: Online Mendelian Inheritance in Man, Ref: reference.

Table 3.9 Compound heterozygous variants in II-2 in Family 3

Chr:Pos	Ref/Alt Allele	Identifier	MAF	Gene Names	Protein Change	OMIM Disease Association	CADD Score
1:247013595	T/C	rs149899496	0.0007215	*AHCTF1*	K1914E	-	10.92
1:247065904	C/A	-	0	*AHCTF1*	S347I	-	24.7
4:159091752	C/A	-	0	*FAM198B*	R259L	-	12.73
4:159092106	C/T	rs147697286	0.01126	*FAM198B*	G141E	-	14.83
6:111345454	A/G	-	0	*RPF2*	D189G	-	28.9
6:111345474	C/T	-	0	*RPF2*	H196Y	-	23.8
8:68165850	T/C	-	0	*ARFGEF1*	N845S	-	14.92
8:68204202	C/A	rs61753695	0.006731	*ARFGEF1*	D266Y	-	23.3
15:41191665	C/T	-	0.00003057	*VPS18*	R217C	-	28.3
15:41195357	C/T	rs189855795	0.008217	*VPS18*	R914W	-	24.8

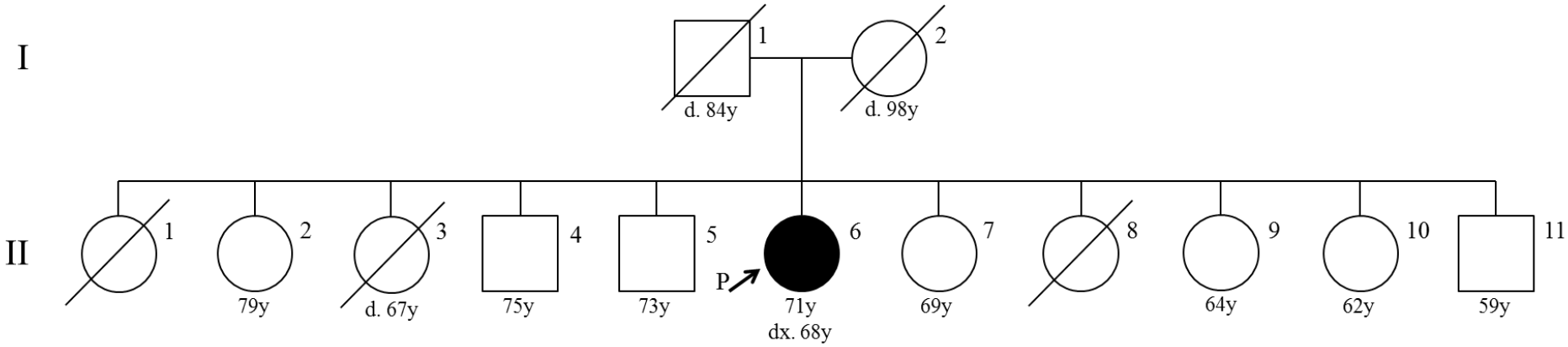
Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, MAF: minor allele frequency (for European ancestry), OMIM: Online Mendelian Inheritance in Man, Ref: reference.

3.5 Family 4

3.5.1 Pedigree and Phenotype

Family 4 has one affected family member (II-6) with a clinical history of IBA (Figure 3.9). II-6 was diagnosed with three IBA, consisting of a giant aneurysm (2.5 cm in size) of the internal carotid artery (ICA), and bilateral aneurysms on the middle cerebral artery (MCA). She was brought to her local emergency department upon blood leaking from her ICA aneurysm, and immediately received surgical clipping for two of her three IBA. The third IBA was also clipped two years later (Table 3.10). Initially it was thought that the hemorrhagic stroke experienced by the proband's sister, II-2, was caused by an IBA, but upon further investigation was found to be caused by a spontaneous posterior fossa hematoma (that showed no signs of vascular involvement).

Figure 3.9 Pedigree of Family 4



"P↗" indicates the proband; "dx." denotes the age at diagnosis; "d." denotes the age at death

Table 3.10 Phenotype of Family 4

Family Member	II-1	II-2	II-3	II-4	II-5	**II-6**	II-7	II-8	II-9	II-10	II-11
Affected (Y/N)	N	N	N	N	N	**Y**	N	N	N	N	N
Sex (M/F)	F	F	F	M	M	**F**	F	F	F	F	M
Age (years)	-	79	-	75	73	**71**	69	-	64	62	59
At diagnosis or screening	-	-	-	-	-	**68**					-
Number of IBA	-	-	-	-	-	**3**	-	-	-	-	2
Location and size of IBA	-	-	-	-	-	**L. MCA (4 mm)** **R. MCA (3 mm)** **L. ICA (2.5 cm)**	-	-	-	-	-
Symptomatic (Y/N)	-	-	-	-	-	**Y**	-	-	-	-	-
Headache											
Dizziness						**Y**					
Other											
SAH (Y/N)	-	-	-	-	-	**Y**	-	-	-	-	-
Age (years), Location						**68, L. ICA**					
Treatment (Y/N)	-	-	-	-	-	**Y**	-	-	-	-	-
Age (years), Location											
Surgical clipping						**70, R. MCA** **68, L. MCA** **68, L. ICA**					
Endovascular coiling											
Other											
Hypertension (Y/N)	-	-	-	-	-	-	-	-	-	-	-
Smoking (Y/N)	-	-	-	-	-	**N**	-	-	-	-	-
Additional Phenotype(s)	Stomach cancer	Posterior fossa hematoma	Stomach cancer	N	N	**N**	St. Vitus' Dance	Stomach cancer	N	N	N

Affected family members are indicated by bold text. Information was not collected, or not applicable, for fields marked by a dash.
IBA: Intracranial berry aneurysm; L. ICA: Left interior carotid artery; L. MCA: Left middle communicating artery; R. MCA: Right middle communicating artery; SAH: Subarachnoid hemorrhage

3.5.2 Exome Sequencing

WES of II-6, filtered following the methods in Chapter 2, produced 900 rare heterozygous or homozygous variants. Family-specific candidate genes were not identified in Family 4.

