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Genetic and clinical heterogeneity of Moyamoya disease

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Thesis

GENETIC AND CLINICAL HETEROGENEITY OF MOYAMOYA DISEASE

by

LUCA FARRUGIA
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Approved by

First Reader
Carl Franzblau, Ph.D.
Professor of Biochemistry

Second Reader
James Meschia, M.D.
Chair of the Department of Neurology
Mayo Clinic Jacksonville
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GENETIC AND CLINICAL HETEROGENEITY OF MOYAMOYA DISEASE

LUCA FARRUGIA

ABSTRACT

Background: Moyamoya Disease (MMD) is a chronic, occlusive cerebrovascular disease characterized by progressive stenosis of intracranial arteries, specifically the internal carotid arteries, and the compensatory formation of an abnormal vascular network at the base of the brain. The exact cause of MMD is still not well understood. Many factors including genetic, environmental, and immunologic have been associated with the disease. \textit{RNF213} is considered the main susceptibility gene, especially in Eastern Asian patients. The founder mutation, p.R4810K, has been associated strongly with MMD, especially in Japan and Korea but has been shown to have low penetrance and has never been described in non-Eastern Asian MMD cases. \textit{RNF213} encodes for an E3 ubiquitin-protein ligase with ATPase activity. It has described to regulate angiogenesis giving rise to the possibility that variants in \textit{RNF213} may play a role in cerebrovascular diseases other than MMD.

Objectives: The aims of this study were to determine if variation in the \textit{RNF213} gene contributes to MMD in a cohort of 15 unrelated patients with MMD of predominantly European descent, to investigate other potential genes implicated in MMD in these 15 patients, and to investigate if \textit{RNF213} also influences more common vascular phenotypes.
**Methods:** Patient history, detailed family history and a blood sample were collected from 15 patients with well-characterized MMD. DNA was extracted from a peripheral venous blood sample, assessed for quality, and DNA concentration quantified by PicoGreen®. The extracted DNA was sent for whole exome sequencing. Genome_GPS_2.0 was used to carry out secondary analysis of sequencing data and all data were stored in Oracle TRC. The files were aligned using Novoalign, variants analyzed using GATK, visualized using IGV and annotated using BioR-Web. Variants of interest were determined using Ingenuity® Variant Analysis™. To determine if *RNF213* also influences more common vascular phenotypes, previously collected and whole exome sequenced samples from the Mayo Clinic Florida Familial Cerebrovascular Diseases Registry were analyzed. Variants in *RNF213* were determined using the same approach as for MMD.

**Results:** Likely pathogenic variants in *RNF213* were found in 13% (2/15) of patients. The p.R4810K variant has been previously published as the founder mutation for MMD in Eastern Asian populations. The affected patient was also of Eastern Asian origin. The other variant, p.R4019C, was found in a European descent case and has been described as a candidate pathogenic variant. Eight variants in five other genes previously associated with MMD were found. Of these, one previously reported variant, p.D455H in *RPTN*, was found in two patients. The other seven have not previously been described in MMD. In the analysis of the potential role for *RNF213* in other cerebrovascular diseases, 54%
(22/41) of African American patients had a non-synonymous, exonic variant in RNF213 with a MAF of less than 3%. A novel RNF213 variant was also found in a Caucasian patient who had a subarachnoid hemorrhage.

**Conclusion:** The p.R4810K variant in RNF213 was confirmed to only be associated with MMD in Eastern Asians and not found in other ethnicities. However, a variant in RNF213 was found in a Caucasian patient suggesting RNF213 may indeed be disease causing in patients of diverse origin. RNF213 may also be implicated in other cerebrovascular diseases suggesting a common pathogenesis while other genes also appear to be involved in the pathogenesis of MMD.
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LIST OF ABBREVIATIONS

AICS……………………………………. Acute Ischemic Cerebrovascular Syndrome
CNV……………………………………… Copy Number Variant
EDAS........................................ Encephalo-Duro-Arterio-Synangiosis
EMS............................................... Encephalo-Myo-Synangiosis
GATK........................................... Genome Analysis Toolkit
GWAS......................................... Genome Wide Association Study
HGMD........................................... Human Gene Mutation Database
HLA............................................. Human Leukocyte Antigen
ICA............................................. Internal Carotid Artery
ICH............................................ Intracerebral Hemorrhage
LD............................................... Linkage Disequilibrium
MCA........................................... Middle Cerebral Artery
MMD........................................... Moyamoya Disease
MMS........................................... Moyamoya Syndrome
MMSE........................................... Mini Mental State Exam
ncRNA......................................... non-coding RNA
NF1............................................ Neurofibromin 1
NFE............................................. Non-Finish European
NIHSS.......................................... NIH Stroke Scale
OMIM.......................................... Online Mendelian Inheritance in Man
RNF213......................................... Ring Finger Protein 213
SAH................................................................. Subarachnoid Hemorrhage
SNP.............................................................. Single Nucleotide Polymorphism
STA. ............................................................. Superficial Temporal Artery
TIA................................................................. Transient Ischemic Attack
TIEG................................. Transforming Growth Factor-Beta-Inducible Early Growth
VCF............................................................... Variant Call Format
WES............................................................. Whole Exome Sequencing
INTRODUCTION

Moyamoya disease (MMD) is a chronic, occlusive cerebrovascular disease characterized by progressive stenosis of intracranial arteries, specifically the internal carotid artery (ICA), and the compensatory formation of an abnormal vascular network at the base of the brain, typically sprouting off the circle of Willis (Suzuki 1969). Occlusion of the ICA generally occurs bilaterally, but unilateral involvement does not rule out a diagnosis of MMD (Kelly 2006). Although the formation of the abnormal vascular network has been considered to be a mechanism to compensate for insufficient blood flow through the ICA it is not clear that that is the case, as a study has shown that neovascularization can precede significant occlusion (Kim 2014). Diagnosis of MMD is commonly made first from the appearance of diminished flow in the ICA and additional collateral flow on MRI vascular studies. The diagnosis is confirmed on conventional catheter-based cerebral angiography, which provides better characterization of basal collaterals or “Moyamoya vessels”. Moyamoya syndrome (MMS) is the presence of MMD characteristics in conjunction with a different, more well-defined pathological condition.

Disease History

In 1957 Moyamoya-like characteristics were first reported in Japan by Dr. K. Takeuchi and Dr. K. Shimizu. The term “Moyamoya” was first introduced in
1969 by Dr. Jiro Suzuki. The name is Japanese for “a puff of smoke”, chosen because of the appearance of the basal collaterals associated with the disease on angiogram. The first surgical bypass for a MMD patient occurred in 1972 and was performed by Dr. Gazi Yaşargil on a four-year-old boy. The boy’s right-sided weakness and speech disturbance was improved at the two-year follow-up (Donaghy 1972).

