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Common Genetic Polymorphisms in Moyamoya and Atherosclerotic Disease

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Widmung

Meinen Eltern

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Abbreviations

µm: Micrometer

A: Adenine

AANAT: Arylalkylamine N-acetyltransferase

ACI: Arteria carotis interna

ANRIL: Antisense non-coding RNA in the INK4 locus

ASA: Acetylsalicylic acid

BAIAP2: Brain-specific angiogenesis inhibitor 1-associated protein 2

BFGF: Basic fibroblast growth factor

C: Cytosine

CAD: Coronary artery disease

CAV: Cardiac allograft vasculopathy

CDKN: Cycline dependent kinase

CEPH/CEU: Centre d`etude du polymorphisme human

CELSR2: Cadherin EGF LAG seven-pass G-type receptor 2

CHB: Han Chinese in Beijing

CHD: Coronary heart disease

CI: Confidence interval

cM: Centimorgan

CT: Computer tomography

CXCL12: Chemokine (C-X-C motif) ligand 12

dNTPs: Desoxynucleotides (-tri-phosphates)

ddNTPs: Dideoxynuclotides (-tri-phosphates)

DNA: Desoxyribonucleic acid

DNAI2: Dynein, axonemal, intermediate polypeptide 2

ECM: Extracellular matrix

EDAS: Encephaloduroarteriosynangiosis

EDTA: Ethylenediaminetetraacetic acid

ELN: Elastin

EMAS: Encephalomyoarteriosynangiosis

EMS: Encephalomyosynangiosis

EST: Expressed sequence tag

Et al.: Et alii / and others

EVPL: Envoplakin

FGF: Fibroblast growth factor

FMMD: Familiar Moyamoya Disease

G: Guanine

GWAS: Genome-wide association study

HCNGP: A transcriptional regulator protein

HGF: Hepatocyte growth factor

HIF: Hypoxia inducible factor

HLA: Human leukocyte antigen

HN1: Hematological and neurological expressed 1

HWE: Hardy-Weinberg-equilibrium

IA: Intracranial aneurysm

IBD: Identical-by-descent

ICA: Internal carotid artery

LD: Linkage disequilibrium

LDL: Low density lipoprotein

LIMK1: LIM domain kinase 1

LOD: Logarithm of the odds

Mb: Mega bases

MI: Myocardial Infarction

MIA3: Melanoma inhibitory activity family, member 3

MLS: Maximum LOD score

MMD: Moyamoya disease

MMP: Matrix metallo proteinase

MRA: Magnetic resonance angiography

mRNA: Messenger ribonucleic acid

MTHFD1L: Methylenetetrahydrofolate dehydrogenase-1-like protein

NADP: Nicotinamide adenine dinucleotide phosphate

NF: Neurofibromatosis

nFMMD: Non-familiar Moyamoya disease

NPL: Non-parametric logarithm of the odds

OR: Odds ratio

PCR: Polymerase chain reaction

PDGF: Plateled derived growth factor

Pro: Proline

PSR: Phosphatidylserine receptor

PSRC1: Proline/serine-rich coiled-coil 1

RAB40B: Ras-related protein Rab-40B

RAC3: Ras-related C3 botulinum toxin substrate 3

rs: RefSNP = Reference SNP

SDF-1: Stromal cell derived factor 1

SGSH: N-sulfoglucosamine sulfohydrolase

SMC: Smooth muscle cell

SNP: Single nucleotide polymorphism

SORT1: Sortilin 1

Sp1: Specificity protein1 (transcription factor)

SPRI: Solid phase reversible immobilization

SYNGR2: Synaptogyrin 2

T: Thymine

TAAD: Familial thoracic aortic aneurysms and dissections

-ter: Terminal region

TGF: Transforming growth factor

TIA: Transient ischemic attack

TIEG: Transforming growth factor beta inducible early growth response

TIMP: Tissue inhibitor of metalloproteinase

UTR: Un-translated region

VSMC: Vascular smooth muscle cell

WTCCC: Wellcome trust case-control consortium

1. Introduction

1.1 Preface

In this doctoral thesis common genetic variations between Moyamoya disease and atherosclerotic disease in a European cohort are discussed. After an overview on the current knowledge on Moyamoya disease, recent findings on genetic influence factors and our results will be discussed.

1.2 General information on Moyamoya Disease

1.2.1 Description and history

Moyamoya Disease (MMD) is a rare disease with bilateral steno-occlusive changes of terminal portions of the internal carotid arteries (ICA) and their main branches within the circle of Willis, associated with the formation of diffuse fine collaterals (Moyamoya vessels). The typical pathology led to the former description of the disease as a "spontaneous occlusion of the circle of Willis" [21]. The mentioned abnormal vascular networks appear hazy in angiographic imaging, explaining the origin of the name Moyamoya, which is Japanese and means puffy, hazy, obscure or vague.

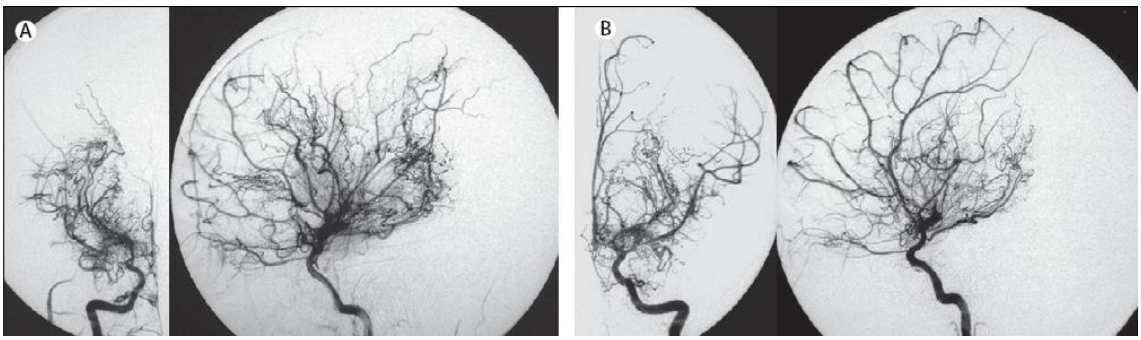


Figure 1: Digital subtraction angiography of a patient with MMD. A) right and B) left internal carotid artery. Source: Kuroda et al. [67]

The Moyamoya appearance was first reported by Takeuchi and Shimizu in 1957 as a hypoplasia of the ICA [140]. Ever since various reports followed until Suzuki proposed the term “Moyamoya disease” in 1969 and classified it into six progressive stages, concerning the progress of the narrowing and the amount of collateral vessels [130].

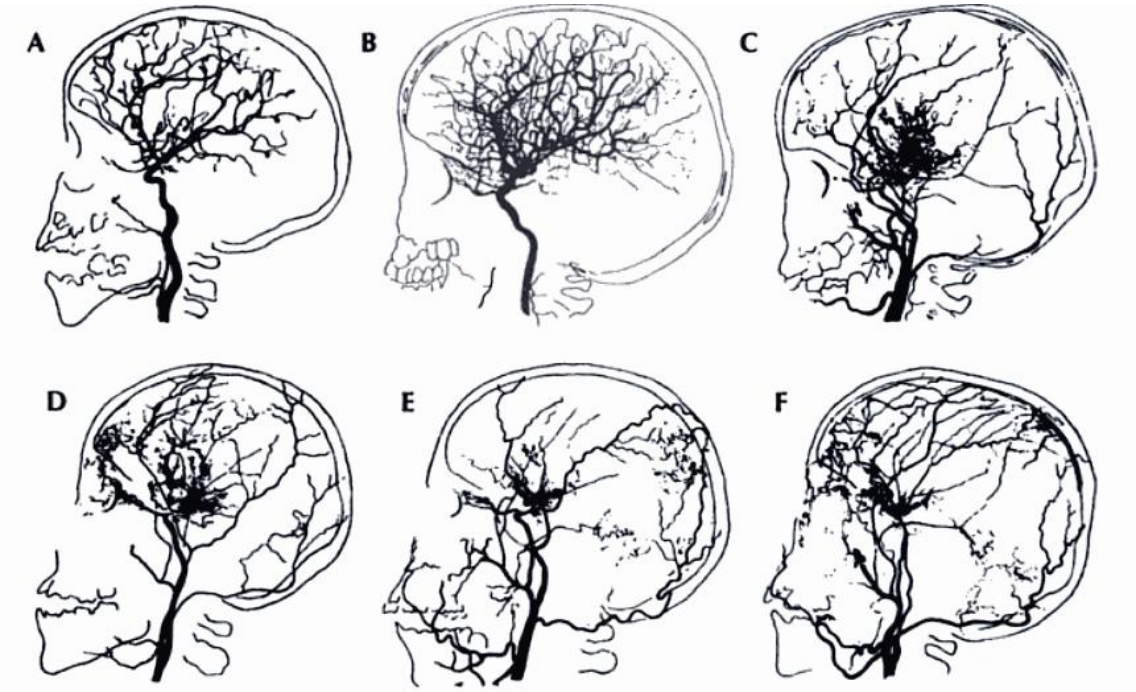


Figure 2: Stages of MMD; A = stage I, B = stage II, C = stage III, D = stage IV, E = stage V, F = stage VI. Source: Ikezaki et al. [47]

Stage	Description
I	***Narrowing of the carotid fork.*** Only the carotid fork stenosis is observed.
II	***Initiation of Moyamoya.*** The carotid fork stenosis and very slight basal Moyamoya are found. Dilatation of all the main cerebral arteries is also observed.
III	***Intensification of Moyamoya.*** Remarkable Moyamoya vessels are seen at the base of the brain. Such Moyamoya vessels are individually quite thick and distinct. Moreover, the disappearance of the middle and anterior cerebral arteries begins to be noticeable.
IV	***Minimization of Moyamoya.*** The Moyamoya vessels become narrow and form a poor network at the base of the brain. Neither the anterior nor the middle cerebral artery is visualized. Moyamoya vessels at the ethmoidal sinus (ethmoidal Moyamoya) conversely increase.
V	***Reduction of Moyamoya.*** Moyamoya vessels at the base of the brain are further narrowed and localized to the vicinity of the carotid siphon. There is also an increase in the collateral pathways from the external carotid arterial system.
VI	***Disappearance of Moyamoya.*** All the main arteries arising from the internal carotid arterial system and Moyamoya vessels completely disappear in angiograms. The cerebrum is maintained only by means of blood flowing from the external carotid or the vertebral arterial system.

Table 1: Suzuki's six stage classification for Moyamoya. Suzuki et al. [130], Ikezaki et al. [47].