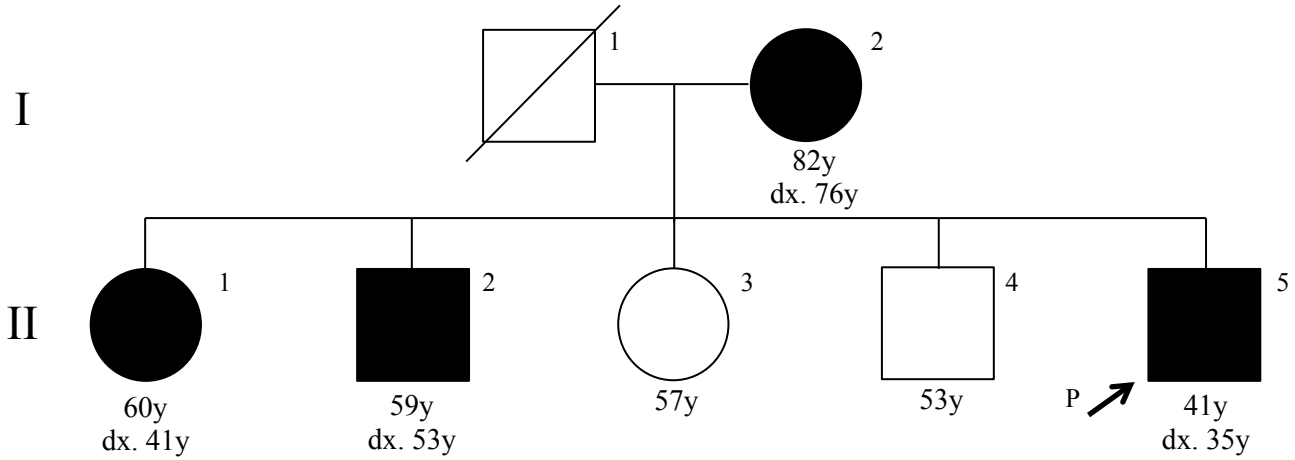
3.6 Family 5

3.6.1 Pedigree and Phenotype

Family 5 has a history of FIA spanning two generations and is of European ancestry. Family 5 has four affected and two unaffected members, all of who have been screened clinically with MRI or CT scans (Figure 3.10). The proband (II-5), was diagnosed by MRI at 35 years of age after being referred because of his family history. At the time of his diagnosis, the proband's older sister (II-1) and older brother (II-2) had already experienced SAH from a ruptured IBA. II-1 was diagnosed with two IBA, both of which have subsequently ruptured and were treated surgically (information on specific intervention was not collected). II-2 also has two IBA, one on the middle cerebral artery (MCA) and one on the posterior communicating artery (Pcom). His initial diagnosis was made after the MCA aneurysm ruptured. Both aneurysms were treated with endovascular coiling; however the Pcom aneurysm recurred and required surgical clipping when it ruptured three years after its initial coiling. Since the diagnosis of three of her children, I-2 received screening and was found to have a small IBA 3 mm in size.

All family members, excluding II-3, have hypertension, which is being controlled by medication. Affected brothers II-2 and II-5 both have a history of smoking, although II-5 has subsequently ceased. II-2 and II-5 developed recurrent seizures since the surgical treatment for their IBA.

Figure 3.10 Pedigree of Family 5



"P↗" indicates the proband; "dx." denotes the age at diagnosis

Table 3.11 Phenotype of Family 5

Family Member	**I-2**	**II-1**	**II-2**	II-3	II-4	**II-5**
Affected (Y/N)	**Y**	**Y**	**Y**	N	N	**Y**
Sex (M/F)	**F**	**F**	**M**	F	M	**M**
Age (years)	**82**	**60**	**59**	62	53	**41**
At diagnosis or screening	**76**	**41**	**53**	-	-	**35**
Number of IBA	**1**	**2**	**2**	-	-	**1**
Location and size of IBA	**3mm**	**unknown** **1.2 mm**	**R. Pcom** **R. MCA**	-	-	**R. MCA (6 mm)**
Symptomatic (Y/N)	**N**	**Y**	**N**	-	-	**N**
Headache		**Y - Severe headache at time of SAH**				
Dizziness						
Other						
SAH (Y/N)	**N**	**Y**	**Y**	-	-	**N**
Age (years), Location		**41** **56**	**53, R. MCA** **56, R. Pcom**			
Treatment (Y/N)	**N**		**Y**	-	-	**Y**
Age (years), Location						
Surgical clipping		**41** **56**	**56, R. Pcom (recurred)**			**36, R. MCA**
Endovascular coiling		**-**	**53, R. MCA** **53, R. Pcom**			
Other						
Hypertension (Y/N)	**Y - controlled by medication**	**Y - controlled by medication**	**Y - controlled by medication**	N	Y - controlled by medication	**Y - controlled by medication**
Smoking (Y/N)	**N**	**Y**	**-**	-	-	**Y**
Additional Phenotype(s)	**Epilepsy**	**-**	**-**	-	-	**Epilepsy**

Affected family members are indicated by bold text. Information was not collected, or not applicable, for fields marked by a dash.
IBA: Intracranial berry aneurysm; R. MCA: Right middle communicating artery; R. Pcom: Right posterior communicating artery; SAH: Subarachnoid hemorrhage

3.6.2 Exome Sequencing

WES of the proband, II-5, filtered following the methods in Chapter 2, produced 770 rare heterozygous variants. The proband screened positive for a rare variant, c.2056C>T (MAF = 0.0 in people of European ancestry), in thrombospondin type 1 domain containing protein 1 (*THSD1*) (Table 3.12). This variant is predicted to lead to the protein change p.R686W in the canonical transcript, and p.R633W in the alternate transcript. Sanger sequencing confirmed the variant in the proband, but it was not found to segregate with disease in the remaining family members (Appendix G). Interestingly, the affected mother (I-2) was not found to carry the p.R686W mutation in blood, suggesting that the variant was transmitted on the paternal allele. The affected sister (II-1) and both unaffected siblings (II-3 and II-4) also carried p.R686W.

While lack of segregation eliminates p.R686W as the single mutation leading to Family 5's Mendelian presentation of FIA, the possibility that it could be a significant risk allele for aneurysm formation cannot be eliminated. Notably, II-5 was also found to have a rare, non-synonymous variant in polycystin 1 (*PKD1*), predicted to cause the amino acid change p.C508F (Table 3.12 Candidate variants in Family 5). Although it is unlikely that a pathogenic variant in *PKD1* would lead to late onset aneurysm formation with no clinical kidney phenotype, p.C508F was tested for segregation within Family 5. It was not found to cosegregate with FIA (Appendix H). Although loss of function variants are relatively rare in relation to the size of *PKD1*, ~97% of the missense variants in *PKD1* on ExAC have a MAF less than 0.01. To our knowledge there is no functional relationship between *PKD1* and *THSD1*.

Neither the *PKD1* variant nor the *THSD1* variant has enough genetic evidence to be considered the single genetic cause of IBA in Family 5. At time of writing exome sequencing on

DNA from I-2, II-1, and II-2 are being completed. A candidate list of genes for this family will be made once data from these exomes are available.

Table 3.12 Candidate variants in Family 5

Chr:Pos	Ref/Alt Allele	Zygosity	MAF	Gene Names	Protein Change	OMIM Disease Association	CADD Score
16:2158482	C/A	het	0	*PKD1*	p.C508F	Autosomal Dominant Polycystic Kidney Disease	26.5
13:52952049	G/A	het	0	*THSD1*	p.R686W	Intracranial Aneurysms	15.3

Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, het: heterozygous, MAF: minor allele frequency (for European ancestry), OMIM: Online Mendelian Inheritance in Man, Ref: reference.

3.7 Comparative Analysis of Families

Following the analysis strategy outlined in Chapter 2, rare, potentially pathogenic variants identified through next-generation sequencing in one affected family member from each of Families 1- 5 were overlapped. The affected individuals being compared range in age from 12 years to 68 years at diagnosis, and their aneurysms vary in size and location (Table 3.13). Genes that had rare variants in three or more families were selected for manual annotation and literature search. A total of 38 variants affecting four genes were found to overlap across any four out of five families. None of these four genes present as strong candidates. When we lowered the threshold to variants that overlapped across any three out of our five families, 116 variants overlapped in 32 genes. Of these 32 candidates, three genes, *ASTN2*, *HSPG2*, and *ITGB4* all have been annotated to have a disease association and/or a function relevant to the development or maintenance of vasculature. Thus, these were selected as the top candidates from this list of 32 genes.