Figure 1: Dr. Jiro Suzuki and One of the Earliest Angiograms of MMD (Suzuki 1969)

**Demographics**

MMD is primarily seen as an Eastern Asian disease most common in Japan, Korea, and China. The incidence in Japan and Korea (1-2 per 100,000 (Baba 2008, Ahn 2014)) is about 20 times higher than in the U.S. (0.086 overall and 0.28 in Asian Americans (Uchino 2005)) and has a prevalence of 10.5-16 per 100,000 in Japan and Korea (Baba 2008, Ahn 2014). Females are about twice as likely to develop MMD as males with a ratio of about 1.9:1 (Ahn 2014). Interestingly, MMD presents in a bimodal distribution with peaks of incidence in the 1st and 4th decades of life dividing the disease into early and late onset
The increased incidence in females as compared to males is less pronounced in the first decade of life than it is in the second peak in the fourth decade.

**Figure 2: Bimodal distribution of MMD Incidence and Prevalence** (Ahn 2014)

**Etiology**

The exact cause of MMD is still not fully understood. Genetic, environmental, and immunologic factors have been associated with MMD. Based on inheritance patterns, it is thought to be hereditary and passed on in an autosomal dominant manner with incomplete penetrance (Mineharu 2006). About 10-15% of cases appear to be familial in Japan (Wakai 1997, Baba 2008). Risk factors for developing the disease include being of East Asian descent, female, age (due to the peaks of onset in the first and fourth decades of life), and genomic imprinting as it has been shown that the disease is more likely to develop if inherited from the mother (Mineharu 2006).
**Disease Pathology**

Pathologically MMD presents with intimal thickening in the walls of the terminal portions of the ICA bilaterally due to hyperplasia of smooth muscle cells. Proliferating intima may also contain lipid deposits. Affected vessels have an irregular, wavy elastic lamina, degeneration of media, and decreased diameter. Intraluminal thrombi can accompany the intimal thickening creating significant arterial occlusion. The anterior, middle, and posterior cerebral arteries that emanate from the circle of Willis may also show varying degrees of stenosis or occlusion (Takekawa 2004).

**Six Angiographic Stages**

Dr. Suzuki and Dr. Takeuchi proposed 6 stages of MMD progression (Table 1). Progression through the stages is commonly observed in children but not as frequently in adults. Clinical symptoms do not appear to associate strongly with specific stages (Houkin 1996). Along with the minimization of Moyamoya vessels characterizing stage 4, sometimes dural and pial collateral vessels begin to form.

**Table 1: Suzuki Stages of MMD** (Adapted from Suzuki 1969)

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<th>Stages</th>
<th>Angiogram Appearance</th>
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<tr>
<td>1</td>
<td>Narrowing of Terminal ICA (generally bilaterally)</td>
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<td>5</td>
<td>Reduction of Moyamoya vessels and significant ICA occlusion</td>
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<tr>
<td>6</td>
<td>Absence of Moyamoya vessels and complete ICA occlusion</td>
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**Early versus Late Onset**

Ahn et al (2014) demonstrated how the two peaks in age of development separate MMD into early and late onset. Early onset MMD develops in the first 10 years and ischemic symptoms generally predominate. Initial symptoms include headaches, hemiplegia, and seizures and tend to lead to transient ischemic attacks (TIA) or cerebral infarcts. (Scott 2004). Late onset MMD develops in the fourth decade and the typical collateral vessels occur less frequently. Over time cerebral perfusion is reduced, which may lead to significant cerebral damage and cognitive and neurological decline. Ischemic symptoms predominate in adults, but they are significantly more likely to suffer from hemorrhage than children (Han 1997).

**Treatment**

Currently no pharmacological therapies are available to reverse or halt progression of the steno-occlusive arterial changes in MMD as the optimal target for intervention is not known. Instead, treatment is aimed at limiting the risk of strokes through arterial bypass or the creation of a new blood supply to the affected areas of the brain. The commonly used surgical procedures are direct or indirect revascularization techniques.

Direct revascularization techniques involve arterial bypass with the most common being a superficial temporal artery – middle cerebral artery bypass (STA-MCA). To complete the procedure the surgeon isolates the STA, pulls back
the temporalis muscle, makes an incision in the dura, and then creates an anastomosis between the STA and MCA (Karasawa 1978). The risk of hyperperfusion is relatively high (28%) post-operation and manifests as temporary neurologic deterioration (Kim 2008). Direct bypass is less commonly done in children due to their small vessel size.

In contrast, indirect revascularization techniques aim to create an environment that promotes angiogenesis in affected regions of the brain. For example in Encephalo-duro-arterio-synangiosis (EDAS), opened dura mater is flipped into the subdural space to generate more contact between the cortex and outer side of the dura, using the middle meningeal artery as the source to form a new blood supply (Matsushima 1990). A similar procedure, Encephalo-myosynangiosis (EMS), attaches the temporalis muscle directly to the surface of the brain and uses the deep temporal artery as the source to form a new blood supply (Karasawa 1977). Other indirect methods include multiple burr hole surgery, pial synangiosis, and omental transplantation (Kawaguchi 1996, Scott 2004, Havlik 1992). In certain cases, EDS and EMS procedures are combined to maximize the potential for angiogenesis. Direct and indirect techniques have also been performed together (Kim 2006). Age, hemorrhagic or ischemic presentation, and collateral presence are some of the key factors in determining the choice of treatment. The superiority of direct versus indirect revascularization techniques on a long term scale is still heavily debated and resolution of the issue requires larger studies with longer follow up.
Prognosis

There is no singular prognosis for MMD. In some cases neurologic deterioration occurs rapidly while in others the disease develops over decades with occasional TIAs. Progression or lack of depends on the extent of occlusion and the rate at which it occurs, the formation of basal collaterals which provide sufficient blood flow to salvage brain tissue, and the patient’s neurologic state at time of treatment. If untreated over half of patients experience significant disease progression while fewer than 3% of patients had disease progression after surgery. Surgical treatment prior to a major stroke event results in the best prognosis. As typical of a progressive disease, untreated patients are likely to have further arterial occlusion leading to stroke and mental decline and potentially death due intracerebral hemorrhage (Choi 1997, Han 1997, Hallemeier 2006).

Genetics of MMD

Due to its increased prevalence in East Asia, most large-scale genetic analysis of MMD has been done in Japan and South Korea. Over the last twenty years many candidate genes have been proposed.