In 1997, the Research Committee on the Spontaneous Occlusion of the Circle of Willis (Moyamoya Disease) published guidelines for the diagnosis of MMD. According to these the disease is defined by a stenosis or occlusion of the terminal portions of the ICA or the proximal areas of the anterior or middle cerebral arteries and abnormal vascular networks in the arterial territories near the occlusive or stenotic lesions [22]. Definite cases are only diagnosed if the stenosis is bilateral, unilateral lesions may be diagnosed as probable MMD. Probable cases in childhood are liable to progress to bilateral cases within one or two years, whereas adult unilateral cases tend to remain the way they are [44]. Some patients with known systemic diseases, cerebrovascular diseases

with atherosclerosis, autoimmune diseases, meningitis, brain neoplasia, Down's syndrome, Neurofibromatosis, head trauma, history of cerebral insults, or after cerebral irradiation [67] may present with stenotic or occlusive changes in their major cerebral arteries which may result in collaterals looking angiographically similar to those in MMD. For these cases terms like: quasi Moyamoya disease, Moyamoya syndrome or Moyamoya-like vasculopathy are used [46]. If untreated, Moyamoya disease often has a very poor clinical outcome. Affected children suffer ischemia and transient ischemic attacks (TIAs), whereas adults often present with severe intracranial hemorrhages due to a rupture of the thin collaterals [25].

1.2.2 Etiology

The etiology of MMD is widely unknown. Besides genetic influences, which will be discussed later (Paragraph: Genetics in Moyamoya disease), different assumptions on the etiology of MMD have been published, but none were proven [67]. Epidemiological studies indicated that infection in the head and neck might be implicated in the development of Moyamoya disease, although no specific infectious pathogens have been identified so far [152]. Other than that, histopathological findings such as an increase of different cytokines and growth factors, for example basic fibroblast growth factor (bFGF) or platelet derived growth factor (PDGF), have been described and discussed widely in several publications [165].

As one of the few proven possible risk factors for sporadic MMD, Ullrich et al. [145] published the outcome of 345 children after cranial irradiation in their childhood. The results showed that 12 children developed MMD. This number equals a prevalence of 3.5%, which is much higher than in the general population. So far, more than 60 cases of Moyamoya after cranial irradiation have been reported in the literature [145].

1.2.3 Epidemiology

Epidemiologic data on MMD show a strong variance due to the small incidence, major population differences and possible false positive diagnoses. The following data provide an overview on the major studies.

Epidemiologically MMD has a much higher incidence in Japan, Korea and other Asian countries with 1 out of 1 Million persons per year being affected in Japan, whereas the occurrence in other ethnicities shows lower rates [21].

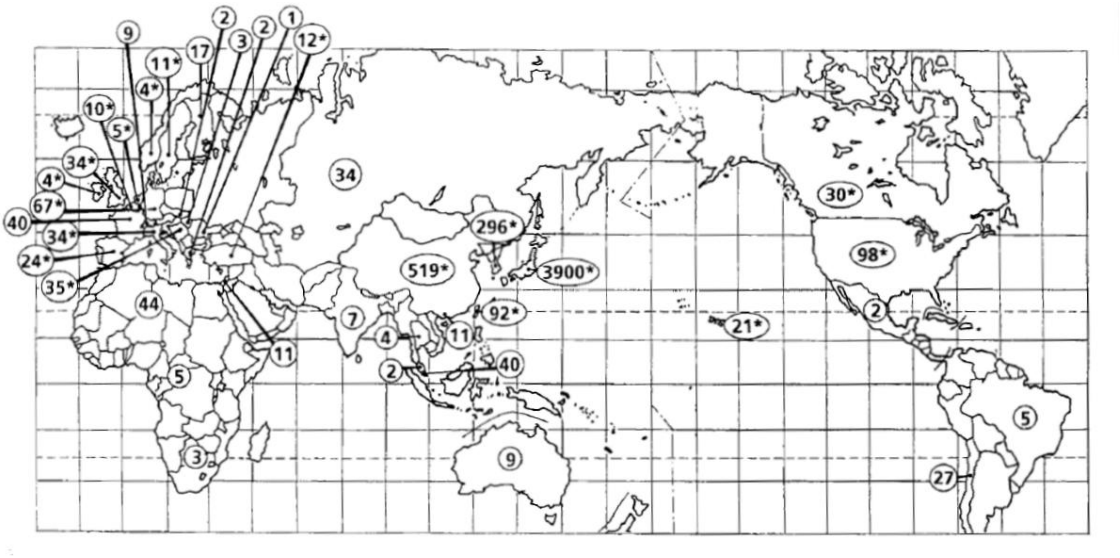


Figure 3: Worldwide distribution of MMD in 1997 (Total Moyamoya cases in literature); Source: Yonekawa et al. [164]

According to the latest numbers of 2008, a total of 7500 Japanese are affected by MMD [51]. A distribution in all age groups, with a two peak predomination in onset at the age group under 10 years and between 30-40 years with a female to male ratio of 1,7:1, was described [21, 48]. With a prevalence of approximately 3 cases per 100,000 children, it is the most common pediatric cerebrovascular disease in Japan [122].

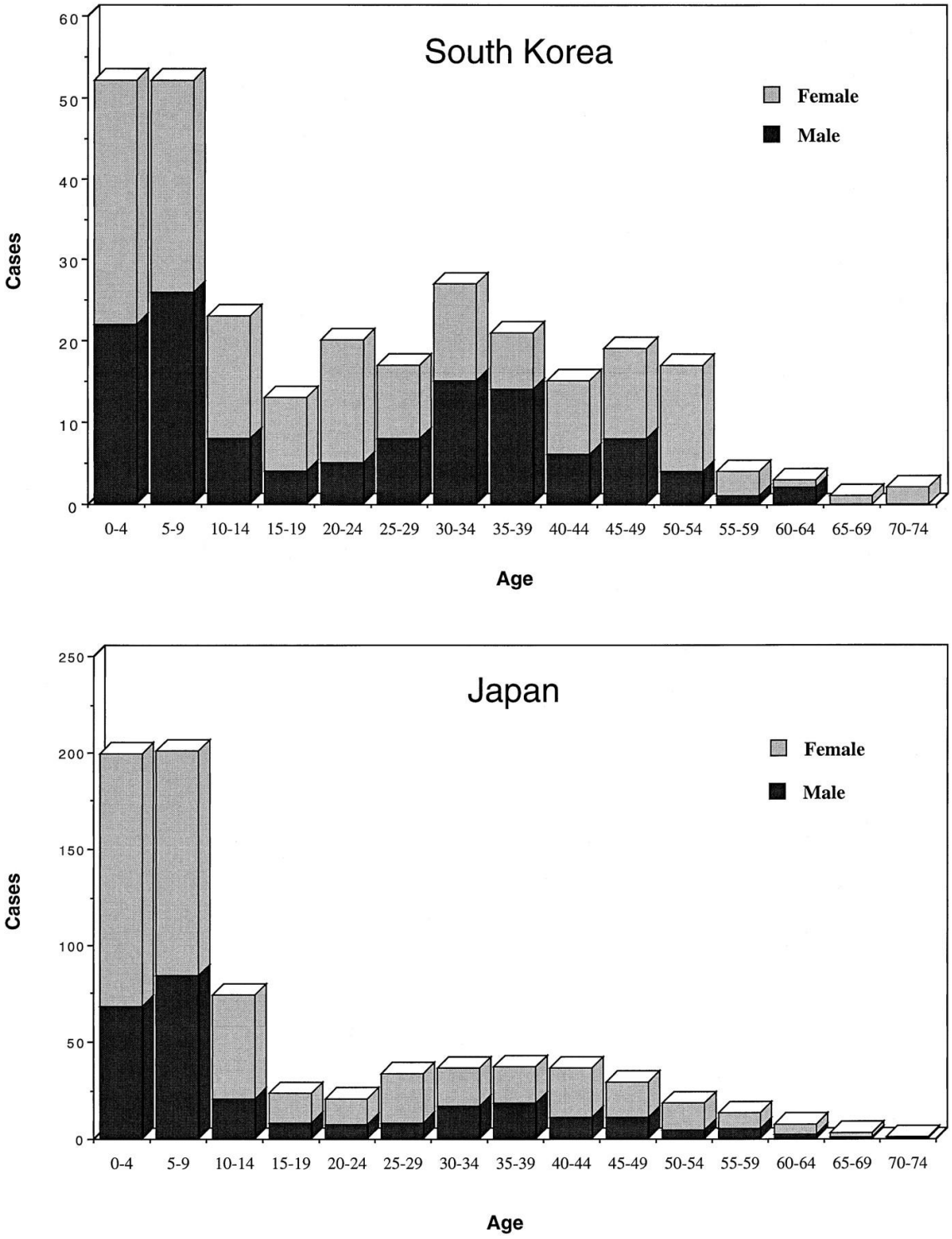


Figure 4: Age distribution in MMD (292 Korean and 731 Japanese cases) Source: Ikezaki et al. [48]

A nationwide epidemiological survey in Japan, published by Wakai et al. [146] in 1995, showed a higher incidence than reported before. In this study the incidence was 0.35 and the prevalence was 3.16 persons affected out of 100.000.

Baba et al. [8] showed a 3-fold increased incidence rate compared to the data published by Wakai et al. [146] as a result of an “all-inclusive survey” on Hokkaido, one of Japan’s major islands in 2008. The numbers showed a detection rate of 0.94 patients per 100.000 persons and a prevalence of 10.5 patients per 100.000 people in Japan.

Numbers from the U.S. (Washington State and California) presented by Uchino et al. [144] in 2005, showed an incidence of 0.086 per 100.000 persons per year in this region.

Comparing the data, we assume an average incidence rate of 1-2 patients per 100.000 in Japan and a 10-fold decreased rate (0.1-0.2 per 100.000) in non Asian-countries. The age of onset of MMD related symptoms was described concordantly by most authors as two peaks at the age of under 10 years and between 30 and 40 years of age (figure 4). The risk of developing MMD was described as being almost two times higher in females than in males [48].

A high incidence of familial occurrence is seen in MMD and accounts for 9-15% of all known cases [23, 100, 162]. So far, 172 familial cases in 76 pedigrees have been reported, including 38 parent-offspring pairs of 16 pedigrees and 128 sib pairs in 51 pedigrees [91-92]. Familial cases show differences in comparison to sporadic cases [67, 92]:

- The ratio of women to men was 5.0 in familial cases, but around 1.6 in sporadic cases.
- The mean age at onset was 11.8 years in familial cases but 30.0 years in sporadic cases.
- The parents presented with symptoms of Moyamoya disease at 22–36 years of age (mean 30.7 years), whereas their offspring presented with

symptoms at 5–11 years of age (mean 7.2 years), which suggests anticipation in familial Moyamoya disease (FMMD).

1.2.4 Pathological findings

A narrowing of the skull base arteries (especially at the terminal portions of the ICAs) mainly due to an intimal thickening caused by non-inflammatory lipid deposits and a migration of smooth muscle cells (SMCs) from the tunica media to the tunica intima (fibrocellular thickening) has been described in MMD patients. In addition a thinning and/or duplication of the internal elastic lamina to a neointima is very common in MMD vessels, making the overall histopathological resemble to the changes seen in atherosclerotic vessels [6, 42, 72, 79, 132-133, 138-139, 160]. The microscopic structure of the thin Moyamoya collaterals are comparable to small dilated perforating arteries, which are in some part newly developed and in the other preexisting [66, 73]. The major histopathological changes consist of fibrin deposits in the wall, fragmented elastic laminae and the formation of microaneurysms, which are probably responsible for the increased number of hemorrhages in MMD. Because of their thin walled structure, collapsed vessels with microthrombi can be found, possibly responsible for ischemia seen in patients with MMD [97, 160].