Table 3.13 Phenotype of affected individuals sequenced from Families 1 - 5

Family	1	2	3	4	5
Family Member	III-5	II-1	II-2	II-6	II-5
Affected (Y/N)	Y	Y	Y	Y	Y
Sex (M/F)	M	M	M	F	M
Age (years)	61	55	12	71	41
At diagnosis or screening	53	40	12	68	35
Number of IBA	2	3	4	3	1
Location and size of IBA	unknown	L. MCA R. MCA L. Pcom	Acom (4 mm)	L. MCA (4 mm) R. MCA (3 mm) L. ICA (2.5 cm)	R. MCA (6 mm)
Symptomatic (Y/N)	N	N	N	Y	N
Headache					
Dizziness				Y	
Other					
SAH (Y/N)	N	Y	Y	Y	N
Age (years), Location		40, L. MCA 47, L. Pcom	12, Acom	68, L. ICA	
Treatment (Y/N)	Y	Y	Y	Y	Y
Age (years), Location	53, unknown			70, R. MCA	36, R. MCA
Surgical clipping		40, L. MCA	Acom (4 mm)	68, L. MCA	
Endovascular coiling		47, L. Pcom		68, L. ICA	
Other					
Hypertension (Y/N)	Y - post-treatment, controlled by medication	N	N	-	Y - controlled by medication
Smoking (Y/N)	-	-	N	N	Y
Additional Phenotype(s)	-	Long-QT Syndrome	N	N	Epilepsy

Information was not collected for fields marked by a dash.
Acom: Anterior communicating artery; IBA: Intracranial berry aneurysm; L. ICA: Left interior carotid artery; L. MCA: Left middle communicating artery; R. MCA: Right middle communicating artery; R. Pcom: Right posterior communicating artery; SAH: Subarachnoid hemorrhage

3.7.1 Candidate Genes with Rare Variants in Four Families

In four out of the five families presented in this thesis, 38 variants overlapped in four genes: *DST*, *TTN*, *DNAH1*, and *CRIPAK*. As only a limited number of genes fell into this analysis category, and all have been included in this thesis despite being relatively poor candidates functionally.

Dystonin (*DST*) contains rare variants in Families 2, 3, 4 and 5 (Table 3.14). *DST* codes for the protein, bullous pemphigoid antigen 1 (BPAG1), a component of the cytoskeleton in epithelial cells. Homozygous mutations in DST cause epidermolysis bullosa simplex, a condition characterized by skin blistering.[197]

Titin (*TTN*) contains rare variants in Families 1, 2, 4, and 5 (Table 3.15). Titin is a sarcomere protein found in cardiac, skeletal, and smooth muscle. In humans it is associated with myopathies, cardiomyopathies, and muscular dystrophies.[170,198-200] *TTN* is an extremely large gene containing 363 exons.[201] Due to its size the mutational target space of *TTN* is huge and is statistically more likely than other genes to contain a rare variant.

Dynein Axonemal Heavy Chain 1 (*DNAH1*) contains rare variants in Families 1, 2, 4, and 5 (Table 3.16). *DNAH1* is a ciliary protein that is critical for the function of sperm flagellum. Homozygous mutations in *DNAH1* lead to impaired sperm motility and male infertility.[202]

Cysteine rich PAK1 inhibitor (*CRIPAK*) contains rare variants in Families 2, 3, 4, and 5 (Table 3.17). CRIPAK is expressed in many tissue types, where it negatively regulates PAK1. Knock-down of *CRIPAK* by small interfering RNA in a breast cancer cell line led to increased cytoskeletal remodeling though the increased activity of PAK1.[203] *CRIPAK* has not been implicated in any disease at this time.

Table 3.14 Variants in *DST*

Family	Chr:Pos	Ref/Alt	MAF	Zygosity	Protein Change	CADD Score
2	6:56481627	T/C	0.00004989	het	p.R20565H	14.57
3	6:56485496	C/G	0.005227	het	p.E1112D	18.15
	6:56569113	T/C	0.002996	het	p.I248V	18.54
4	6:56485243	G/A	0.00008246	het	p.R1197C	20.8
5	6:56481130	C/T	0.001898	het	p.A2379T	11.21

Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, MAF: minor allele frequency.

Table 3.15 Variants in *TTN*

Family	Chr:Pos	Ref/Alt	MAF	Zygosity	Protein Change	CADD Score
1	2:179449204	T/C	0.0004544	het	p.I12819V	14.67
	2:179542447	C/A	0	het	p.V10154F	21.3
	2:179658136	C/A	0	het	p.E511*	37
2	2:179419672	C/T	0.00004989	het	p.R20565H	23.4
4	2:179544347	T/C	0.000008845	het	K9971E	20.2
	2:179410271	G/T	0.00000828	het	R29288S	23.2
5	2:179612315	T/C	0.0009906	het	p.M252V	11.87

Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, MAF: minor allele frequency.

Table 3.16 Variants in *DNAH1*

Family	Chr:Pos	Ref/Alt	MAF	Zygosity	Protein Change	CADD Score
1	3:52392645	C/A	0	het	p.F1386L	18.99
2	3:52397098	A/T	0.002393	het	p.S1728C	25.6
	3:52379582	C/T	0.0002741	het	p.R506W	33
	3:52396410	C/T	0.00675	het	p.R1663C	34
4	3:52398865	G/T	0.00002499	het	p.R1783L	26.7
	3:52356727	C/T	0.0002329	het	p.P90L	32
5	3:52378570	A/G	0.0015	het	p.K451E	20.4

Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, het: heterozygous;
MAF: minor allele frequency.

Table 3.17 Variants in *CRIPAK*

Family	Chr:Pos	Ref/Alt	MAF	Zygosity	Protein Change	CADD Score
2	4:1388867	A/C	0.007825	het	p.I190L	0.063
3	4:1388786	G/C	0.00003212	het	p.R146P	0.001
	4:1388976	C/T	0.001073	het	p.A226V	0.611
4	4:1388886	A/G	-	het	E196G	11.06
	4:1388769	G/A	0.0001821	het	R157H	16.76
5	4:1388852	T/C	0.00004991	het	W185R	0.437

Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, het: heterozygous; MAF: minor allele frequency.

3.7.2 Candidate Genes with Rare Variants in Three Families

In three out of the five families 116 variants overlapped in 32 genes. These candidates were further filtered down by gene function or previous association to FIA to three top candidate genes, *ASTN2*, *HSPG2*, and *ITGB4*.

Astrotactin 2 (*ASTN2*) contains rare variants in Families 1, 2, and 4 (Table 3.18). Polymorphisms in *ASTN2* have been associated with risk for a number of conditions, including attention deficit hyperactivity disorder (ADHD) and Alzheimer's disease.[204,205] Functionally, ASTN2 is thought have a role in glial-guided neuronal migration by forming a complex with and regulating ASTN1, a known ligand in this process. A heterozygous variant in *ASTN2* that causes the protein change p.T1083I (MAF = 0.024 in Japanese populations) was previously found to segregate fully in one consanguineous Japanese family, and partially with three additional Japanese families affected by FIA.[103] Family 2 carries this same mutation. However, this variant appears to be a polymorphism in the Japanese population, and may be present in Family 2 due to ancestral founder effects. Genome data on First Nations individuals at a population level would be needed to calculate the true MAF of p.T1083I in Family 2, in which a *CCM2* variant was also identified.