Initially linkage analysis was performed on familial MMD cases to try to identify potential chromosomal regions of interest and modes of inheritance. Ikeda et al published the first genetic locus study in 1999. In this study 16 Japanese families (37 MMD patients, 77 individuals total) were reported on.
Linkage analysis revealed an association at chromosome 3p24.2-p26 in the MMD patients (Ikeda 1999). This region had previously been associated with Marfan syndrome and the von Hippel–Lindau disease gene responsible for hemangioblastoma. The Ikeda study paved the way for further genetic MMD research.

In 2000 Inoue et al published a MMD study investigating a genetic association between the disease and human leukocyte antigens (HLA). The linkage analysis performed on 20 affected Japanese sibling pairs found linkage to chromosome 6q25 (Inoue 2000). A Korean study (Han 2003) replicated these results in 2003. Also in 2000, Yamauchi et al. performed linkage analysis on 24 Japanese families (56 MMD patients). Because the characteristic bilateral terminal occlusion of the ICA typical to MMD has sometimes been seen in neurofibromatosis type 1, the study investigated linkage between MMD and chromosome 17 (Neurofibromin 1 (NF1) located at chromosome 17q11.2).

Although no association was found between NF1 and MMD, linkage was shown to the telomeric region of chromosome 17q25. Epidermal growth factor receptor-binding protein 2, integrin-β4 subunit, and tissue inhibitor of metalloproteinase 2, all located in adjacent regions of this chromosome, were proposed as candidate genes. It was also concluded from this study that the disease is most likely inherited in an autosomal dominant fashion with low penetrance (Yamauchi 2000).
In 2004, Sakurai et al investigated 12 Japanese families with 12 sibling pairs in a genome-wide linkage analysis (Seven of the 12 families were used in the previously described Inoue et al study). Markers on the previously reported 3p, 6q, and 17q regions with association with MMD did not reach significant linkage levels but instead investigators found novel linkage to chromosome 8q13-24 and suggestive linkage to chromosome 12p13-p12. Transforming growth factor-beta-inducible early growth (TIEG), located on 8q was proposed as a candidate gene (Sakurai 2004).

In 2008 Mineharu et al performed genome-wide linkage analysis on 15 extended Japanese families (53 MMD patients) after identifying the autosomal dominant with incomplete penetrance inheritance pattern in these families in 2006. The study identified a major gene locus on chromosome 17q25.3 that, although also in the telomeric region, does not overlap with the 17q25 region linked in the Yamauchi 2000 study. Associations in the 3p and 8q regions were not replicated. BAIAP2, TIMP2, RAC3, and RAB40B were proposed as candidate genes (Mineharu 2008). Liu et al (2010) continued with the work of Mineharu, adding two more families to the 15 already included. Linkage analysis demonstrated an increased association in the 17q25.3 region. Further investigation via fine mapping narrowed signal to a 2.1 Mb region. After sequencing and segregation analysis, a single nucleotide polymorphisms (SNP) in Raptor (ss161110142 G/A), a gene associated with tissue hypertrophy, appeared of greatest interest. A large scale, multiethnic, case-control study
investigating this and six other *Raptor* SNPs was carried out in Japanese (90 cases vs. 384 controls), Korean (41 cases vs. 223 controls), Chinese (23 cases and 100 controls), and Caucasian (25 cases and 164 controls) populations. The study concluded that ss161110142 increased susceptibility to MMD in East Asian populations but is not present in the Caucasian samples.

A breakthrough in the search for a susceptibility gene in MMD came from two separate studies in 2011 with the identification of Ring Finger Protein 213 (*RNF213*) as the susceptibility gene and p.R4810K as the founder mutation. Kamada et al published the first MMD genome-wide association study (GWAS) using 72 Japanese patients (64 sporadic cases and 8 probands of MMD families) and 45 controls. A strong association was identified in the same 17q25.3 region as previously described. Investigating further, a locus-specific association study was performed in the 17q25.3 region. This revealed a strong association of SNPs in the 3' end of *RNF213*. Mutational analysis of *RNF213* demonstrated the same missense mutation (p.R4810K) in 19 of 20 Japanese MMD families and 46 of 63 sporadic cases. In Japanese controls, six out of 429 subjects carried this mutation while in Caucasians none of the 400 controls or five MMD subjects studied was a carrier. As a result of this study, p.R4810K in *RNF213* was characterized as a founder mutation in MMD.

At around the same time, Liu and Mineharu (2011) continued their investigation into genetic association in MMD and produced the same results. Genome-wide linkage analysis and fine mapping in eight 3-generation families
revealed a 1.5 Mb locus in the 17q25.3 region with strong association to MMD. After exome analysis in the region and then Sanger sequencing, the variant p.R4810K in *RNF213* (same as Kamada) was identified with the strongest association. The variant was sequenced in East Asian and Caucasian populations and found in 145 of 161 (90%) of Japanese cases, 30 of 38 (79%) Korean cases, 12 of 52 (23%) Chinese cases, and once again not found in a Caucasian population. Five additional variants in *RNF213* (p.D4863N, p.E4950D, p.A5021V, p.D5160E and p.E5176G) in East Asian cases and four variants (p.N3962D, p.D4013N, p.R4062Q and p.P4608S) in Caucasian cases were also found. None of the additional nine variants seen were found in controls. All 10 variants identified were located at the 3’ end of *RNF213*. The study concluded that *RNF213* is a susceptibility gene for MMD.

Both of these studies noted the low penetrance of the founder mutation in MMD. A majority of carriers never develop the disease. It therefore seems likely that undiscovered modifiers, genetic and/or environmental, have a significant role in disease onset.

*RNF213*

*RNF213* is now considered to be the main susceptibility gene for MMD, especially among Japanese, Korean, and Chinese populations. However, MMD does not appear to be a monogenic disorder but instead a disorder resulting from the combination of several genetic and environmental factors. It is also possible
that unknown genetic modifiers play a significant role in the etiology of MMD. This is indicated by the substantially higher prevalence of the p.R4810K variant in East Asian populations compared to the actual prevalence of MMD.

Structurally the RNF213 protein contains AAA domains associated with ATPase activity and a RING finger domain which is a specialized type of Zn-finger that binds two atoms of zinc (Liu 2011). Functionally RNF213 is an E3 ubiquitin-protein ligase that potentially plays a role downstream in the non-canonical Wnt signaling pathway by targeting and degrading NFAT1. This pathway is critical for maintenance and remodeling of vasculature. By inhibiting NFAT1, RNF213 expressed in endothelial cells appears to prevent the inhibition of vessel regression (Scholz 2016).