1.2.5 Symptoms

Children with Moyamoya disease frequently suffer transient ischemic attacks (TIAs) with symptoms of hemi- or monoparesis, epileptic attacks and sensory impairment on alternating sides, but rarely present intracranial hemorrhage. If the ischemia results in cerebral infarction or atrophy, patients may show fixed neurological deficits and mental retardation [28, 95, 120]. In adults, MMD manifestations are characterized by a much higher rate of intracranial hemorrhage, mostly at the common sites of the basal ganglia, ventricular system, medial temporal lobes, and the thalamus [129], which is known to lead to a worsening of prognosis due to the hemorrhages' locations, including lethal outcomes [21]. For many years fine collaterals were accounted for being the source of the hemorrhage, but newer studies suggest adjacent aneurysms to

play the main role [122]. The leading clinical manifestations, including a comparison between children and adults, are listed in the table below.

Clinical manifestation	Children (0-9years) n=431	Adults (30-39 years) n=235
Hemorrhage	21	161
Epilepsy	107	11
Infarction	137	40
Transient ischemic attack	194	23
Others	23	8

Table 2: Clinical manifestations in adults and children; Source: Handa et al. [28]

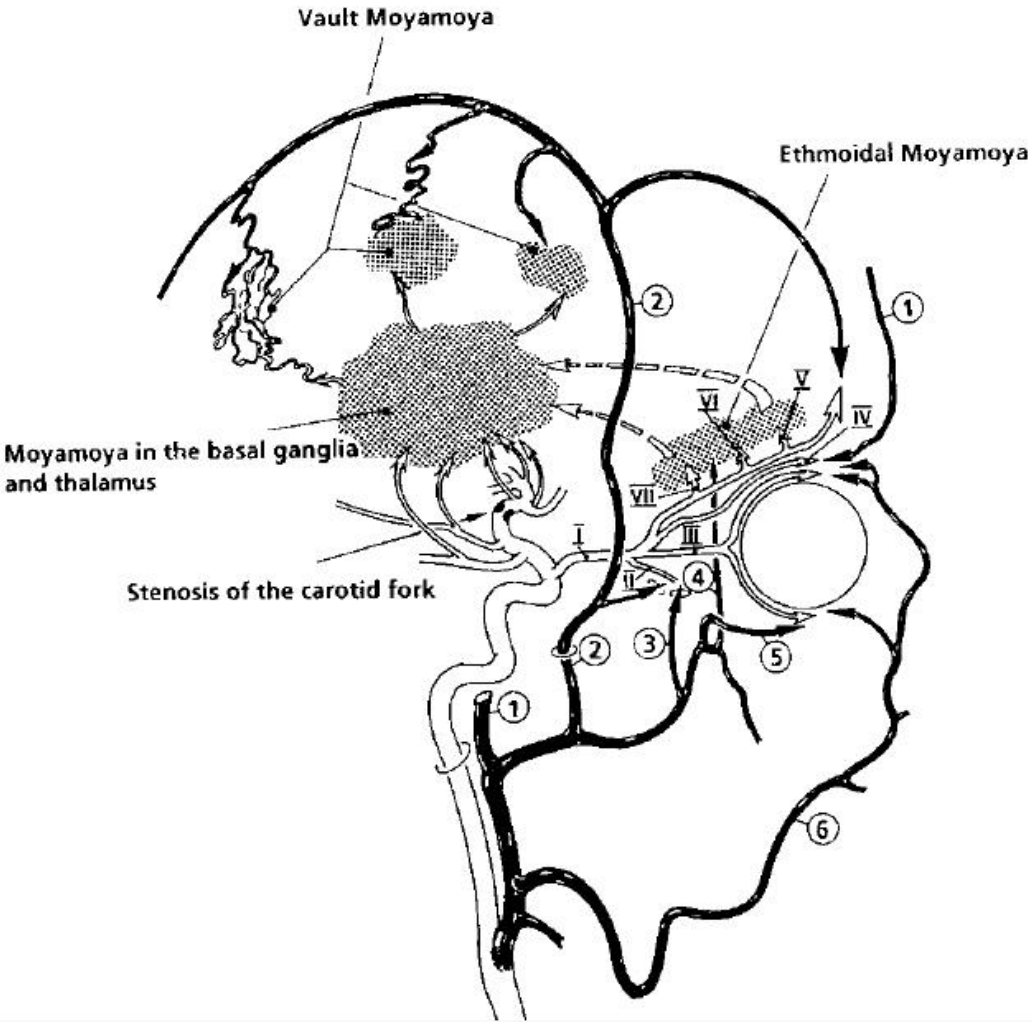
The main minor symptoms in patients with MMD are medication resistant migraine-alike headaches, which may even persist in up to 63% after surgery [125]. The main major symptoms such as motor paresis, headache, convulsion and others are listed in the table below, also comparing the differences between children and adults.

Symptoms	Children (0-9years) n=431	Adults (30-39 years) n=235
Motor paresis	338 (78%)	83 (35%)
Disturbance of consciousness	56 (13%)	123 (52%)
Headache	37 (8.6%)	137 (58%)
Convulsion	113 (26%)	25 (11%)
Psycho-organic syndrome	7 (1.6%)	17 (7.2%)
Speech disturbance	83 (19%)	32 (14%)
Sensory disturbance	44 (10%)	24 (10%)
Involuntary movement	7 (1.6%)	1 (0.4%)
Mental retardation	38 (8.4%)	8 (3.4%)
Visual disturbance	15 (3.5%)	11 (4.7%)
Visual field defect	9 (2.1%)	13 (5.5%)

Table 3: Symptoms in a group of 666 MMD patients; Source: Handa et al. [28]

1.2.6 Diagnostic work-up

MMD is diagnosed by radiographic studies. Because of the disease's rareness, especially in non-Asian countries, several studies are often needed until the correct diagnosis can finally be confirmed. Direct radiological signs are the typical "puff of smoke" collaterals surrounding a narrowed artery, whereas indirect signs may show cerebral hypoperfusion or multiple infarctions. Symptomatic patients are often primarily examined by a cerebral computer tomography (CT), where signs of ischemia can be found. To clarify these findings, usually a magnetic resonance angiography (MRA) is conducted, whereas the most suggestive findings for MMD are diminished flow voids in the internal carotid and middle and anterior cerebral arteries, associated with prominent collateral flow limitation in the basal ganglia and thalamus. These changes are characteristic for MMD [129].



1 = superficial temporal artery, 2 = middle meningeal artery, 3 = deep temporal artery, 4 = sphenopalatinal artery, 5 = infraorbital artery, 6 = facial artery, I = ophthalmic artery, II = lacrimal artery, III = supraorbital artery, IV = frontal middle and dorsal nasal artery, V = anterior meningeal artery, VI = anterior ethmoidal artery, VII = posterior ethmoidal artery

Figure 5: Various types of Moyamoya networks; Source: Yonekawa et al. [164]

As MRA is a non-invasive technique with no radiation exposure and excellent diagnostic results, it has become the gold standard making the diagnosis [12, 60, 110, 136, 153-156]. Nevertheless a cerebral, full four vessel series angiography is needed for exact evaluation of the small collaterals [129]. The pathological changes seen in the angiographic imaging of vessels altered by

MMD is classified into six progressive stages, beginning with slight stenosis without any collaterals at the first stage and ending at the sixth stage with a total ICA stenosis where the cerebral perfusion is only possible through the now very prominent collaterals (table 1) [130].

Further evaluation of the disease is possible by performing electroencephalography or cerebral blood flow studies by transcranial doppler ultrasonography, xenon enhanced CT, positron emission tomography, or single photon emission computed tomography with acetazolamide challenge, whereas all these techniques may give additional information, but are of no diagnostic value in comparison to the MRA and angiography [65, 70, 74, 88, 90, 128-129, 137, 141]. An algorithm of diagnosis for MMD is given in table 6.

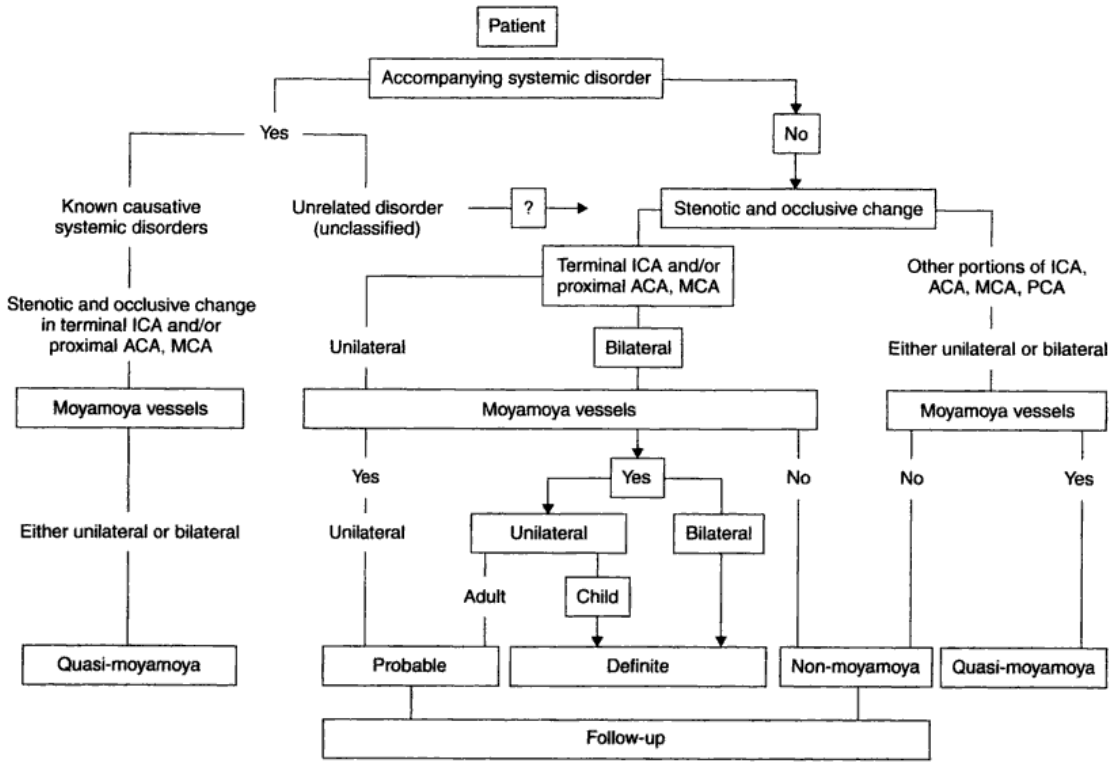


Figure 6: Algorithm of diagnosis; Source: Ikezaki et al. [45]

1.2.7 Therapy

To this day, no therapeutic option is known for healing MMD. Several possibilities of ameliorating the disease's progress are known. The conservative treatment consists of the antiplatelet effect of acetylsalicylic acid (ASA), preventing microthrombotic events which could lead to TIAs or ischemia [95, 120-121, 123]. Calcium channel blockers ease headaches and supposedly lower the rate and severity of TIAs [120, 129].