Integrin beta 4 (*ITGB4*) contains rare variants in Families 1, 4, and 5 (Table 3.19). Integrins are transmembrane proteins that maintain cell-cell, or cell-cytoskeleton, adhesion, as well as propagate signals received from inside or outside the cell. Beta 4 (β4) is known to adhere the basement membrane to the cytoskeleton in endothelial cells of adult vasculature. During angiogenesis β4 is negatively regulated. However, an overexpression of β4 is able to initiate migration of nearby β4-negative endothelial cells during angiogenesis.[206,207] Knock-out of ITGB4 in mice leads to death shortly after birth due to epithelial defects.[208] Induction of cerebral

aneurysm by surgically increasing hemodynamic stress in rats led to the upregulation of 15 genes, including *Itgb4*, over a three month period of time.[209]

Heparan sulfate proteoglycan 2 *(HSPG2)* contains rare variants in Families 1, 2, and 4 (Table 3.20). *HSPG2* has been implicated in FIA through familial mapping studies and a previous candidate gene association study. Linkage analysis of a large Dutch pedigree and large European pedigree both mapped an FIA susceptibility locus to the chromosomal region 1p36.13-p34.3. Analysis of 44 candidate genes in 328 Dutch individuals with intracranial aneurysms associated SNPs found in *HSPG2*. This was replicated in another Dutch cohort of 310 individuals.[210] *HPSG2* encodes the protein perlecan, which is an extracellular matrix component in many mesenchymal tissue types, including vascular tissue.[211,212]

Hspg2 whole gene knock-out mice are usually embryonic lethal due to cartilage and/or cranial abnormalities (e.g. exencephaly) or die shortly after birth from respiratory failure.[213] Gustafsson *et al*. found that about 40% of embryonic mice with *Hspg2* knock-out had dilated microvasculature that had hemorrhaged in the brain and skin by embryonic day 12.5.[214] Perlecan has both heperan sulfate and glycosaminoglycan side chains. *In vitro* the glycosaminoglycan side chain inhibited VSMC binding to the perlecan core, while the heparan sulfate side chains were able to bind FGF1 and FGF2 and propagate FGF2 signaling. In endothelial cells, adhesion to perlecan was not affected by the presence of glycosaminoglycan side chain, and the heparan sulfate side chains bound and promoted signalling of both FGF1 and FGF2.[215] Mice with deficiency of the heparin sulfate side chain (homozygous deletion of exon 3 in *Hspg2*) survived through embryonic development and appeared healthy. However, upon skin puncture, 6-week-old mice showed delayed wound healing. The basement membrane of the vasculature was not affected, but FGF-2 mediated angiogenesis was impaired.[216] Zebrafish with perlecan knock-

down via translation-blocking morpholinos displayed decreased extension of vessel sprouts from the dorsal aorta into the tail and trunk. In humans, homozygous or compound heterozygous mutations in *HSPG2* cause Schwartz-Jampel syndrome, an autosomal recessive condition characterized by osteochondrodysplasia, myotonia, and dysmorphic facial features.[217]

HSPG2 and *ITGB4* are interesting candidate genes based on gene function and animal models, while *ASTN2* has not yet been associated with vascular development or function. Based on both gene function and previous association to FIA, HSPG2 has the strongest evidence for causality supporting it – however further work is required before it can my labeled as causative. The specific mutations seen in our family will need to be tested with an animal model (such as CRISPR/Cas9 knock-in in a zebrafish model) to determine their *in vivo* effect.

Table 3.18 Variants in *ASTN2*

Family	Chr:Pos	Ref/Alt	MAF	Zygosity	Protein Change	CADD Score
1	9:119495803	C/A	0.0	het	Exon 12 +1	26.8
2	9:119249734	G/A	0.002238	het	p.T1083I	24.8
4	9:120177201	C/A	0.0	het	p.A6S	23

Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, het: heterozygous, MAF: minor allele frequency.

Table 3.19 Variants in *ITGB4*

Family	Chr:Pos	Ref/Alt	MAF	Zygosity	Protein Change	CADD Score
1	17:73736078	G/A	0.00006211	het	p.R791H	33
4	17:73733712	G/T	0.006933	het	p.C736F	29.7
5	17:73728022	G/A	0.001166	het	p.G365S	3.574

Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, het: heterozygous, MAF: minor allele frequency.

Table 3.20 Variants in *HSPG2*

Family	Chr:Pos	Ref/Alt	MAF	Zygosity	Protein Change	CADD Score
1	1:22178643	C/T	0.0005131	het	p.G2270R	33
2	1:22222455	G/T	0.001927	het	p.D34E	8.884
4	1:22186149	T/C	0.000008351	het	p.S1735G	21.2

Alt: Alternate, CADD: combined annotation dependent depletion; Chr:Pos: chromosome:position, het: heterozygous, MAF: minor allele frequency.

Chapter 4: Discussion

4.1 Summary of Findings

The thesis describes our initial findings from an ongoing research project. In Chapter 3: we presented the results of our genetic investigations into the underlying cause of FIA in five families, using various combinations of next-generation sequencing, whole-genome SNP microarray, and Sanger sequencing techniques. We presented data on both family-specific candidate genes and candidate genes selected through overlap of sequence variants between families. Our findings are summarized in Table 4.1.

At this time, our study was not able to find sufficient evidence to associate a specific gene as the single cause of FIA in any one family or in a subset of families. The lack of conclusive associations within our cohort, as well as the lack of conclusive phenotypic associations from previous familial exome sequencing studies, coupled with the most recent finding that the first gene associated with FIA, *THSD1*, is estimated to account for approximately 1% of all intracranial aneurysm cases, indicates that familial intracranial aneurysms is a genetically heterogeneous condition.

Comparison with the genetic architecture of other known aneurysm phenotypes can be can provide parallel information. Pathogenic mutations in *ACTA2* account for 15% of cases of familial thoracic and aortic aneurysms/dissections (FTAAD).[218] Theoretically, if coding mutations in a specific gene accounted for a similar proportion of FIA cases, then studies like the FIA Study (with over 300 families), should have identified such a gene. Thus, it is likely that mutations in many different genes can lead to FIA, and that each gene accounts for only a small percentage of cases.

Notably, the rare variants presented in our study have varying CADD scores. CADD is an algorithm for SNVs and indels that combines multiple annotation tools to compare simulated variants to "fixed" or real variants at every base in the genome. Some of the algorithms combined in CADD include genomic annotation from the UCSC Genome Browser, conservation scores from GERP, and predicted effect on protein function by SIFT and Polyphen. The CADD scores reported in this thesis are scaled in comparison to all other possible SNVs in the genome. Variants with a CADD score higher than 10 are within the top 10% of variants unlikely to be seen in the human genome, whereas variants with a CADD score greater than 20 are in the top 1% of variants unlikely to be seen in humans. Most known pathogenic variants annotated to disease have a scaled CADD score between 30 and 40.[219] For this reason, candidate variants in our study with a CADD score greater than 20 have been ranked more highly as potentially pathogenic, compared to variants with lower CADD scores. Moving forward, candidate genes that have rare variants with scaled CADD scores greater than 20 in multiple families will be prioritized for animal studies over candidate genes with variants that have lower CADD scores.

Table 4.1 Summary of Candidate Genes in Families 1 - 5

Family	1	2	3	4	5
Family Specfic Candidate Genes	*NOTCH4, CFB, PIK3C2G, HLA-DRB1, DDR1*	*CCM2*	*CA5A, C2orf16, VPS18, AHCTF1, FAM198B, RPF2, ARFGEF1*	-	*THSD1, PKD1*
Overlap Analysis Candidate Genes	*TTN, DNAH1, ASTN2, ITGB4, HSPG2*	*TTN, DNAH1, CRIPAK, DST, ASTN2, HSPG2*	*DST, CRIPAK*	*TTN, DNAH1, CRIPAK, DST, ASTN2, ITGB4, HSPG2*	*TTN, DNAH1, CRIPAK, DST, ITGB4*

At the cellular level, we hypothesize that intracranial berry aneurysms form through "two-hit kinetics." This process requires two pathogenic mutations to arise in the same gene (or pathway of genes) within the same cell in order for disease to manifest. My idea is based on the two-hit hypothesis of cancer initiation first put forward by Knudson in 1971.[220] Since that time

numerous other diseases have been found to have a two hit-mechanism, including the renal cyst and intracranial aneurysm formation in patients with ADPKD.[221,222] At a population level, individuals would acquire these "hits" throughout their lifetime; phenotypically, their IBA would appear sporadic, developing only from cells with two "hits." Since the majority of FIA pedigrees show a dominant inheritance of disease, I suggest that affected FIA family members inherit the first "hit" as a Mendelian allele and acquire the second "hit" as a somatic mutation during their lifetime. As is seen in familial cancer syndromes, such as familial retinoblastoma,[223,224] the predisposing inherited "hit" significantly increases the chances of obtaining two "hits," leading to high penetrance in these families, acting recessively at the cellular level while appearing dominant at the phenotypic level. This model also explains why familial cases would present at a younger age and with more severe disease compared to patients with sporadic IBA (as discussed in Section 1.3).