Figure 3: Proposed Role of RNF213 in Angiogenesis (adaptation of Scholz 2016)

Liu et al (2011) used a zebrafish model to assess the effect of loss of RNF213. Injecting RNF213 morpholinos into early stage zebrafish embryos that knocked down gene expression showed abnormal blood vessel sprouting and irregular vessel diameter.
It is still debated whether p.R4810K causes gain of function or loss of function in RNF213, but the consensus seems to be growing that inflammatory signals and altered immune response play significant roles in RNF213 expression and could be the key modifier along with RNF213 mutation in MMD (Kobayashi 2015, Ohkubo 2015).

Recent Findings in Multiethnic MMD Populations

Although the susceptibility variant for MMD, p.R4810K in RNF213, has been well described in East Asian populations, it has yet to be seen in any other race-ethnic group. Liu et al (2011) described a few other mutations in RNF213 in Caucasian MMD subjects but most non-East Asian populations do not display mutations in the gene. Therefore other genes need to be explored. Shoemaker et al (2015) performed whole-exome sequencing (WES) and case control analysis on three population subsets: East Asian/Southeast Asian/Pacific Islander (70 subjects), Caucasian (136 subjects), and multiethnic non-founder mutation carrying (182 subjects). As expected, the founder mutation was only found in subjects of East Asian descent. In the Caucasian subset the most significant finding was a p.P562L mutation in ZXDC in 14.7% of the cases. The study calls the mutation potentially causative for MMD, but notes its relatively high frequency in controls (4%) as indication of very low penetrance. Other candidate genes the study detected in the Caucasian population included RPTN (p.D110H), CD46.

Kobayashi et al (2015) recently directly sequenced *RNF213* in 19 Slovakian and Czech MMD patients. Once again the founder mutation was not seen in a Caucasian population but four other *RNF213* variants were identified (p.R4019C, p.E4042K, p.V4146A, and p.W4677L).
SPECIFIC AIMS

The role of \textit{RNF213} still in MMD is not entirely clear and it seems likely that other genes and environmental factors are involved in the pathogenesis. Studies have previously shown the strong association of p.R4810K in \textit{RNF213} with MMD in East Asian populations but not in other populations. Other studies have described different variants in \textit{RNF213} and alternative candidate genes in non-Eastern Asian populations that require validation. \textit{RNF213} has also recently been associated with other cerebrovascular diseases. The extent this gene is involved in non-MMD diseases and if it is implicated in all ethnicities is still unknown.

The purpose of this paper is to determine if variation in the \textit{RNF213} gene contributes to MMD in a cohort of 15 predominantly Caucasian patients with MMD, to investigate other potential genes implicated in MMD in these 15 patients, and to investigate if variation in \textit{RNF213} also influences more common vascular phenotypes.
METHODS

Mayo Clinic Registry and Study Eligibility and Enrollment

Patients with a personal or family history of cerebrovascular disease and healthy controls are asked to participate in the Mayo Clinic Florida Familial Cerebrovascular Diseases Registry (IRB# 08-003878), which has received approval from the Mayo Clinic Institutional Review Board. The primary goal of the study is to identify causal gene mutations in cerebrovascular disease and identify key relationships between these genes and cerebrovascular conditions. Vascular Neurologists prospectively identify patients with one of 23 eligible diagnoses from inpatient and outpatient services at Mayo Clinic Florida. Family members are encouraged to participate to enhance efforts in identifying pathogenic genes and variants. Healthy stroke-free controls are concurrently enrolled. Participants enrolled in person have a structured history and physical examination that includes assessment of demographic variables, stroke risk factors, stroke symptoms, secondary and tertiary diagnoses (if applicable), vital signs, neurological impairment (NIH Stroke Scale (NIHSS)), mental status (Mini Mental State Exam (MMSE)), and functional status. Stroke symptoms are assessed using the 8-item Questionnaire for Verifying Stroke-Free Status.

Age at time of DNA sample collection, sex, race, and ethnicity are recorded. Race and ethnicity were defined according to self-report using standard Office of Management and Budget definitions. Country of origin and years of education were also noted.
The presence or absence of arterial hypertension, diabetes mellitus, atrial fibrillation (either chronic or intermittent), cigarette smoking (active or former and pack-years), and coronary artery disease (history of myocardial infarction, coronary stenting or bypass, and angina pectoris) are recorded. Height, weight, blood pressure, and heart rate are measured. Blood pressure is taken from the left arm with the patient in a resting state (at least 10 minutes after any physical exertion) using a sphygmomanometer. Mid-abdominal circumference was measured halfway between the lower border of the ribs, and the iliac crest in a horizontal plane (cm).

Two 10 mL EDTA purple top tubes of venous blood for each consented participant are collected. Blood samples are hand-carried from the site of collection to the investigator's laboratory in a sterile, biohazard, sealed container. De-identified barcoded samples are stored at -80°C. If a positive family history is indicated the relational mySQL database MD running in Linux OS requires a Progeny Unique Pedigree Number. Progeny is a relational database in Sybase, maintained on a central server to which clients have password-protected access (Progeny Software, LLC; http://www.progeny2000.com). The program is used to maintain pedigree relationships, along with clinical and genetic data.

DNA was extracted from the blood, assessed for quality, and re-extracted if necessary. DNA integrity was assessed by gel electrophoresis and DNA concentration quantified by PicoGreen®. PicoGreen® dsDNA Quantitation
Reagent is an ultra-sensitive fluorochrome that selectively binds double-stranded DNA (dsDNA) and when bound displays enhanced fluorescence.

For the purpose of this study, the extracted DNA of 15 unrelated samples with a diagnosis of MMD (Table 2) was sent for WES.

**Table 2: Summary of MMD Subject Demographics**

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<thead>
<tr>
<th></th>
<th>Average (Min - Max)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>39.9 (23 - 58)</td>
</tr>
<tr>
<td><strong>Age at stroke</strong></td>
<td>34.1 (14 - 54)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>3:12</td>
</tr>
<tr>
<td><strong>Family History of MMD</strong></td>
<td>2/15</td>
</tr>
<tr>
<td><strong>MMSE</strong></td>
<td>28.7 (23 - 30)</td>
</tr>
<tr>
<td><strong>NIHSS</strong></td>
<td>1.3 (0 - 4)</td>
</tr>
</tbody>
</table>

Data on the role of *RNF213* mutations in other cerebrovascular vascular diseases was pulled from the previously done WES results of those in the Registry.

**Whole Exome Sequencing**

2.1 µg of genomic DNA was used from each proband to perform exome sequencing with the Sure Select V4 + UTR exome capture kit (Agilent Technologies, Santa Clara, CA, USA) following manufacturer’s standard protocol. The sequencing was carried out at the Mayo Clinic Bioinformatics Core (Rochester, MN, USA) which performed sequencing on a HiSeq 2000 sequencer (Illumina, San Diego, CA, USA). The Mayo Clinic Bioinformatics Core (Rochester, MN, USA) also performed alignment and base calling using their standard pipelines.
**Alignment and Variant Calling**

Genome_GPS_2.0 was used to carry out secondary analysis of sequencing data (Figure 4). All data were stored in Oracle TRC.