Surgical treatment lowers the frequency of disease related symptoms and complications. All surgical approaches have the common goal of improving the blood flow to hypoperfused regions by using the external carotid circulation as the donor supply [95, 123].

The main techniques can be divided in two groups, direct and indirect bypasses. Direct anastomosis between the superficial temporal artery and the middle cerebral artery instantly achieves an improvement in the local hypoperfused region, but offers several surgical disadvantages, such as too short vessels and diffuse postoperative strokes.

Indirect anastomotic procedures consist of three major techniques. [40, 61, 81]

- For an encephaloduroarteriosynagiosis (EDAS) the superficial temporal artery is dissected free for small distance of a few centimeters and then sutured to the open dura mater. A variation to this technique describes the suture of the vessel´s adventitia directly to the pia mater, in order to overcome one barrier, the arachnoid mater, to improve revascularization [129].
- Encephalomyosynangiosis (EMS) describes the process of placing the dissected temporal muscle on the surface of the brain in order to stimulate the development of collateral vessels.
- Both techniques combined are called encephalomyoarteriosynangiosis (EMAS).

Several other, less frequently used, procedures such as drilling burr holes [41, 124], inversion of or splitting parts of the dura mater [16, 58], cervical sympathectomy and omental transposition have been described [30, 55-56, 81-82, 96, 115, 131, 143, 166-167]. The outcome of the operative procedures may

be connected to the amount of angiogenic factors, such as basic fibroblast growth factor, which are significantly elevated in the cerebrospinal fluid of patients with MMD [78, 87, 135].

1.3 Genetics in Moyamoya disease

Several factors, such as familial occurrence of MMD in 9-15% of all cases, a much higher incidence in Asian countries and the reported coincidence of MMD with other genetically caused diseases may indicate a strong genetic component in the disease's development [23, 67, 92, 100, 129, 162].

In 1999, Ikeda et al. [43] performed a "total genome search" with the DNA of 16 families (77 members, 28 males and 49 females). For 37 of the 77 individuals the diagnosis was confirmed according to the guidelines for the diagnosis of Moyamoya disease set by the Research Committee on Spontaneous Occlusion of the Circle of Willis of the Ministry of Health and Welfare of Japan [48]. The first trial with eight families covered 371 polymorphic microsatellite markers spanning all 22 autosomes. Two point LOD scores were calculated under the assumption of either autosomal recessive inheritance or autosomal dominant inheritance (incomplete penetrance). The findings showed a logarithm of the odds (LOD) score greater than one on the loci D3S2387 (LOD= 1.493576 at a recombination fraction of 0.1 (nonparametric LOD [NPL] score 2.31, information content 0.848)) and D3S3050 (LOD=3.184154 at a recombination fraction of 0.01 (NPL score 1.80, information content 0.892)). Nonparametric linkage analysis showed a NPL score of 11.96 at loci between D3S2387 and D3S3050 and an information content of 0.785–0.892. These two loci are both on chromosome 3p24.2-p26.

For the study of the eight additional families, four markers (D3S2387, D3S3050, D3S1560, and D3S1304) at 3p24.2-p26 were used. A nonparametric analysis revealed a significant linkage at D3S3050 (NPL score 3.46, information content 0.851).

In the genetic region at 3p24.2-p26, genes associated with the Marfan Syndrome, a disease with weakness of the connective tissue, and the von

Hippel Lindau Syndrome, a disease associated with hemangioblastomas, has been described before [14, 69]. These associations make this a region of high interest concerning vascular diseases.

Inoue et al. [50] stated an association between the Human Leukocyte Antigen (HLA) and MMD. For further investigation, a linkage study with 15 microsatellite markers on chromosome 6, where the *HLA* gene is located, was performed on 20 affected siblings and their families [49]. All patients were Japanese individuals with diagnosed definite Moyamoya disease according to the 1995 guidelines of the Research Committee on the Spontaneous Occlusion of the Circle of Willis of the Ministry of Health and Welfare, Japan [22]. The marker D6S441 on Chromosome 6 showed a positive linkage with an identical-by-descent (IBD) value of one for 12 sibling pairs and two for eight sibling pairs (IBD: 0:12:8). Sharing of the identical allele was evaluated in 19 families, in terms of the haplotype of these adjoining markers. In 16 of these 19 families (82%), D6S441 was shared between the affected members; in two families, it was not. Sharing of the allele could not be determined in one family because of low heterozygosity.

Yamauchi et al. [163] assumed the association between the gene for Neurofibromatosis type 1, located on Chromosome 17q11.2 and MMD [151]. Yet in more than 50 cases MMD in patients with NF1 has been described [9, 19, 68, 100, 150]. Yamauchi et al. [163] assumed that the NF1 protein increases the growth of endothelial and smooth muscle cells in the internal carotid arteries and their branches. Although this assumption could not be confirmed, several indications for an association of MMD with genes on Chromosome 17q25 were found. Linkage analysis was performed with samples of 56 MMD patients out of 24 families. A two-point linkage analysis under the assumption of a dominant transmission model gave a maximum LOD score of 3.11 at the recombination fraction of 0.00 for the marker at locus D17S939. Furthermore the examination of 22 microsatellite markers from the 9-cM region between D17S785 and D17S836 showed LOD scores up to 4.58 in multipoint linkage studies, showing a possible association between MMD and 17q25. With the “affected pedigree

member" method, the linkage was confirmed at a significantly low P value (*P* <0.00001).

Sakurai et al. [111] performed a linkage analysis with 428 microsatellite markers screening 12 nuclear families with MMD affected sib pairs (46 members). All patients (24) were diagnosed with definite Moyamoya disease. The results showed significant evidence for linkage to Chromosome 8q23 (D8S546) with a maximum LOD score (MLS) of 3.6 and suggested evidence to Chromosome 12p12 (D12S1690) with a MLS score of 2.3. This study also presented MLS scores of 1.7, 1.6 and 1.3 for the formerly described markers on Chromosomes 3p, 6q and 7q [43], [49], [163], which was not quite enough to reach suggestive level for linkage, probably as a result of the smaller sample size in this study. Considering disease related genes in the newly found regions with positive linkage, the transforming growth factor-beta-inducible early growth response (*TIEG*) gene, located on 8q22.3, may be a possible candidate gene for the genesis of MMD.

Nanba et al. [93] performed sequence analysis of the 9-cM region between D17S785 and D17S836 on Chromosome 17q25, that was described as a susceptible region for a MMD gene by Yamauchi et al. [163] before. The DNA of one family with familiar MMD (9 members, 4 affected), which showed a close linkage to 17q25 in former analysis, was screened [163]. At the time of the study 65 genes were identified in this region, out of which the authors picked nine to be sequenced. *DNAI2* Dynein, axonemal, intermediate polypeptide 2 (formation of cell skeleton); *AANAT* Arylalkylamine N-acetyltransferase (Expressed in the brain, pineal gland, eyes, and kidneys); *PSR* Phosphatidylserine receptor (recognizing the surface of apoptotic cells); *HCNGP* Transcriptional regulator protein (Transcriptional regulator protein); *HN1* Hematological and neurological expressed 1 (Expressed in the hematopoietic cells and the brain of mice); *SGSH* N-sulfoglucosamine sulfohydrolase (Relation of central nervous disorders); *SYNGR2* Synaptogyrin 2 (Transferring substances through membranes); *EVPL* Envoplakin (Cell

attachment); *TIMP2* Tissue inhibitor of metalloproteinase 2 (maintenance of extracellular matrix). This sequence analysis could not detect any mutations related to MMD in the nine genes nor a novel candidate gene using the expressed sequence tag (EST) technique. Although there have been no MMD related findings, Chromosome 17q25 stays susceptible for a possible MMD gene, due to the fact, that linkage analysis was only able to narrow down the susceptible region to 9Mbs, out of which only a small fraction was analyzed.

Kang et al. [54] investigated single nucleotide polymorphisms (SNPs) and the DNA sequences of exons, introns and promoter regions of the Tissue Inhibitor of Metalloproteinase (*TIMP*) *2* and *4* genes, which span 3p24.2-p26 and 17q25, both susceptible loci for MMD genes [43, 163]. The study was performed with the DNA of eleven Korean familiar MMD patients (FMMD), 50 non familiar MMD patients (nFMMD) and 50 non MMD controls. The most significant result of this study was a higher presence of a G/C heterozygous genotype at position -418 (GenBank reference sequence U44381; FMMD versus non-familial MMD: odds ratio = 9.56, 95% confidence interval = 1.85–49.48, P = 0.005; and FMMD versus non-MMD: odds ratio = 10.50, 95% confidence interval = 2.02–54.55 P = 0.001). This polymorphism is located at the third base of a Sp1 binding site in the promoter region of *TIMP2*, underlining the suggestion of 17q25 being a susceptible locus for MMD. TIMPs are proteins interacting with Matrix Metalloproteinases, which in case of malfunctioning may lead to changes in the extracellular matrix (ECM), erroneous smooth muscle cell (SMC) migration and secretion in the arterial walls [53]. Additionally to the maintenance of the ECM, TIMPs / MMPs are also known to play a major role in vascular injury and repair processes [18, 29, 127]. Especially at the main locations of arterial thickening in MMD vessels, at the terminal portions of the internal carotid artery, shear stress is very high. Therefore these spots seem to be a reasonable location for continuing lesions and repair processes which could lead to an intimal thickening in case of functional defects [102].

Mineharu et al. [85] analyzed 15 highly affected families with at least three MMD patients. Overall, 52 familiar MMD patients were included in this study for reasons of determining the inheritance pattern and clinical characteristics of FMMD. The results showed an autosomal dominant mode of inheritance with incomplete penetrance for FMMD.

In 2008, Mineharu et al. [84] published the results of a parametric genome-wide linkage analysis (382 markers on all 22 autosomes and 18 on the X-chromosome) of 15 Japanese families with 55 affected patients. Additionally a total of 17 microsatellite markers were genotyped at 12.5-Mb intervals at the 17q25-qter linkage region. Under the aspect of a broad classification of MMD, a maximum multipoint LOD score of 8.07 and 6.57 under a narrow classification at Chromosome 17q25.3 (D17S704) was found. In 14 out of the 15 families a segregation of the disease haplotype was observed in all affected individuals. Additionally, after defining the candidate interval to a distance of about 3.5 Mb between D17S1806 and the telomere of 17q by an informative crossover and selecting four candidate genes (*BAIAP2, TIMP2, RAC3, RAB40B*) out of 94 known genes in this region, a mutation analysis on respectively one member of five families was performed without showing any significant mutations related to MMD. The change of the *TIMP2* promoter SNP at position -418, which was significant in Korean MMD patients [54], could not be replicated in this study with Japanese MMD patients.