4.2 Strengths

Our inclusion of cases with only non-syndromic FIA or IBA has enriched our cohort for patients more likely to have a strong genetic contribution to their aneurysm formation. The exome sequencing, and exome sequencing plus microarray, strategies outlined in Chapter 2: are well-validated methods for identifying genetic associations with disease, and have been used by large consortia such as FORGE Canada.[171] We identified strong family-specific candidate variants as well as strong candidate genes from our overlap analysis. Though intuitively appealing, it is difficult to disentangle our own assessment of candidacy from the intrinsic "narrative potential of the human genome," whereby a plausible argument can be made for the involvement of almost any gene in any process, particularly one as complex as vasculogenesis.

Nevertheless, the single successful study to date used an approach similar to ours in a much larger group of patients, demonstrating proof-of-principle to our overall approach.

4.3 Limitations

A significant limitation to this research is the number and size of families enrolled in our cohort. Linkage mapping in Family 1 produced numerous candidate chromosomal regions, which could have been reduced in number if clinical information and a DNA sample were available on an affected member distantly related to the nuclear family. Our linkage analysis also depended on the assumption that family members who were not diagnosed by brain imaging are truly unaffected, and that they will not develop an intracranial aneurysm later in life. The two youngest unaffected individuals were screened at 48 years and 50 years old, both earlier than the youngest diagnosis of FIA in their family, raising the possibility that the assumptions on which our mapping data are based may be inaccurate.

Using Sanger sequencing to test for variant segregation within a family also relies on family members being diagnosed correctly as affected or unaffected. This problem has been noted in studies of familial forms of other common, complex and late-onset diseases such as Parkinson's disease. When genes or variants have an age-dependent penetrance, they may be discarded from the analysis prematurely due to phenotypic misclassification.[225]

Our research also had some intrinsic technical limitations, because exome sequencing is unable to detect large CNVs, epigenetic variation, or non-coding variation such as deep intronic variants that might be important in disease.[226] Notably, the most up-to-date build of the human genome (GRCh38.p7) recognizes 20,441 coding genes and 22,219 noncoding genes, suggesting

that the "mutational target space" of RNA-only genes may exceed that of protein-coding genes, at least with respect to the number of targets.[227,228]

4.4 Future Research Directions

4.4.1 Cohort Recruitment and Sequencing

Based on my finding that FIA is likely extremely heterogeneous, further enrollment of additional FIA families will be critical to identify the gene(s) that are pathogenic for FIA and IBA when mutated with a disease-causing variant. Next-generation sequencing of the proband from each new family to compare with the sequencing data of probands from the other families in the cohort should continue. This strategy allows for the most cost-effective accumulation of genetic information on FIA; coupled with Sanger sequencing, it would still allow for variant segregation to be tested within families. In families containing more distant affected relatives separated by multiple meioses, exome sequencing with rare variant comparisons between distantly-related affected relatives can be a powerful method to filter the number of shared candidate variants.

4.4.2 Animal Models

In addition to preliminary functional work in human and yeast cell lines, evidence from one (or more) animal models is generally considered essential to assess the impact of candidate variants *in vivo*. Though worms (*C. elegans*) and flies (*D. melanogaster*) offer powerful methods of screening for genetic function, the fact that their circulatory systems are not easily comparable to that of humans renders them less valuable in the context of this phenotype. However, the circulatory systems of fish and rodents share significant similarities to human circulatory

systems, such that these two model organisms are used frequently for functional validation of genes implicated in vascular biology.

4.4.2.1 Zebrafish

Zebrafish are often used to study cardiovascular and cerebrovascular conditions. Zebrafish embryos are transparent during development, so the embryonic vasculature can be viewed both easily and directly.[229] Zebrafish models of aortic aneurysm genes have clearly displayed hemorrhage of malformed blood vessels.[230,231] As well, 82% of human disease genes have a zebrafish orthologue, indicating that we are likely to be able to interrogate our candidate genes using this model.[232] Using morpholino oligonucleotides (MO) specific to the transcript of the fish orthologue, we could knock-down the function of our candidate gene through complementary binding of the MO to the transcript. This knock-down experiment allows us to model the effects of a non-functional gene. To validate whether the transcripts containing the variants found in our FIA families are sufficient to carry out the function, we could proceed to rescue experiments. In such an experiment, co-injection of the wild-type transcript should restore a normal phenotype to the MO-bearing zebrafish, whereas transcripts containing a damaging variant would not rescue the fish phenotype.[223]

4.4.2.2 Mice

Intracranial aneurysms do not develop in mice naturally, and must be induced surgically or by a combination treatment of elastase and induced hypertension.[209,233] Despite this, knock-out of many of the candidate genes discussed in Chapter 3 does disrupt vascular function in mice. It is possible that because mice do not develop intracranial aneurysms without intervention, even

knock-out of true aneurysm-causing genes in mice might not recapitulate the human phenotype exactly (as was seen with *THSD1,* where the mice developed spontaneous SAH but did not have detectable aneurysms). Additionally, many candidate genes cause embryonic lethality in mice when knocked-out constitutively, so mouse validation may require studies of embryonic vasculature, or of an endothelial-specific knock-out model. As intracranial aneurysms are usually a late-onset condition, an inducible knock-out model that removed the candidate gene after development might allow visualization of this vascular phenotype without causing embryonic lethality. This could be accomplished with a tamoxifen-dependent Cre-loxp system.[225]

4.4.3 Functional Experiments

Candidate genes that meet our threshold for sufficient genetic evidence for association with intracranial aneurysms may have little, or no, supporting functional evidence at the time they are found (as was the case with *THSD1*). In such a situation, it would be practical to proceed with screening of a human vascular cell line (such as a human umbilical venous endothelial cells, HUVECs) for expression of the candidate transcript (and possibly for absence of the mutant transcript) prior to animal modeling. Co-immunoprecipitation assays could then be used to identify binding partners of the candidate protein, if none were already known. To test the effect of the variants seen in the families in our cohort, a relatively simple assay, such as the yeast-two hybrid assay could be used to test protein-protein binding. A benefit of using this assay is that cDNA of human wildtype and mutant transcripts and of the known binding partners can be cloned easily into yeast, allowing protein interactions to be modeled. These experiments would help characterize the role of the candidate protein in vasculature.

4.5 Significance of the Research

Knowing the frequency and relative penetrance of variants causing or contributing to the formation of IBA in families is important for understanding the pathophysiology of this disease. While the recent discovery of *THSD1* provides some insight into these questions, more gene discoveries will be necessary to describe the genetic architecture of FIA. Solving the genetic cause of aneurysms in rare families like these will identify key proteins that are necessary for healthy brain arteries in the general population. Such knowledge will also improve patient screening methods, and aid in the development of novel therapeutics.