**Genome_GPS 2.0**

![Genome_GPS 2.0 workflow diagram]

**Figure 4: Genome_GPS 2.0 workflow**

Genome_GPS is the comprehensive secondary analysis pipeline for next-generation sequencing data at Mayo Clinic. Secondary analysis entails four steps: alignment, single nucleotide and small insertion/deletion variant calling, structural variation discovery, and annotation. From the Illumina HiSeq platform, 100 base-pair paired end reads are aligned to hg19 using Novoalign (Novocraft Technologies, Malaysia). Quality of sequencing chemistry is evaluated using FastQC (Babraham Bioinformatics). After alignment, PCR duplication rates and
percent reads mapped on target are used to assess the quality of the sample preps. Realignment and recalibration steps are implemented in the Genome Analysis Toolkit (GATK) (McKenna 2010). All germline variant calling (both single nucleotide and small insertions and deletions) is jointly called through GATK Haplotype Caller and GenotypeGVCF walkers. Where possible, samples with pedigree files will also undergo PhaseByTransmission to correct potential genotype calling errors. Somatic single nucleotide variations (SNVs) are genotyped using SomaticSniper (Larson 2012), whereas insertions and deletions are called by GATK Somatic Indel Detector (Mckenna 2010). Each variant in coding regions are functionally annotated by SnpEff (Cingolani 2012), as well as ClinVar (Landrum 2014), dbNSFP (Liu 2011), Online Mendelian Inheritance in Man (OMIM) (Hamosh 2005), and the Human Gene Mutation Database (HGMD) (Stenson 2013) to predict biological effects. Each variant is also annotated with allele frequency from the Exome Aggregation Consortium (Robinson 2015). Variants of significant interest are visually inspected using Integrative Genome Viewer (Robinson 2011) and then biologically validated using standard molecular biology techniques. Prioritization of mutations for validation (those that are likely to have the most dramatic effect on protein function), is based on several factors including: how many times a gene was mutated in different cases, how deleterious the mutations are likely to be (nonsense mutation versus synonymous), etc. SNP annotation was performed with SNP & Variation Suite
8.4.2 (Golden Helix Inc., Bozeman, MT, USA). Variants were called according to standard nomenclature (den Dunnen 2001).

**Variant Filtering**

After aligning and variant calling, each sample’s data was sent back in a variant call format (VCF) file. The VCF files were uploaded into Ingenuity® Variant Analysis™ (http://www.ingenuity.com/variants), a commercial variant analysis software. This is a program for filtering and prioritizing variants. Ingenuity Variant Analysis resulted in generation of a filtered list of the most significantly associated genes with each case.

Starting with 286,143 variants spanning 22,439 genes, variants were kept with call quality at least 20 in cases and outside the top 5% most exonically variable 100 base regions in healthy public genomes (1000 Genomes). Variants are excluded with an observed allele frequency greater than or equal to 3% of the genomes in the 1000 genomes project, NHLBI ESP exomes, or ExAC Frequency. Variants are kept that are experimentally observed to be associated with a phenotype meaning scoring Pathogenic, Possibly Pathogenic or Disease-associated according to HGMD. Frameshift, in-frame indel, or stop codon change or missense variants are kept as well as those that disrupt splice site up to 2 bases into intronic regions or are predicted to disrupt splicing by MaxEntScan. Finally, variants that are known or predicted to affect the inputted “Gene List” are kept.
The “Gene List” (Table 3) was created by identifying genes from GWAS studies on stroke and monogenic disorders that compromised vessel integrity or were known to result in stroke or other types of vascular events.

**Table 3: Gene List Used for Filtering**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCC6</td>
<td>CD36</td>
<td>EDNRA</td>
<td>FUT8</td>
<td>LTBP4</td>
<td>NOTCH2</td>
<td>RBBP8</td>
<td>TGFB3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABO</td>
<td>CD46</td>
<td>EFEMP2</td>
<td>GDF2</td>
<td>MAP2K1</td>
<td>NOTCH3</td>
<td>RNF213</td>
<td>TGFB1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE</td>
<td>CECR1</td>
<td>ELN</td>
<td>GLA</td>
<td>MFAP5</td>
<td>NR3C1</td>
<td>ROPN1</td>
<td>TGFB2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTA2</td>
<td>CLU</td>
<td>ENG</td>
<td>GUCY1A3</td>
<td>MTCP1</td>
<td>NTM</td>
<td>RPTN</td>
<td>TREX1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACVR1L</td>
<td>CNNM2</td>
<td>EPHX2</td>
<td>HABP2</td>
<td>MTHFR</td>
<td>PCNT</td>
<td>SH2B3</td>
<td>TTR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH18A1</td>
<td>COL1A1</td>
<td>F2</td>
<td>HBB</td>
<td>MYH11</td>
<td>PDCD10</td>
<td>SKI</td>
<td>TWIST1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE</td>
<td>COL3A1</td>
<td>F5</td>
<td>HDAC9</td>
<td>MYLK</td>
<td>PDIA4</td>
<td>SLC26A11</td>
<td>YY1AP1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP</td>
<td>COL4A1</td>
<td>FBLN5</td>
<td>HTRA1</td>
<td>NAPSA</td>
<td>PHACTR1</td>
<td>SLC2A10</td>
<td>ZFHX3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP7A</td>
<td>COL4A2</td>
<td>FBN1</td>
<td>IL12RB2</td>
<td>NCF1</td>
<td>PITX2</td>
<td>SMAD3</td>
<td>ZFDC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCC3</td>
<td>COL5A1</td>
<td>FBN2</td>
<td>JAG1</td>
<td>NF1</td>
<td>PLOD3</td>
<td>SOX17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10orf88</td>
<td>COL5A2</td>
<td>FGA</td>
<td>KALRN</td>
<td>NFATC2</td>
<td>PRKCH</td>
<td>STARD13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBS</td>
<td>CRP</td>
<td>FGB</td>
<td>KL</td>
<td>NINJ2</td>
<td>PRKG1</td>
<td>TCF7L2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCM2</td>
<td>CTC1</td>
<td>FOXF2</td>
<td>KRT1</td>
<td>NOS3</td>
<td>RASA1</td>
<td>TGFB2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Genes highlighted in gray have been previously associated with MMD*
RESULTS

Relevant Clinical Information

Two of the MMD subjects were Asian (Korean and Filipino), one was African American, and the remaining 12 were Caucasian. Six of the 15 had an ischemic stroke, four had a TIA, eight of the 12 known had family history of stroke, nine of the 15 had hypertension, and five of the nine known had family history of hypertension.