Guo et al. [27] published the association of heterozygous mutations in the *ACTA2* gene in twenty families (127 members) with familial thoracic aortic aneurysms and dissection (TAAD). Exact phenotyping of these families revealed a high incidence of vascular diseases like coronary artery disease, stroke or Moyamoya disease. Five of the 127 examined patients suffered an early-onset (between five and forty-six years) stroke, caused by Moyamoya disease. Interestingly the genotyping revealed a common mutation at the position R258H/C in seven members of three unrelated families with early onset

stroke and MMD, leading to the assumption that *ACTA2* mutations may lead to ischemic strokes.

In 2009, Liu et al. [75] expanded their in 2008 reported linkage analysis by two Japanese families increasing the LOD score from 8.07 to 9.67 on 17q25.3. This region contains 40 genes, out which 4 (*CARD14, Raptor, AATK, BAHCC1*) were chosen for sequencing in four unrelated affected individuals. As result the polymorphism ss161110142 G/A in the *Raptor* gene (position -1480 from the transcription site of the Raptor gene) appeared to be of interest because of all four sequenced affected individuals were found to be heterozygous. This SNP was genotyped in 34 families showing that every affected individual carried the rare A allele. All affected individuals showed complete segregation, the calculated two point LOD score was 14.2 ($P = 3.89\ 9\ 10^{-8}$). As the last stage, a case-control study on ss161110142 G/A 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) patients with non-FMMD was performed. As result, the rare allele (A) was much more frequent in the cases (26% Japanese, 33% Korean, and 4% Chinese) than in controls (1, 1, and 0%, respectively) and was associated with an increased odds ratio of 52.2 (95% confidence interval 27.2–100.2) ($P = 2.59\ 10^{-49}$). However, the rare A allele was not detected in the Caucasian samples. 21 of the 25 Caucasian samples were also part of our study.

The table below gives an overview of all genetic studies on MMD up to date.

Author	Samples	Study	Screening	Findings on Chromosome	Finding/Marker	Result	Adjacent genes
Ikeda et al. 1999	37 affected persons out of 16 families	Linkage analysis	Total genome search for 371 polymorphic microsatellite markers spanning all 22 autosomes	3p24.2-p26	D3S3050	NPL score 3.46	Marfan Syndrome, Hippel Lindau Syndrome
Inoue et al. 2000	20 affected sibling pairs and their families	Linkage analysis	15 Microsatellite markers on Chromosome 6	Chromosome 6	D6S441	IBD: 0:12:8; Shared Haplotype among 16 of 19 families (82%)	*HLA*
Yamauchi et al. 2000	24 families containing 56 patients with MMD	Linkage analysis	22 Microsatellite markers on Chromosome 17q25	17q25	D17S939; 9-cM region between D17S785 and D17S836	D17S939: LOD score of 3.11; 9-cM region between D17S785 and D17S836: LOD scores up to 4.58	NF1
Sakurai et al. 2004	12 nuclear families, containing 12 affected sibling pairs	Linkage analysis	428 microsatellite markers	8q23, 12p12	D8S546, D12S1690	8q23 (D8S546): MLS score 3.6 ; 12p12 (D12S1690): MLS score of 2.3	*TIEG*
Nanba et al. 2005	1 affected family with 9 members, 4 affected	Sequence analysis, bioinformatics (EST)	9 candidate genes from the 9-cM region betweenD17S785–D17S836 in chromosome 17q25	none	none	No MMD related variations were found	*DNAI2, AANAT, PSR, HCNGP, HN1, SGSH, SYNGR2, EVPL, TIMP2*

Author	Samples	Study	Screening	Findings on Chromosome	Finding/Marker	Result	Adjacent genes
Kang et al. 2006	11 familiar MMD patients vs. 50 non familiar MMD patient controls, 50 healthy controls	Sequencing and SNP analysis	*TIMP 4 and TIMP2* on chromosomes 3p24.2-p26 and 17q25	17q25	G/C SNP at *TIMP2* promoter region, position -418 (rs8179090)	G/C heterozygous genotype at position - 418 in nine of 11 patients with FMMD, in 16 out of 50 non-familial MMD controls, and in 14 out of 50 non-MMD controls (FMMD vs. non-familial MMD: OR= 9.56; 95% confidence interval, 1.85–49.48; P =0.005; and FMMD vs. non-MMD: OR= 10.50; 95% confidence interval, 2.02–54.55; P=0.001)	*TIMP2, TIMP4*
Mineharu et al. 2006	15 highly affected families; 52 familiar MMD patients	Determination of inheritance pattern by studying affected families	none	none	none	Autosomal dominant mode of inheritance with incomplete penetrance	none
Mineharu et al. 2008	15 highly affected families; 55 familiar MMD patients	Genome-wide parametric linkage analysis	382 markers on all 22 autosomes and 18 on the X-chromosome	17q25.3	D17S704; Candidate interval 3.5 Mb between D17S1806 and the telomere of 17q	Maximum multipoint LOD score of 8.07 (broad classification) and 6.57 (narrow classification)at Chromosome 17q25.3 (D17S704)	*BAIAP2, TIMP2, RAC3, RAB40B*

Author	Samples	Study	Screening	Findings on Chromosome	Finding/Marker	Result	Adjacent genes
Guo et al. 2009	20 families (127 members) with familiar TAAD, including five patients with MMD	Linkage analysis, association studies, sequencing	Whole genome, *ACTA2*	10q23.3	R258H/C mutation in Exon 7 of ACTA2	Mutation at the position R258H/C in seven members of three unrelated families with early onset stroke and MMD	*FAS*, LOC100132116, *STAMBPL1*
Liu et al. 2009	37 families from Japan and Korea; 90Japanese, 41 Korean, 23 Chinese, 25 Caucasian non-FMMD samples	Segregation analysis followed by a case control study on one SNP	Linkage: Genome-wide, as described in Mineharu et al. 2008; Candidate gene sequencing on 17q25.3 (CARD14, *Raptor, AATK*, BAHCC1); Case control study on ss161110142 G/A with nFMMD	17q25.3	ss161110142 G/A in the *Raptor* gene	Increasing the LOD score from 8.07 to 9.67 on 17q25.3; Increased frequency of the rare A allele in the Asian cases with an odds ratio of 52.2 (95% confidence interval 27.2–100.2) (P=2.59*10^-49)	*CARD14, Raptor, AATK, BAHCC1*

Table 4: Genetic findings in MMD

1.4 Determination of Single Nucleotide Polymorphisms

As described, genetics seem to be the most promising approach for a better understanding of MMD. Seventeen SNPs were genotyped for association with Moyamoya. The study started with an extensive literature review on previous genetic and histopathological findings on MMD. Previous findings were limited to the description genetic loci as regions of interest for MMD [27, 43, 49, 75, 84-85, 93, 111, 163], and two specific polymorphisms (ss161110142, TIMP2 promoter -418bp (rs8179090)) on 17q25 [54, 75]. Concerning histopathological features of MMD, several publications described and discussed similarities between MMD and atherosclerotic changes [6, 42, 72, 79, 132-133, 138-139, 160]. Based on these assumptions we analyzed histopathologic and genetic findings on atherosclerosis. We found that both diseases share similarities in microscopic changes and pathological mechanisms, even all the way back to one of the key pathways of atherogenesis, described in 1977 by Ross et al.[108]: “The response to injury hypothesis”. The genetic knowledge on atherosclerotic diseases was much more elaborate than on MMD. Several genome-wide association studies (GWAS) reported different loci and SNPs as being strongly associated with different forms of atherosclerotic diseases [31, 83, 113, 142]. We used this knowledge to design our study trying to replicate SNPs, which have been described to be associated with atherosclerosis, in patients with Moyamoya disease. We also included the SNP in the promoter region of *TIMP2* which has been described as being associated with MMD by Kang et al. [54]. TIMPs are known to play a role in the repair processes after vascular injury [18, 29, 127] and therefore support our thesis on atherogenesis-alike processes in MMD. We also included four SNPs from a locus containing the *Elastin* gene, which were described by Akagawa et al. [2] as being associated with intracranial aneurysms. MMD has been described together with intracranial aneurysms (IAs) [3, 62, 77, 89, 98] and secondly, according to Karnik et al. [57], Elastin plays a key role in the signaling of vascular proliferative diseases where abnormal proliferation and migration of vascular smooth muscle cells into the arterial lumen leads to neointimal formation and vascular stenosis. These processes are explained in detail later.

In conclusion, we picked out SNPs associated with vascular abnormalities, mainly atherosclerosis, and genotyped them in our MMD samples, as we think that common genetic variations between MMD and atherosclerosis may be likely.

The SNPs were summarized in four groups:

- *Genetic Polymorphism in the TIMP2 gene*: Describing the SNP reported in the study by Kang et al. [54] concerning the *TIMP2* gene in MMD patients.
- *Genetic Polymorphisms related to the Elastin gene*: Describing SNPs reported by Akagawa et al. [2] concerning polymorphisms in the region of the *Elastin* gene in patients with intracranial aneurysms.
- *Genetic Polymorphisms related to Atherosclerotic Diseases*: Describing SNPs reported by Samani et al. [113], Helgadottir et al. [32] and McPherson et al. [83], concerning polymorphisms associated with atherosclerotic disease.
- *Genetic Polymorphisms related to Atherosclerotic Cardiac Allograft Vasculopathy*: Describing SNPs reported by Tambur et al. [142], concerning Cardiac Allograft Vasculopathy.

1.4.1 Genetic Polymorphisms related to the TIMP2 gene

Although histopathological pathways leading to MMD are still not understood, the most common change seems to involve an excessive migration of SMCs from the tunica media to the tunica intima, leading to a thickening of the arterial wall [63]. Matrix metalloproteinases (MMP), regulated by tissue inhibitors of matrix metalloproteinases (TIMP), play an important role concerning the mobilization of SMCs and turnover of ECM components, as well as in the formation of a neointima and a thickened tunica intima caused by SMCs. Besides their physiological function, MMPs and TIMPs are responsible for the activation of SMCs to synthesize ECM components and for the induction of intimal hyperplasia after vascular injury and the following repair processes [18, 29, 127]. At the most frequent spots of MMD, the terminal ICAs, continuing shear stress caused by high blood flow may lead to an increase of micro injuries

and subsequent repair processes, in which a malfunction of the MMP/TIMP system may lead to stenosis and in turn to MMD [54]. The genes for *TIMP4* and *TIMP2* are located at 3p25 and 17q25. Both chromosomal locations are linked to familiar MMD [43, 163]. Kang et al. [54] investigated these genes in a Korean population with MMD searching for mutations and found an associated in the promoter region of *TIMP2* at an SP1 binding site. A decreased amount of this proteinase may lead to intimal thickening in patients with MMD [54]. Although the genetic findings in the promoter region of *TIMP2* (promoter-418bp; rs8179090) could not be replicated as shown in the latest study by Mineharu et al. [84], we decided to examine this SNP in our study population.