4.5.1 Screening Families

A successful outcome of this project would be identifying a genetic cause for FIA. This information could benefit families both clinically and psychologically. As screening of families is not recommended until at least two members are confirmed to have intracranial aneurysms and most IBA are asymptomatic, diagnosis of FIA often begins with two or more family members experiencing SAH. This traumatic experience is often accompanied by worry and stress that remaining family members have inherited the predisposing risk factor (pers. comm. from study families). As has been seen when ending the "diagnostic odyssey" of patients with rare genetic diseases, diagnosing families genetically as well as clinically may lessen the stress of having a familial disease.[234]

Many affected participants in this study have one or more children who are at increased risk for FIA. As mentioned in the Introduction (Section 1.3), relatives of people affected by SAH are more likely to develop an FIA, more likely to rupture, and more likely to have a poor outcome, when compared to sporadic cases of IBA. Therefore, at-risk relatives from families

who carry pathogenic variants in FIA-associated genes could receive genetic testing to determine whether or not they are predisposed genetically to intracranial aneurysm formation. For families in whom the onset of FIA is in adulthood, genetic testing could be done at the age of majority, at the discretion of the family member undergoing testing. Family members positive for the pathogenic rare variant could then seek early screening, and family members negative for the pathogenic rare variant would no longer need to worry about having a genetic predisposition for IBA. Family members who were already diagnosed would have a confirmed genetic diagnosis, and would be able to discuss with their physician the option of early intervention to have their aneurysms treated with clipping or endovascular coiling at a smaller size than would otherwise be recommended, potentially reducing morbidity and mortality.

4.5.2 Screening Unrelated Families and Sporadic Cases

With a specific gene in hand, other at-risk individuals and families could be tested to discover if their aneurysms were caused by variants in the same gene. Eligible groups would include other families with FIA, people who have more than one IBA, people who have only one other affected family member, and children and adolescents who have early onset-IBA. All of these situations would translate findings from this project usefully outside of the original discovery cohort.

4.5.3 Treatment

A long-term outcome of this project would be the development of a new treatment for FIA/IBA. There are no pharmaceuticals specifically being used to prevent IBA. Having a proven causative gene would unlock new ways to investigate the pathogenesis of IBA, enabling

development of new therapies by pharmaceutical companies. Notably, many pharmaceutical targets have been chosen via rare versions of common disease that highlight key components of disease pathogenesis.[235-237] Examples include statins that target LDLR (for heart disease and stroke),[238] and sulfonylureas that target KCNJ11/ABCC8 (for diabetes).[239] In these cases, the drugs are being prescribed or developed for use in the general population with common disease, not only for use in the subpopulation with rare mutations. Similarly, we expect that therapeutics developed from our future findings would not only aim to treat families with FIA, but also the 3% of the general population diagnosed with IBA.

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Appendices

Appendix A Phenotype Collection Form

FIA Phenotype Collection Form

Your Name (Surname, Given Name): _____
Date of Birth: _____
Current Age:_____
Date of Completion of Questionnaire:_____

1. Has a doctor ever told you that you have a brain aneurysm (or more than one)?

   ☐Yes, one ☐Yes, more than one – I have had _____ brain aneurysms ☐No

2. If you answered "No" how many times have you had a screening test for intracranial aneurysms? I have been screened_____ times.

3. What imaging technology was used to conduct each screening test?

Type of test (CT, MRI, MRA)	Date done (Da/Mo/Ye)	Age (years)	Name of Hospital	Result

4. How old were you at the time of diagnosis of your FIRST aneurysm or diagnosis?_____

5. What was the name of the doctor (e.g. neurologist, neurosurgeon) who made the diagnosis?

6. Where are the medical records located?_____

1

7. What was the result of each screening test (number and size of aneurysms)?

8. Did you experience any of the following symptoms BEFORE your diagnosis?
   - Headaches ☐Yes ☐No
   - Dizziness ☐Yes ☐No
   - Other_____ ☐Yes ☐No

9. What treatment(s) did you receive for your aneurysm(s)?
   - Surgical clipping ☐ Yes ☐ No
   - Endovascular coiling ☐ Yes ☐ No
   - Other: _____

10. Were there any complications or neurological problems from your treatment(s)?

11. Did you experience any of these symptoms AFTER your diagnosis or treatment?
   - Headaches ☐Yes ☐No
   - Dizziness ☐Yes ☐No
   - Other_____ ☐Yes ☐No

12. Do you, or have you, ever smoked? ☐Yes ☐No

13. Do you have hypertension? ☐ Yes ☐ No Medication? _____
    ☐ Before diagnosis ☐ After diagnosis ☐ Both

14. Are you taking any medications? ☐Yes ☐No

15. Have you ever had a stroke or a bleed from any of your aneurysms? ☐Yes ☐No

If "Yes," please give details below:

16. Do you have aneurysms elsewhere in your body (e.g. arms, legs, abdomen, heart, aorta)?
☐Yes ☐No

17. Has a doctor ever told you that you have Polycystic Kidney Disease? ☐Yes ☐No

If "Yes," please give details below:

18. Has a doctor ever told you that you have a connective tissue disorder? ☐Yes ☐No

If "Yes," please give details below:

19. Do any of your relatives have brain aneurysms now or in the past? ☐Yes ☐No

20. If "Yes," please take pedigree on opposite side of the page.
(Include consanguinity, ancestry, age of diagnosis, cause of death, number of aneurysms, first and last names etc.)

Appendix B Primer sequences used in qPCR validation of the CNV disrupting *DMBT1*.

Primer	Sequence	T_m (°C)
5' Region Forward	CTGGTTATCTGGTGGCTCTGG	57.4
5' Region Reverse	CAAACCAATGTTGCGGCACT	57.4
Deleted Region Forward	AGCTGGCAGTAGTGGACAAGA	58.2
Deleted Region Reverse	GCCTTCAGTCCACCCTATGTC	57.0
H6PD Forward	GGTGGATAGATGCAGAAACAAGGA	56.8
H6PD Reverse	TATGAATGTGTAACTGCTGGAGGTCTT	57.7

T_m: Predicted melting temperature.

Appendix C Primer sequences used in PCR validation of candidate variants in Family 1.

Primer	Sequence	T_m (°C)
RYR1 Forward	GTAGTGTCCATGTGGGCAGATTC	57.6
RYR1 Reverse	CCAGCCCTAACCCTTGATATTGATA	56.1
BSN Forward	CCCACAGCCGGGTACGAC	61.0
BSN Reverse	ATCACCCCTGGCTGCCATTA	59.0
VAT1 Forward	CTACCCCCTCCCATATTATGCC	56.7
VAT1 Reverse	CCCCTGCTTATGGGTGTCTTG	57.9
*SLC7A9*_1 Forward	GTGCTGACACCTGCCTTACC	58.4
*SLC7A9*_1 Reverse	GAGGGCGTCCATCTTCCG	58.2
*SLC7A9*_2 Forward	CAGTGGAAGGGCGTTTGGT	58.2
*SLC7A9*_2 Reverse	CTCCAGGGCTTTGCTGAAAAC	56.8
HSPB6 Forward	GGCAATGGAAGTGGTCGAGT	57.6
HSPB6 Reverse	AGGAGCAGGATGGAGATCCC	58.5
ARHGAP33 Forward	TCATTGCCCTGCCAGAACC	58.1
ARHGAP33 Reverse	GATCGTGGTGGGCAGTAGC	58.3
FRY Forward	GCCCCCATTACAGGGACTTT	57.4
FRY Reverse	TGCTCAAGTTGAGAGCACCTTAG	56.8

T_m: Predicted melting temperature.

Appendix D Primer sequences used in PCR validation of candidate variants in Family 2.

Primer	Sequence	T_m (°C)
CCM2 Forward	CTGGGTGCTGCCTGCTTTTTAAAC	59.3
CCM2 Reverse	CTATACTCCCCACCTGGGTGGAA	59.9

T_m: Predicted melting temperature.