Table 4: MMD Patients Clinical Information

<table>
<thead>
<tr>
<th>Proband No.</th>
<th>Gender</th>
<th>Age at Stroke</th>
<th>Stroke Family History</th>
<th>MMD Family History</th>
<th>Laterality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>45</td>
<td>No</td>
<td>No</td>
<td>Bilateral</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>14</td>
<td>No</td>
<td>No</td>
<td>Bilateral</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>17</td>
<td>Yes</td>
<td>Yes</td>
<td>Bilateral</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>40</td>
<td>Yes</td>
<td>No</td>
<td>Unilateral</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>45</td>
<td>Yes</td>
<td>Yes</td>
<td>Unilateral</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>30</td>
<td>Unknown</td>
<td>No</td>
<td>Bilateral</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>33</td>
<td>Unknown</td>
<td>No</td>
<td>Bilateral</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>54</td>
<td>Yes</td>
<td>No</td>
<td>Unilateral</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>41</td>
<td>Yes</td>
<td>No</td>
<td>Bilateral</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>37</td>
<td>No</td>
<td>No</td>
<td>Bilateral</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>20</td>
<td>Yes</td>
<td>No</td>
<td>Bilateral</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>44</td>
<td>Unknown</td>
<td>No</td>
<td>Unilateral</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>22</td>
<td>No</td>
<td>No</td>
<td>Unilateral</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>35</td>
<td>Yes</td>
<td>No</td>
<td>Bilateral</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>35</td>
<td>Yes</td>
<td>No</td>
<td>Bilateral</td>
</tr>
</tbody>
</table>
Identification of Genetic Variants in MMD Cases

WES was performed on all 15 MMD cases to determine associated genetic variants. Each sample’s variants were analyzed individually and filtered through Ingenuity. Remaining variants were analyzed manually using HGMD, OMIM, Alamut, ExAC, and previous research publications in order to determine their potential relevance. The effect of the variants on protein function was assayed using two prediction algorithms: Polyphen-2 and SIFT both obtained from Alamut.

Variants in RNF213

A total of 13.3% (2/15) MMD cases two carried mutations in RNF213. A total of 8.3% (1/12) Caucasian subjects carried a RNF213 variant. In confirmation of the previous literature, the founder mutation p.R4810K in RNF213 was identified in the only subject of Eastern Asian ancestry. The geographic region specific nature of this mutation was also confirmed, as it was not present in any of the other cases including the other Asian American subject who is of Pacific Islander descent.

The other RNF213 variant found was p.R4019C in a Caucasian subject. This variant has been previously described in other Caucasian MMD cases (Liu 2011, Cecchi 2014, Kobayashi 2016). This variant is very rare, only seen in Caucasian and Latino populations and has a minor allele frequency (MAF) of less than 0.1%. P.R4019C is predicted to be Probably Damaging and Damaging
by PolyPhen-2 and SIFT. Conservation of arginine in position 4019 is seen in mammals except rodents.

**Significant Non-RNF213 Variants Identified**


### Table 5: Significant MMD Variants Identified

<table>
<thead>
<tr>
<th>dbSNP</th>
<th>Chromosome</th>
<th>Gene</th>
<th>Amino Acid Change</th>
<th>ExAC MAF (overall)</th>
<th>ExAC MAF (per ethnicity)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>East Asian</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs112735431</td>
<td>17</td>
<td>RNF213</td>
<td>p.R4810K</td>
<td>0.04%</td>
<td>0.39%</td>
</tr>
<tr>
<td>rs78198420</td>
<td>1</td>
<td>IL12RB2</td>
<td>p.N271Y</td>
<td>0.05%</td>
<td>0.64%</td>
</tr>
<tr>
<td><strong>Caucasian</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs139265462</td>
<td>17</td>
<td>RNF213</td>
<td>p.R4019C</td>
<td>0.05%</td>
<td>0.08%</td>
</tr>
<tr>
<td>rs143744326</td>
<td>1</td>
<td>RPTN</td>
<td>p.D455H</td>
<td>2.49%</td>
<td>3.59%</td>
</tr>
<tr>
<td>rs189546119</td>
<td>1</td>
<td>RPTN</td>
<td>p.Q459*</td>
<td>0.13%</td>
<td>0.03%</td>
</tr>
<tr>
<td>rs2307153</td>
<td>1</td>
<td>IL12RB2</td>
<td>p.G465D</td>
<td>1.41%</td>
<td>2.12%</td>
</tr>
<tr>
<td>rs142692233</td>
<td>3</td>
<td>ZXDC</td>
<td>p.A126V</td>
<td>8.93%</td>
<td>20%</td>
</tr>
<tr>
<td><strong>African American</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs149963292</td>
<td>3</td>
<td>ZXDC</td>
<td>p.A53E</td>
<td>1.40%</td>
<td>17.07%</td>
</tr>
<tr>
<td>rs112996264</td>
<td>19</td>
<td>NAPSA</td>
<td>p.L408V</td>
<td>0.24%</td>
<td>3.26%</td>
</tr>
<tr>
<td><strong>Pacific Islander</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1447000814</td>
<td>7</td>
<td>PDIA4</td>
<td>p.D54H</td>
<td>0.06%</td>
<td>0.007%</td>
</tr>
</tbody>
</table>

The p.D455H mutation in *RPTN* has been replicated in a previous MMD study (Shoemaker 2015) which showed the variant in 14.7% (10/68) of Caucasian cases. This study found the variant in 16.7% (2/12) of Caucasian cases. P.D455H has a MAF of 3.6% in Non-Finish European (NFE) populations.
(ExAC) and is predicted to be Benign and Tolerated by PolyPhen-2 and SIFT. Conservation of aspartic acid in position 455 is seen in mammals except rodents. A novel nonsense mutation (p.Q459*) in RPTN was also found in this study in one Caucasian case. P.Q459* is very rare with a MAF of less than 0.1% in NFE populations (ExAC) and the glutamine position at 459 is conserved in mammals.

The p.N271Y mutation in IL12RB2 has been studied before for its role in IL-12 mediated immune response (de Paus 2013) but never shown in a MMD case before. This variant was seen in the East Asian subject who also had the p.R4810K founder mutation in RNF213. P.N271Y has a MAF of 0.6% in East Asian populations and is predicted to be Possibly Damaging and Tolerated by PolyPhen-2 and SIFT. The asparagine at position 271 is conserved in mammals except canines. Another mutation in IL12RB2 (p.G465D) was found in a Caucasian case. P.G465D has a MAF of 2.1% in NFE populations (ExAC) and is predicted to be Benign and Tolerated by Polyphen-2 and SIFT. Aspartic acid at position 465 has been shown to be tolerated in one species of primate. Both of these IL12RB2 mutations are in non-coding RNA (ncRNA) gene regions.