1.4.2 Genetic Polymorphisms related to the *Elastin* gene:

Several publications focused on histopathological findings and differences, such as intimal thickening, hyperplasia, and irregularities in the internal elastic lamina of peripheral and intracranial vessels of patients with MMD in comparison to non-MMD samples [6, 42, 72, 79, 132, 138-139, 160]. Concordantly, all authors described significant intimal thickening with multilayered elastic fibers and smooth muscle cells, whereas the tunica media and tunica intima showed a decrease of these cells. Takebayashi et al. [138] described this phenomenon closer by describing 15-30 SMC layers in the compact cellular zone near the lumen in arteries of patients with MMD, but only few scattered SMCs would be expected. Takebayashi et al. [138] also described the tunica media in normotensive control patients to have an average thickness of 80µm with multiple layers of SMCs, whereas the arteries of MMD patients show an average tunica media thickness of 70µm and that they only have two to six smooth muscle cell layers. Also ultrastructural changes in the tunica media were described as “moth eaten” changes by Takebayashi et al. [138]. These changes are alike to changes in the arterial wall of hypertensive patients with severe intracranial hemorrhages and to pathological changes seen in atherosclerosis. Similar changes were recently described in a study by Takagi et al. [133].

According to Karnik et al. [57] Elastin plays a key role in the signaling of vascular proliferative diseases where abnormal proliferation and migration of

vascular smooth muscle cells into the arterial lumen leads to neointimal formation and vascular stenosis. Karnik et al. [57] showed that: 1) In vitro Elastin responsible for the inhibition of vascular smooth muscle cell (VSMC) proliferation comparing *ELN* (+/+) with *ELN* (-/-) cells and artificially supplying exogenous recombinant tropoelastin, which is the precursor protein of Elastin. 2) Elastin is needed for the correct association of highly organized actin myofilaments, also called actin stress fibers. 3) Elastin controls the migration of VSMCs in vascular proliferative diseases (VSMCs move from the tunica media to the subendothelial space building a neointima) [118]. Karnik et al. [57] stated that an Elastin matrix layer around each concentric layer of VSMCS provides an autocrine signal to these cells to stay in the tunica media and not to migrate towards the newly built neointima. Their study showed that *ELN* (-/-) cells have a much smaller migrational activity, but a much higher (fourfold increased) chemotactical activity towards PDGF, which is thought to be responsible for the subendothelial migration of VSMCs in occlusive vascular lesions [76]. Yamamoto et al. [159] showed differences in the response on mitogenic stimuli (including PDGF) of arterial smooth muscle cells from patients with MMD. The results showed that SMCs from MMD patients migrated and grew faster after being stimulated by PDGF than SMC from controls. This correlates with Karnik's [57] findings concerning a fourfold increased chemotactical activity of VSMCs towards PDGF in *ELN* (-/-) cells under the assumption of a genetic defect in the Elastin gene in MMD patients.
Karnik et al. [57] also showed that Elastin reduces the vascular proliferative response after arterial injury in vivo by using an Elastin coated stent versus a control stent in a porcine model.

The presence of intracranial aneurysms in patients with MMD has been reported [3, 62, 77, 89, 98]. This phenomenon can be explained by an increased blood flow through the collaterals and its negative effect on the walls of the vessels or the fact that genetic aberrations could cause structural malformations [25]. Because of the frequency of a parallel appearance of MMD

and IA we decided to genotype SNPs in the *Elastin* gene, which have been shown to be associated with IAs by Akagawa et al. [2].

Akagawa et al. [2] performed a SNP finemapping of 168 SNPs of the 4.6Mb linkage region around D7S2472 on 7q11 which was reported to show strong linkage with LOD scores >1 in affected Japanese sib pairs with intracranial aneurysms [99], and Utah residents of European origin [20], but not in a study with another Japanese cohort [157]. The results of Akagawa's study showed strong linkage to IAs in a linkage disequilibrium (LD) block spanning the promoter region of *LIMK1* (LIM domain kinase gene 1) and the 3'end of *ELN* (Elastin gene), which had been screened for allelic association with IAs before [37, 99, 109]. The most significant association was observed for the *ELN* 3'-UTR G(+659)C SNP (rs8326) with an odds ratio of 3.11. The *ELN* 3'-UTR (+502)->A insertion SNP (rs34208922) showed a significant association with an odds ratio of 1.38 in the *Elastin* gene. In the promoter region of *LIMK1*, the promoter G(-961)A SNP (rs6460071), the promoter G(-428)A SNP (rs710968) and the promoter C(-187)T SNP (rs6460071) showed strong linkage with odds ratios between 1.61 and 1.93. The 3' UTR region of *ELN* is known to play a key role in posttranscriptional processing of *Elastin* in chicken specimen [33-34] and the *LIMK1* promoter region may play a role in transcriptional activity. For more information on the impact of these polymorphisms, Akagawa et al. [2] performed in vitro and ex vivo analyses, showing the functional impact of the described SNPs. As result, a decrease of transcript levels either through accelerated *ELN* mRNA degradation or through decreased *LIMK1* promoter activity was proven.

Because of described histopathological findings on Elastin in MMD vessels and the coincidence of MMD with IAs, we included the *ELN* and *LIMK1* SNPs rs8326 [C/G], rs34208922 [-/A], rs710968 [C/T], rs6460071 [G/A], described by Akagawa et al. [2] as being associated with IAs, in our study.

1.4.3 Genetic Polymorphisms related to Atherosclerotic Diseases

Several authors described histopathological findings in vessels of patients with MMD as similar to those in atherosclerotic arteries and suggested common mechanisms in the formation of stenosis [1, 80, 134, 159, 161]. An overview of control mechanisms involved in atherogenesis is given in figure 7.

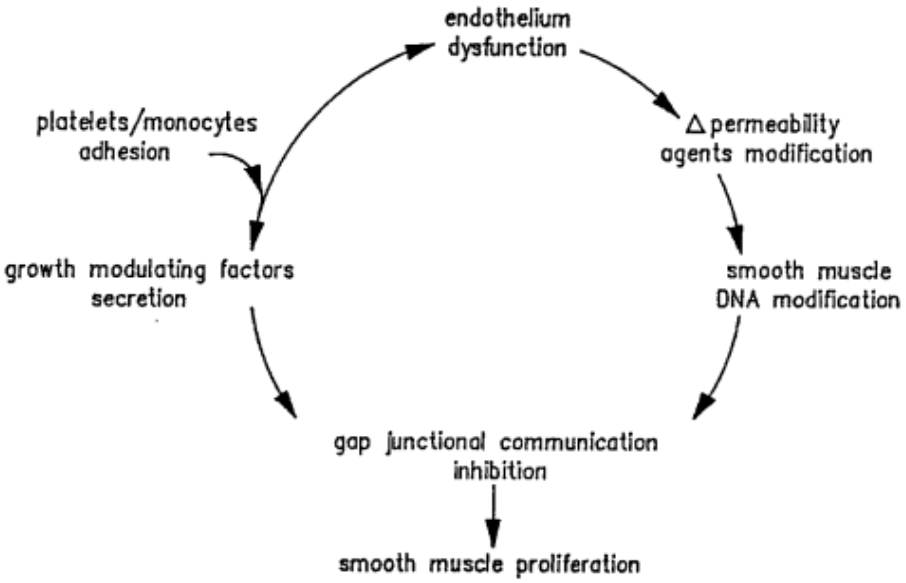


Figure 7: Atherogenesis; Source: Zwijsen et al. [170]

Masuda et al. [80] analyzed immunohistochemical stainings of MMD vessels and described phenotypic modulations in the formation of a neointima of SMCs, which were described among changes in atherosclerotic plaques before [101]. These changes were mainly composed of a SMC-migration-caused thinning of the tunica media, combined with a thickening of the tunica intima and the formation of a neointima. Therefore the author suggested that the migration and proliferation of SMCs in MMD might be due to atherosclerosis common mechanisms.

Yamashita et al. [161] discussed “intimal pads” [39, 97, 160] in MMD vessels as being similar to those seen in the beginning of the genesis of atherosclerotic plaques. He additionally accounts for this by assuming a common mechanism in the pathogenesis of MMD and atherosclerosis according to the “response to injury” process after endothelial damage, which was described by Ross et al.

[108] as one of the important processes in atherogenesis. This process mainly consists of platelets which adhere to denuded intimal surface and induce the migration of SMCs from the tunica media to the tunica intima with chemotactical PDGF.

This goes along with findings by Yamamoto et al. [159], who showed a higher chemotactical sensitivity of SMCs from MMD patients towards PDGF than in SMCs from healthy controls.

Takagi et al. [134] stated that the hypertrophic tunica intima is made of SMCs, as seen in atherosclerotic lesions, and suggested a similar mechanism of SMCs changing their nature while building a neointima similar to that seen in atherosclerotic plaques.

Yamamoto et al. [159] proposed that lower migrational response towards IL1beta (Interleukin 1 beta) and lower mitogenic response towards PDGF in SMCs of MMD patients may be responsible for a delayed repair process in injured arteries. This results in an extended permeability for blood constituents, such as cytokines and growth factors, which are responsible for the repair process. Because of the extended exposition, higher amounts of these mediators could diffuse towards the SMCs and may lead to a thickening of the tunica intima in MMD vessels. This key process in the development of arterial stenosis of vessels induced by MMD was postulated to possibly be connected with the process of overgrowth of SMCs in atherosclerosis after injury.

In conclusion to these histopathological findings and assumptions we decided to investigate genetic regions associated with the genesis of atherosclerosis, in MMD patients. The chosen SNPs were selected by reviewing the literature on genetic studies on atherosclerotic diseases such as coronary heart disease and myocardial infarction [32, 83, 113].

Samani et al. [113] performed a “Genome-wide Association Analysis of Coronary Artery Disease“ with approximately 3,000 cases (Wellcome Trust Case Control Consortium [WTCCC] study and the German MI [Myocardial Infarction] Family Study) and around 4,000 controls investigating more than

300,000 SNPs. The results, including a replication of SNPs in a German cohort, showed several genetic loci associated with coronary artery disease. We picked seven polymorphisms with the strongest association to be genotyped in our study on MMD. The following three SNPs were replicated in the German cohort:

- rs1333049, risk allele C, on Chromosome 9p21.3 with a combined P-value of $3x10^{-19}$. In this region the genes for two cyclin dependent kinase inhibitors (*CDKN2A* and *CDKN2B*) are located, both playing an important role in the cell cycle and through their TGF-β–induced growth inhibition they may be connected to the pathogenesis of atherosclerosis [113]. This SNP's association with coronary artery disease (CAD) was also replicated by Hinohara et al. [36] in a Japanese and a Korean population. Additionally this locus was replicated in two other studies as being associated with coronary artery disease and myocardial infarction [32, 83], making it the most replicated locus in coronary artery disease up to date [113]. SNPs in this region were also described to be associated with intracranial aneurysms and abdominal aortic aneurysms recently [10, 31].