Appendix E Primer sequences used in PCR validation of candidate *THSD1* variant

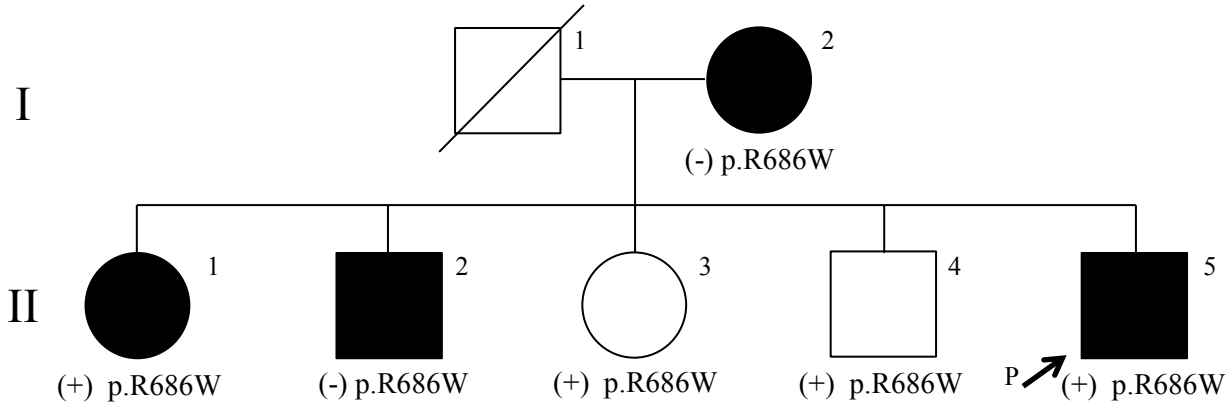
Primer	Sequence	T_m (°C)
THSD1 Forward	CCCAAGGTCTGGTTTCCTCAA	63.5
THSD1 Reverse	GAGTTTCCATGAAGCCAGGCA	64.7

T_m: Predicted melting temperature.

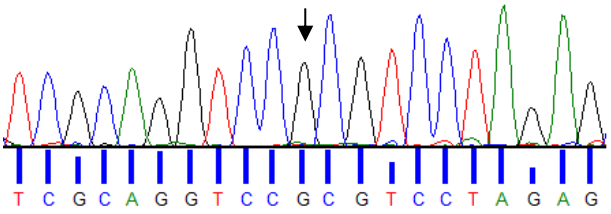
Appendix F Primer sequences used in PCR validation of candidate *PKD1* variant

Primer	Sequence	T_m (°C)
PKD1 Forward	CAGGTACACATGCTCCACTGTT	
PKD1 Reverse	GCTGCCAACCACACCTATG	

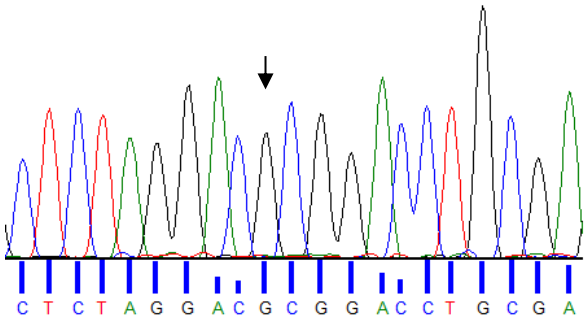
Appendix G Sanger sequencing traces of *THSD1* variant in Family 5



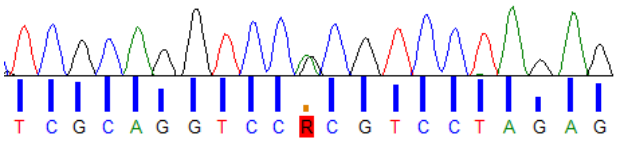
I-2: *THDS1* (+)



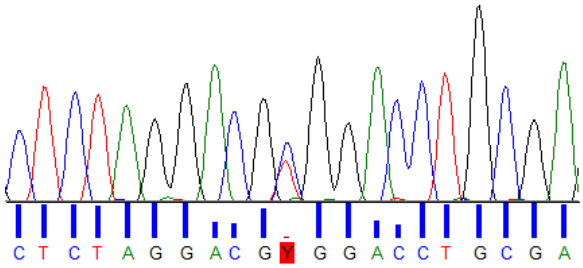
I-2: *THSD1* (-)



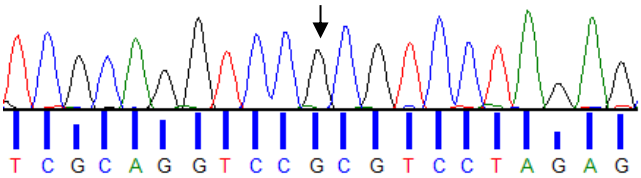
II-1: *THSD1* (+)



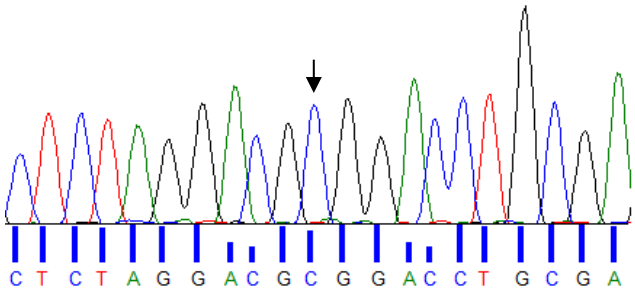
II-1: *THSD1* (-)



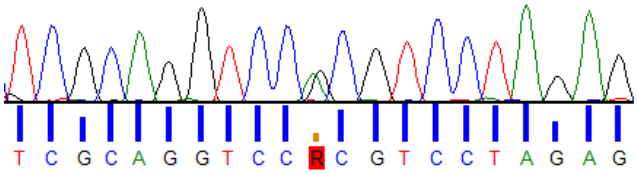
II-2: *THSD1* (+)



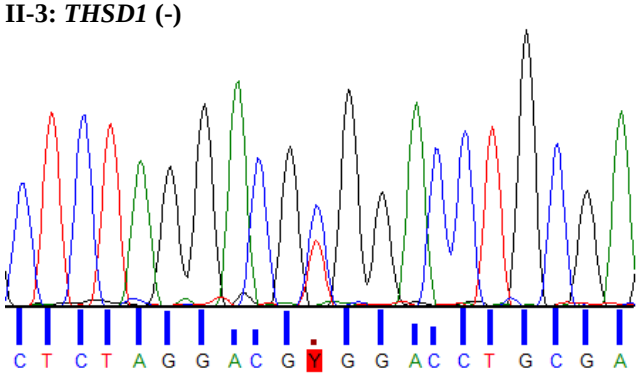
II-2: *THSD1* (-)



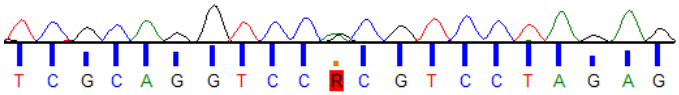
II-3: *THSD1* (+)



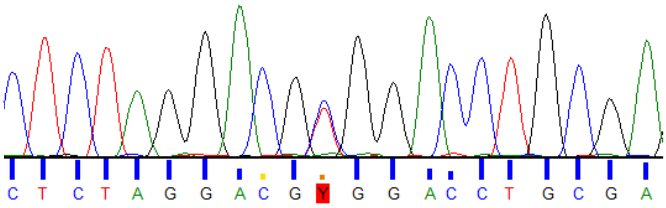
II-3: *THSD1* (-)



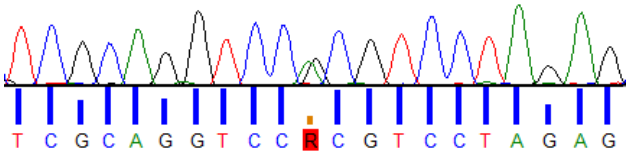
II-4: *THSD1* (+)