Shoemaker et al (2015) found a variant in ZXDC (p.P562L) to be the most enriched in Caucasian MMD cases. In this study, two variants in ZXDC, p.A126V and p.A53E, were identified in a Caucasian subject and an African American subject respectively. Both mutations have relatively high MAF (20% and 17% in their respective populations) although this does not entirely rule out the potential role of the variants as disease modifiers. P.A126V and p.A53E are predicted to
be Benign/Damaging and Benign/Tolerable by Polyphen-2 and SIFT respectively. Alanine at position 126 is conserved in mammals and at position 53 is only conserved in chimps while glutamic acid at this position is tolerated in zebrafish. Both of these ZXDC mutations are in ncRNA gene regions.

Another variant in a gene (NAPSA, p.L408V) associated with MMD in Caucasian samples in the Shoemaker et al (2015) study was found in the African American MMD case. P.L408V has a MAF in African populations of 3.3% and is predicted to be Benign and Tolerated by PolyPhen-2 and SIFT. Leucine at position 408 is conserved in primates.

The last gene from the Shoemaker et al (2015) paper that this study replicated was in PDIA4. The p.D54H variant was found in the Pacific Islander MMD case. This rare variant has a MAF of less than 0.1% in South Asian populations and is predicted to be Possibly Damaging and Damaging by Polyphen-2 and SIFT. Aspartic acid at position 54 is conserved down to roundworms.

**RNF213 in Other Vascular Phenotypes**

Although most notably associated with MMD, RNF213 has been associated with other cerebrovascular diseases as well. WES results from subjects enrolled in the Mayo Clinic Florida Familial Cerebrovascular Diseases Registry were analyzed to investigate this.
A rare, novel variant (p.P1721L) was found in one Caucasian subarachnoid hemorrhage (SAH) case. P.P1721L has a MAF of 1.2% in NFE populations and is predicted to be Tolerated by SIFT. Proline at position 1721 is conserved in mammals as well as in chickens.

Table 6: African American Cerebrovascular Cases and *RNF213* mutation

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>No. of cases with variants</th>
<th>% of cases with variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arteriovenous Malformation</td>
<td>2</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Carotid Arterial Dissection</td>
<td>2</td>
<td>1</td>
<td>50%</td>
</tr>
<tr>
<td>Definite AICS</td>
<td>15</td>
<td>9</td>
<td>60%</td>
</tr>
<tr>
<td>ICH</td>
<td>4</td>
<td>1</td>
<td>25%</td>
</tr>
<tr>
<td>MMD</td>
<td>1</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Perimesencephalic-SAH</td>
<td>2</td>
<td>1</td>
<td>50%</td>
</tr>
<tr>
<td>SAH</td>
<td>11</td>
<td>7</td>
<td>63.6%</td>
</tr>
<tr>
<td>Unruptured Aneurysm</td>
<td>4</td>
<td>3</td>
<td>75%</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>22</td>
<td>53.7%</td>
</tr>
</tbody>
</table>

A recent GWAS of young onset stroke in Caucasians indicated a significant association with two SNPs in *SLC26A11* (Cheng 2016). This gene is located directly next to *RNF213* on chromosome 17q25.3, less than 10kb away. Due to the genes close proximity and therefore potentially having significant linkage disequilibrium (LD) between their variants, it is possible that *RNF213* may be associated with these young onset stroke cases. LD analysis showed significant linkage between many of the SNPs in *SLC26A11* and *RNF213* in both Central European and Japanese populations. Even though the SNPs are not all the same between these populations, the LD patterns in both appeared very similar.
Figure 5: SLC26A11 Hits in Young Onset Stroke GWAS and Location near RNF213 (Cheng 2016)

Figure 6: LD between SLC26A11 and RNF213 in European and Japanese populations
DISCUSSION

Research on MMD has identified some significant genes associated with the disease, but has yet to fully identify the exact genes and number of genes directly implicated in the etiology of MMD. Although most of research has been done in Japan and Korea, where the disease is most prevalent, the genetic heterogeneity between MMD cases in East Asian and the rest of the world indicate that it is important to further investigate the genetics of non-East Asian cases. The purpose of this analysis was to validate and explore novel MMD related genes in a cohort of mainly non-East Asian subjects as well as to examine the role of RNF213 in other cerebrovascular diseases.

Implications of Replicated and Novel Variants in MMD

The founder mutation in RNF213 with MMD in East Asian patients (p.R4810K) and one of the most significantly associated variants in Caucasian cases (p.R4019C) were both replicated in this study. The lack of the founder mutation in the non-East Asian subjects further supports that this variant is specific to the region and not causative in other populations. The presence of p.R4019 in a Caucasian MMD case supports the role of RNF213 in MMD in other populations, but the lack of significant mutations in this gene found in the other 13 cases indicates that other genes and factors are likely involved. Sonobe et al (2014) demonstrated that RNF213 knockout mice did not develop the characteristic Moyamoya abnormal vascular structure therefore also indicating
that solely loss of function of \textit{RNF213} is not sufficient to generate MMD. Kobayashi et al (2015) demonstrated that the combination of overexpression of mutated RNF213, p.R4810K and inflammatory signals significantly led to the suppression of angiogenesis under hypoxic conditions.

Variants in two genes involved in inflammatory response, \textit{IL12RB2} and \textit{ZXDC}, which had previously been associated with MMD (Shoemaker 2015) were replicated in this study. P.N271Y in \textit{IL12RB2} found in the East Asian case with the founder mutation has been previously described to reduce IL-12 response (de Paus 2013). Another variant, P.G465D, was identified in a Caucasian case. This mutation has also never been seen in a MMD case before but is located in the same exon as another IL12RB2 variant (p.Q426H) that was enriched in Caucasian MMD cases (Shoemaker 2015). Functionally the IL12RB2 protein binds with IL2RB1 to form the IL-12 receptor (Presky 1996). Receptor binding leads to downstream production of interferon-\(\gamma\) which been show to inhibit angiogenesis (Sato 1990). This makes combination of founder mutation with the \textit{IL12RB2} mutation very interesting and the role of this gene in MMD should be further explored.

A variant in \textit{ZXDC} was the most enriched in Caucasian MMD cases in a recent large-scale multiethnic study (Shoemaker 2015). This study identified two variants in the gene, in a Caucasian and an African American subject. \textit{ZXDC} has been shown to enhance transcription of MHC Class II genes (Al-Kandari 2007) and the absence of said genes leads to dysfunctional immune response (Villard
Both mutations identified in *ZXDC* had very high MAF in their respective populations and therefore likely can be ruled out as disease causing.