- rs6922269, risk allele A, on Chromosome 6p25.1 with a combined P-value of $2,9x10^{-8}$. This SNP is located in an intron of methylenetetrahydrofolate dehydrogenase (NADP+-dependent) 1–like protein (*MTHFD1L*), which encodes an enzyme being particularly responsible for the synthesis of purine and methionine and may therefore be linked to the plasma homocysteine levels, a possible risk factor for atherogenesis [113].

- rs2943634, risk allele C, on Chromosome 2q36.3 with a combined P-value of $1,61x10^{-7}$. In this region only a pseudogene (*ENSG00000197218*) is described so far.

The combined analysis of SNPs in the study by Samani et al. [113] revealed the association of four further polymorphisms with coronary artery disease:

- rs599839, risk allele A, on Chromosome 1p13.3, with a combined P-value of $4x10^{-9}$
- rs17465637, risk allele C, on Chromosome 1q41, with a combined P-value of $2x10^{-7}$
- rs501120, risk allele T, on Chromosome 10q11.21, with a combined P-value of $9x10^{-8}$
- rs17228212, risk allele C, on Chromosome 15q22.33, with a combined P-value of $2x10^{7}$. This SNP also shows high association with LDL (Low density Lipoproteins) Cholesterol concentrations [112, 114, 147, 149].

Helgadottir et al. [32] performed an association study on myocardial infarction with 4587 cases and 12,767 controls of different ethnicities. Several SNPs showed strong association values within a 190-kb linkage disequilibrium (LD) block on chromosome 9p21, containing the tumor suppressor genes for the Cyclin Dependent Kinase 2 A and B (*CDKN2A* and *CDKN2B*). The proteins encoded in this region are known to play an important role in the regulation of the cell cycle, cell senescence, and apoptosis [64]. After combining data from all case-control-groups, the G allele of the SNP rs10757278 on 9p21.3 showed the strongest association with the disease (OR = 1.28, P = $1.2x10^{-20}$). This genetic region was recently also described by Samani et al. [113] and by Bilguvar et al. [10] to be a susceptible locus for intracranial aneurysms in Japanese and European populations.

McPherson et al. [83] performed a genome-wide association study on coronary heart disease (CHD) with almost 2000 cases and 10.000 controls from three different groups (Ottawa heart study 1 and 2, Atherosclerosis Risk in Communities Study), finding two susceptible loci, rs10757274 and rs2383206, which could be validated with around 2.300 cases and around 10.300 controls (Copenhagen City heart study, Dallas heart study and Ottawa heart study 3). The risk alleles for these SNPs showed a population attributable increase in risk of 10-15%. The findings and two further SNPs could also be replicated by Shen

at al. [126] in an Italian population with myocardial infarction. These SNPs are located on 9p21 near the tumor suppressor genes *CDKN2A* and *CDKN2B*, where Helgadottir at al. [32], Samani et al. [113] and Bilguvar et al. [10] described strong association to myocardial infarction, coronary artery disease and intracranial aneurysms.

1.4.4 Genetic Polymorphisms related to Atherosclerotic Cardiac Allograft Vasculopathy

Cardiac allograft vasculopathy (CAV) is a common problem in patients after heart transplantation. It is the leading cause of death between one and three years after transplantation and accounts for 17% of the deaths after three years [105]. This phenomenon consists of several pathological changes in the walls of the cardiac arteries, leading to a stenosis by fibrous intimal thickening or atherosclerotic plaques, showing close similarities to spontaneous atherosclerosis [148]. Early after transplantation the pathological changes mainly consist of fibrous intimal thickening or vasculitis-like lesions, whereas in later phases focal atherosclerotic plaques and diffuse intimal thickening can be found [148]. Further it has been described, that smaller arteries occlude earlier than the bigger epicardial vessels and that the tunica media tends to be narrower than in controls while the intima shows a distinct thickening, mainly by smooth muscle cells. The genesis of these rapid changes is still not clarified in total. Latest findings suggest a process similar to the Ross “response to injury” hypothesis [108] due to an endothelial injury in the graft’s vessels [24], leading to an immigration of SMCs stimulated by released cytokines and growth factors [103]. Also a non-immunological process has been accounted for the migration of the SMCs. Pinney et al. [103] showed that recipients of a heart from a donor with intracranial hemorrhage have an increased risk of developing a CAV, suggesting that the systemic activation of matrix metalloproteinases 2 and 9 (MMP2 and 9) may lead to the increased migration of SMCs towards the intima. SNPs in the gene of the tissue inhibitor of matrix metalloproteinases 2 (*TIMP2*) showed strong association with MMD in recent studies, underlining a possible coherence [54]. Assuming genetic components in the genesis of CAV, the question whether the malfunction is triggered by the donor organ or by the

recipient or as a combination of both must be discussed. Especially the origin of the excessively growing SMCs in the tunica intima is of great interest. Assumptions that these SMCs are mainly from the donor's media have recently been rebuted suggesting that SMC accumulation could result from adhesion and migration of bone marrow derived circulating SMC progenitors, proposing multiple sites of origin [24, 35, 103]. In addition to the facts on CAV described above, Achrol et al. [1] suggested possible common mechanisms in the genesis of MMD and CAV in a recent review paper on MMD.

Due to these assumptions and the similar processes in the genesis of MMD and CAV/Atherosclerosis, we decided to examine two SNPs that showed strong association with CAV. Tambur et al. [142] performed genetic studies on candidate genes (mainly cytokines and growth factors) for CAV combined with clinical examinations on 59 patients after heart transplantation. The results showed that two SNPs in the *PDGF-B* gene are associated with the clinical outcome of CAV. The SNP at position *PDGF-B* -286 (rs1800818) showed an increased risk of developing early CAV when having the AA phenotype and a decreased risk and a longer survival when having the GG phenotype at this position. Also the SNP at *PDGF-B* -1135 (rs1800817) indicated a protective effect with a significant lower risk of developing an early CAV, if the patient has the CC phenotype.

Although no exact function of associated SNPs in the *PDGF-B* gene mentioned above was described yet, Clemens et al. [13] proposed that an amino acid change in position *PDGF-B* -286 may have an effect on receptor affinity and cell activation of PDGF.

However, PDGF was mentioned as a possible initiator of some processes in the genesis of MMD before [4-5, 159]. It is supposed to play a key role in the migration and replication of SMCs and the resulting thickening of the tunica intima after arterial wall injury in the beginning of the pathogenesis of atherosclerotic changes as well as in vessels with MMD-like changes [104, 106, 159]. Yamamoto et al. [159] discovered different reactions on a PDGF stimulus between MMD SMCs and control SMCs, suggesting that the PDGF needed for

repair processes may fail to repair the wall defect and may lead to a longer retaining of other cytokines, such as fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), in turn leading to a continuing neointimal formation in MMD vessels due to a lack of repair. Aoyagi et al. [4-5] analyzed reactions of VSMCs on PDGF and found an alteration in DNA synthesis and proliferation of MMD VSMCs compared to those of the controls. This finding goes along with the description of a down-regulation of PDGF receptors in VSMCs of MMD vessels. Although it seems to be one reasonable approach explaining the intimal thickening, further research on PDGF and its receptor needs to be done for a better understanding of the development of intimal thickening in MMD vessels.

2. Material and methods

The study was approved by the ethical committee for medical studies at the University of Tübingen (Project-number: 273/2009BO1). Prior to the participation, all participants or their legal guardians gave a written informed consent after being introduced to the experimental procedure verbally or by letter. Every participant was free to ask questions about the procedures verbally, via email, telephone or letter.

2.1 Design of the study

The study was designed as a case/control study, requiring the division of the samples into two populations: Cases and controls.

- Cases: The recruitment of patients was possible by reviewing the database of the university hospital of Tübingen for patients treated for MMD, as well as further MMD patients that contacted us after they had been told of our project by acquaintances who were already participating.
- Controls: This group consists of healthy subjects. The recruitment was done at the Universitätsklinikum Tübingen.

2.2 Cases

Forty MMD patients agreed to participate. This group consisted of twenty-seven (68%) female and thirteen (32%) male participants with age ranging from four to sixty-six years of age (Mean-age: twenty-five years of age).

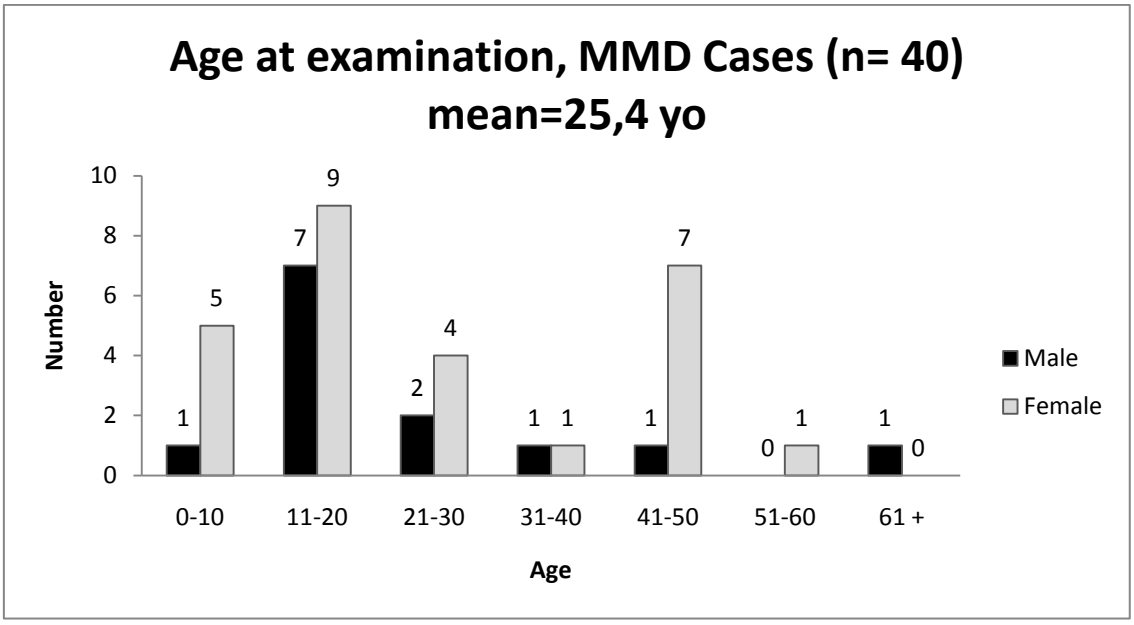


Figure 8: Age at examination, MMD cases

For all participants the age at examination and the age at onset of symptoms were analyzed. The range at the age of the examined patients was from four to sixty-six years, the range of age at onset was from three months to fifty-eight years (Mean: Fifteen years of age). The data described in our examined population is concordant with the data shown in earlier publications with a distribution of MMD in all age groups, but a two peak predomination in onset at the age group under 10 years and between 30-40 years with a female to male ratio of 1,7:1[21, 48]. In our population the female to mal ratio is 2,1:1.