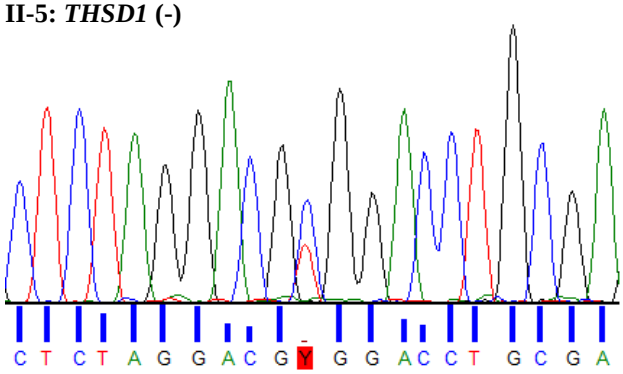
II-4: *THSD1* (-)



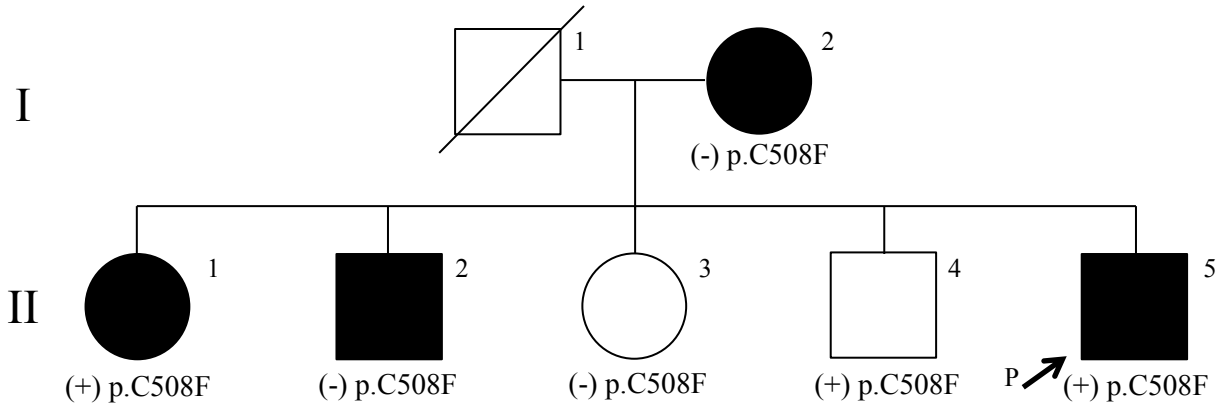
II-5: *THSD1* (+)



II-5: *THSD1* (-)



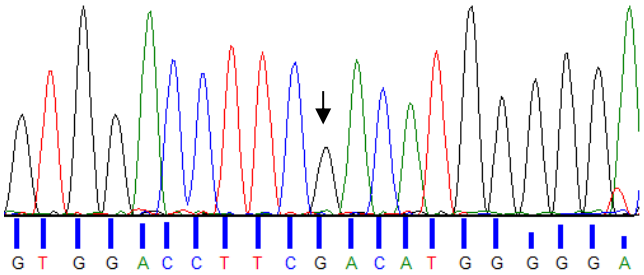
Appendix H Sanger sequencing traces of *PKD1* variant in Family 5



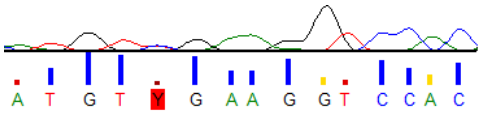
I-2: *PKD1* (+)

(Not Available)

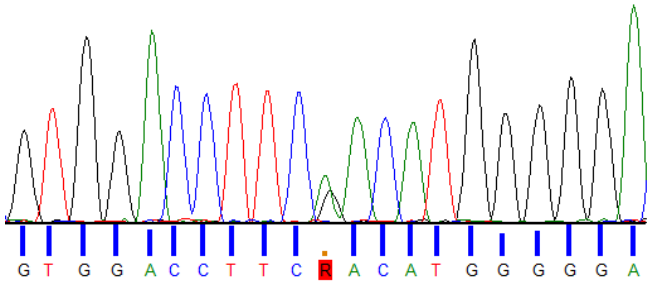
I-2: *PKD1* (-)



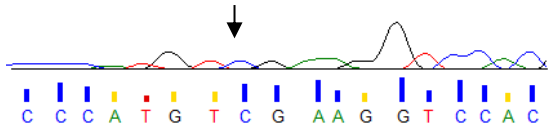
II-1: *PKD1* (+)



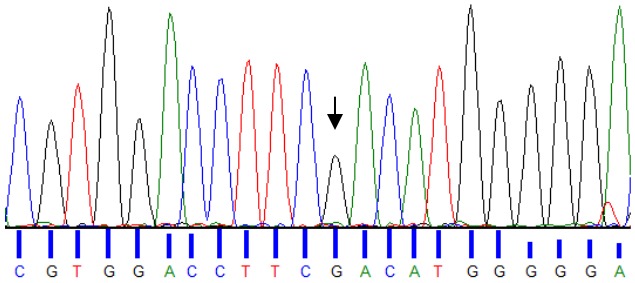
II-1: *PKD1* (-)



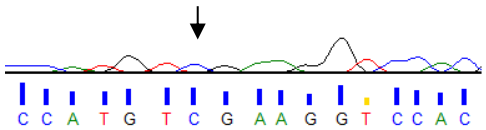
II-2: *PKD1* (+)



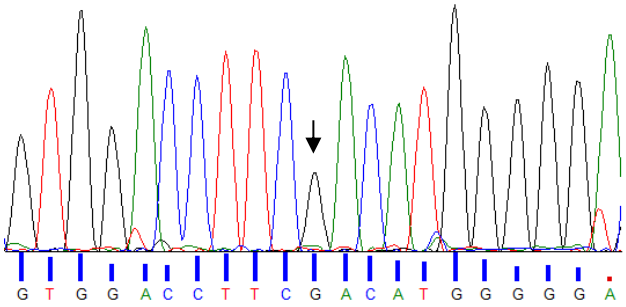
II-2: *PKD1* (-)



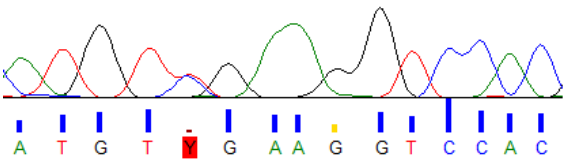
II-3: *PKD1* (+)



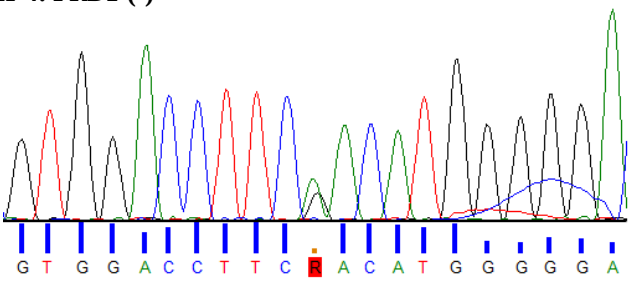
II-3: *PKD1* (-)



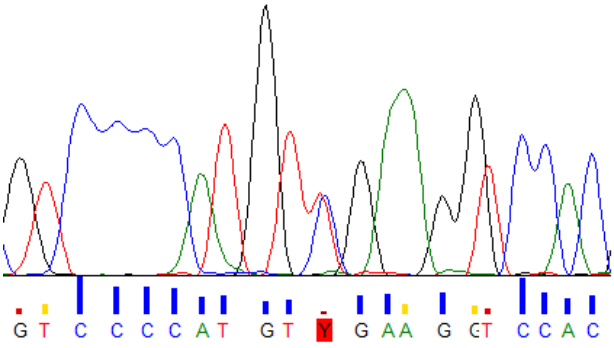
II-4: *PKD1* (+)



II-4: *PKD1* (-)



II-5: *PKD1* (+)



II-5: *PKD1* (-)