Variants in two genes involved in cytoskeletal organization, *RPTN* and *NAPSA*, that had been previously associated with MMD (Shoemaker 2015) were also replicated in this study. The same variant (*RPTN*, p.D455H) identified in the Shoemaker et al (2015) paper was found in two Caucasian cases in this study. The nonsense mutation (p.Q459*), found in separate Caucasian case, is located in the same exon as the previously described p.D455H variant. Variation in this region of *RPTN* seems to be associated with MMD in Caucasian cases and necessitates further research. The p.L408V mutation in *NAPSA* appears to be less interesting due to its frequency in African populations, benign/tolerated predicted scores, and lack leucine of conservation in mammals.

The very rare and normally highly conserved variant found in *PDIA4* in one MMD case could potentially be interesting but as of now its relation to the disease does not seem clear.

Other non-*RNF213* genes that have been previously associated with MMD like *ACTA2* and *GUCY1A3* were not identified in this study. Genes not previously associated with MMD were also investigated but with the small sample size, none appeared to be especially significant.
RNF213 as Risk Factor in More than just MMD

RNF213 has clearly been identified in association with MMD, but it potentially is implicated in other cerebrovascular diseases as well. Recent studies have suggested RNF213 is associated with intracranial major artery stenosis/occlusion (Miyawaki 2013) and intracranial atherosclerotic stenosis (Bang 2015) in East Asian populations and intracranial aneurysms in the French Canadian population (Zhou 2016). Most research on this notion has focused on investigating the association of the MMD founder mutation with other cerebrovascular diseases in East Asian populations. This study supports the potential of RNF213 in non-MMD cases even with non-founder mutations in non-East Asian patients.

Large-scale investigation of RNF213 in vascular disease patients of African descent has not been attempted, but this study indicates a potential need for it. Non-synonymous, exonic mutations of less than 3% MAF in RNF213 were found in over half of the subjects and 17% (7/41) displayed multiple variants. Based on the expected number of alleles in a cohort of this size due to the MAF in African populations (ExAC), four variants could be ruled out as appearing by chance (p.G2611R, p.T3243A, p.D3731G, and p.S4814R). This lowers the number of cases with at least one RNF213 mutations to 20 (48.8%). One of the most interesting variants found (p.Q469H), was present in five cases with four different diagnoses. It is predicted to be Probably Damaging by Polyphen-2. Further research on this mutation is warranted.
Both of the MMD subjects with *RNF213* mutations in this study had a family history of stroke. It would be interesting to sequence the family members with stroke but without MMD for the same mutations. If present then analysis of other non-*RNF213* mutations solely appearing in the MMD case would be of great interest in determining potential modifiers of disease onset.

In regards to the LD found between *SLC26A11* and *RNF213*, sequencing of *RNF213* in young onset stroke patients would be required to determine if the gene is truly associated. It is important to note that more highly associated regions with young onset stroke were reported than the hits in *SLC26A11* in the GWAS (Cheng 2016). If *RNF213* was found to be associated with young onset stroke, it is more likely that it would be in a modifying role as opposed to disease causing. Also, even though *RNF213* seems like the more likely candidate due to its relation with other cerebrovascular diseases, it cannot be ruled out that *SLC26A11* itself plays a significant role.

In the context of clinical and genetic heterogeneity, as sequencing continues to become cheaper, increased WES performed on both common and rare vascular phenotypes will be essential in elucidating the role of *RNF213*. More data on *RNF213* as well as other genes that had previously only been associated with rare phenotypes may show that they are implicated in much more common diseases as well.
**Significance of the Genetic Revolution**

The role of genetics in medicine is exponentially growing as the technology continues to rapidly advance. This can be seen with the price and time it takes for GWAS and WES dropping dramatically and therefore becoming more universally viable as research techniques. For MMD and other poorly understood heterogeneous diseases, this genetic revolution moves toward not only further uncovering disease etiology but also new and more individualized treatment options.

Paired with the expansion of genetics in research are the field’s increasing clinical implications. The importance comes with choice of therapeutics and selecting individuals for clinical trials. Personalized medicine will introduce a treatment or preventative plan that targets an individual’s specific genetic variations. For example although a patient may present with a certain phenotype pertaining to a disease, the presence of a different disease associated genetic variation will lead to a more specified approach than if they had been lumped in with everyone with said phenotype. In the same way multiple clinical diseases presenting with mutations in the same gene could all be given the same therapeutic approach. From this study, the potential role of *RNF213* in multiple cerebrovascular disorders could result in therapy directed at this specific gene/protein pathway in variant carriers. Similarly, the varying genetic landscape of MMD patients indicates that there will not be a single drug able to prevent or halt vessel occlusion in all cases. An individualized approach seems necessary.
To reach this point of understanding MMD etiology and in turn treatment, it is apparent that more research on the genetics of the disease is needed, especially in non-East Asian cases.

**Study Limitations**

This study, in regard to the genetics of MMD and the role of *RNF213* in multiple cerebrovascular diseases, is limited by the small sample size and lack of controls. In part the small sample size is due to the rarity of the disease, especially in the United States. As part of the next step, non-affected family members will be sequenced and used as controls. Also, because this study utilized WES as opposed to whole genome sequencing, copy number variants (CNVs) were not recorded. Another area of concern is that clinical heterogeneity of MMD, especially in regard to unilateral cases and the separation of MMD cases from MMS.

**Implications in Future Research**

Moving forward, continued enrollment and WES of MMD patients at Mayo Clinic Jacksonville is necessary to build a more robust database of variants in MMD. There are already two more patients enrolled since the original 15 samples were sent for WES. DNA of non-affected family members is being collected and will serve as controls to produce more reliable results. CNV analysis may also provide further insight. As the database continues to grow,
more in depth statistical analysis can be performed therefore further validating or invalidating results.

Gaining an understanding of the role of *RNF213*, an ongoing process, requires a multiethnic approach. Currently, nearly all efforts have been focused in Japanese and Korean patients. This analysis not only demonstrates the potential role of *RNF213* in multiple cerebrovascular diseases but also indicates the value in research of the gene in non-East Asian populations, including the understudied African American population. Clearly more research is needed.

The way forward is a global effort. *RNF213* is not limited to MMD and MMD is not just an Asian disease but a worldwide disorder. A coordinated effort is required between clinicians providing high quality imaging for disease confirmation and with geneticists continued sequencing on a multiethnic scale. Broadening the research of *RNF213* to other cerebrovascular diseases will not only likely lead to more robust understanding of its function but also in doing so will elucidate its role in and the etiology of MMD more clearly. The advancements could lead to the development of finely tuned, targeted therapies that would enormously benefit patients worldwide.
REFERENCES