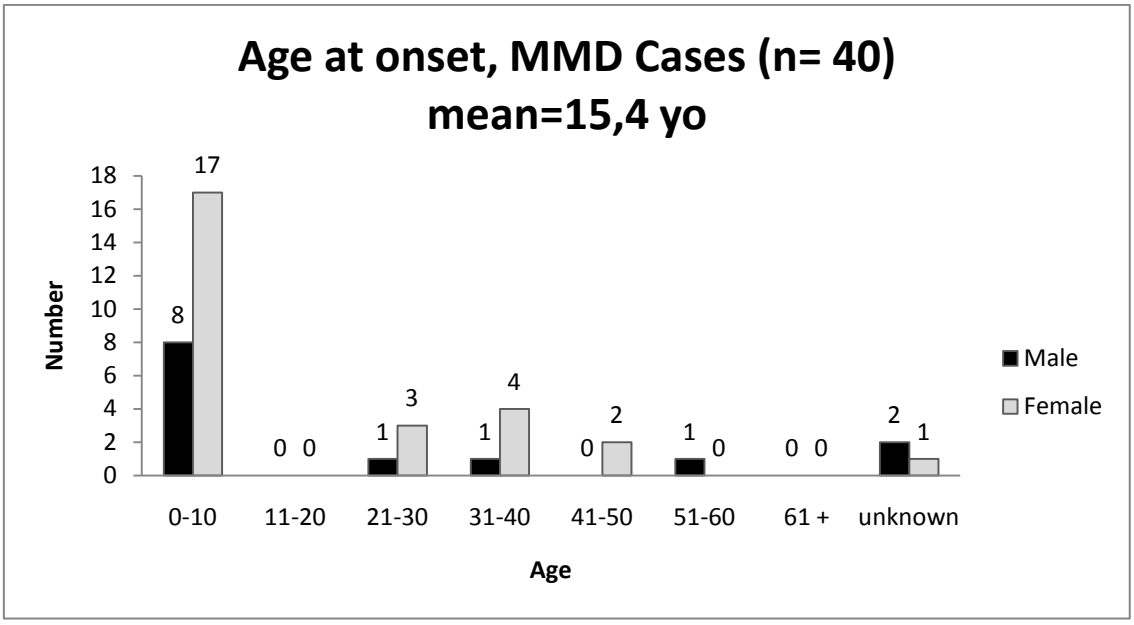


Figure 9: Age at onset, MMD cases

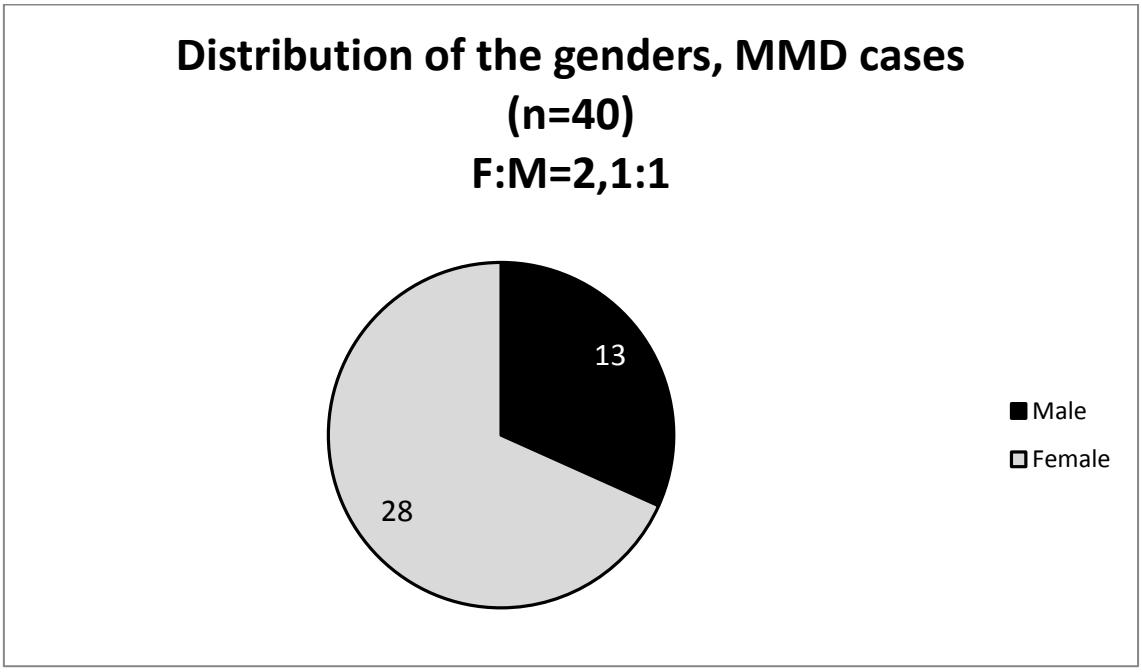


Figure 10: Distribution of genders - cases

All MMD patients were Caucasians from central Europe, their geographical origin is given in the table below:

Ethnicity	Number
German	26
Swiss	7
North Italian	2
Dutch	2
Swedish	1
Czech	1
Austrian	1

Table 8: Geographical origin of MMD patients

In addition to the donation of DNA, all participants were asked to fill out a questionnaire concerning their health and disease history, as well as information on certain diseases in their families. The questions concerned:

- Gender
- Ethnicity
- Primary symptoms
- Primary diagnosis
- History of the disease
- Treatment of the disease
- Recent health problems and medications
- Co-morbidities
- Affected relatives

For the recognition and the treatment of MMD, the primary symptoms are of interest. The diagram below shows the distribution of primary symptoms at the onset of the disease in our cohort. These numbers go along with the findings of Handa et al.[28], who published information from a data bank with 1,500 cases of patients suffering a “spontaneous occlusion of the Circle of Willis”.

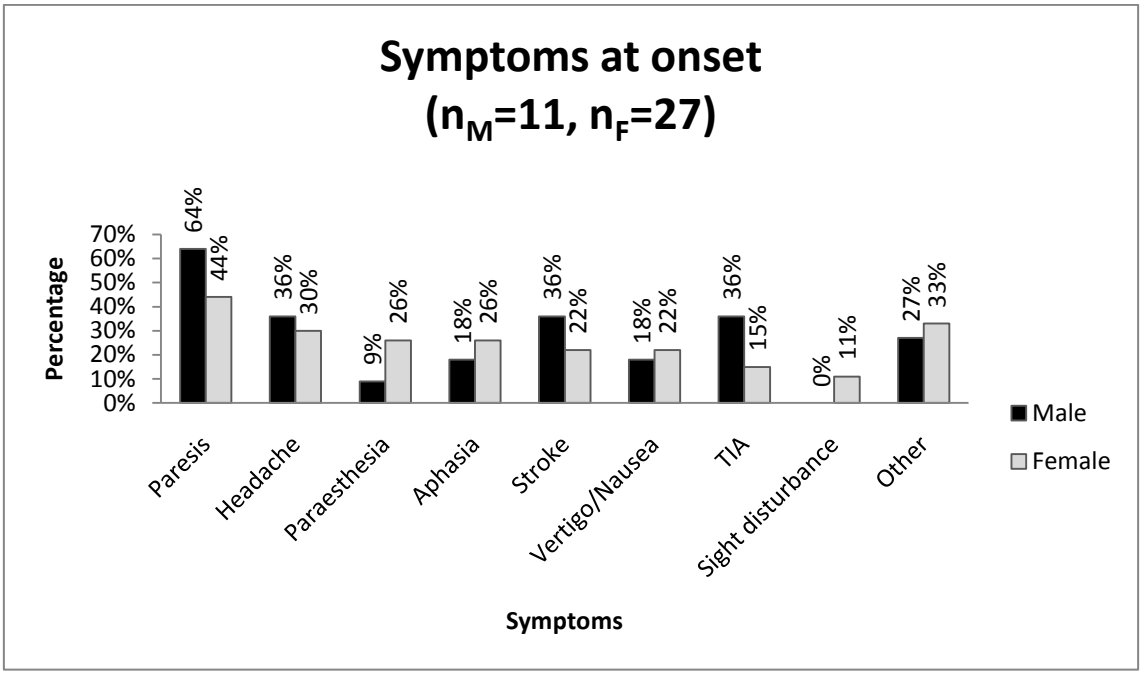


Figure 11: Symptoms at onset

The figures below show the distribution and overall number of the affected vessels. The information on the vessels was available for 27 patients.

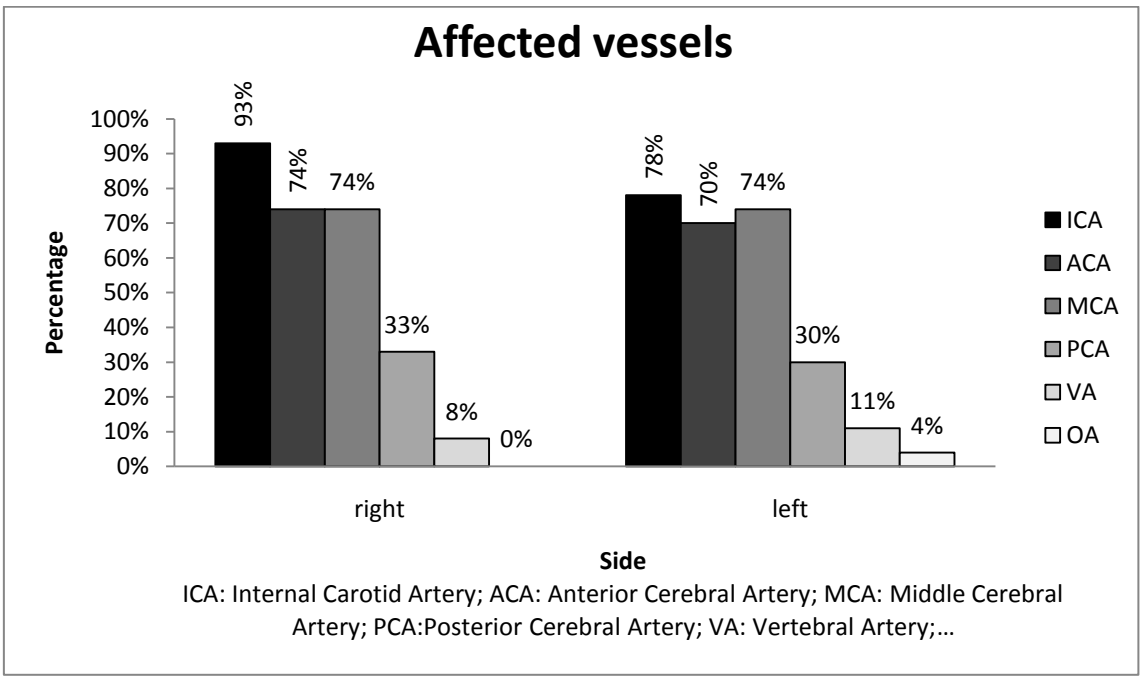


Figure 12: Affected vessels

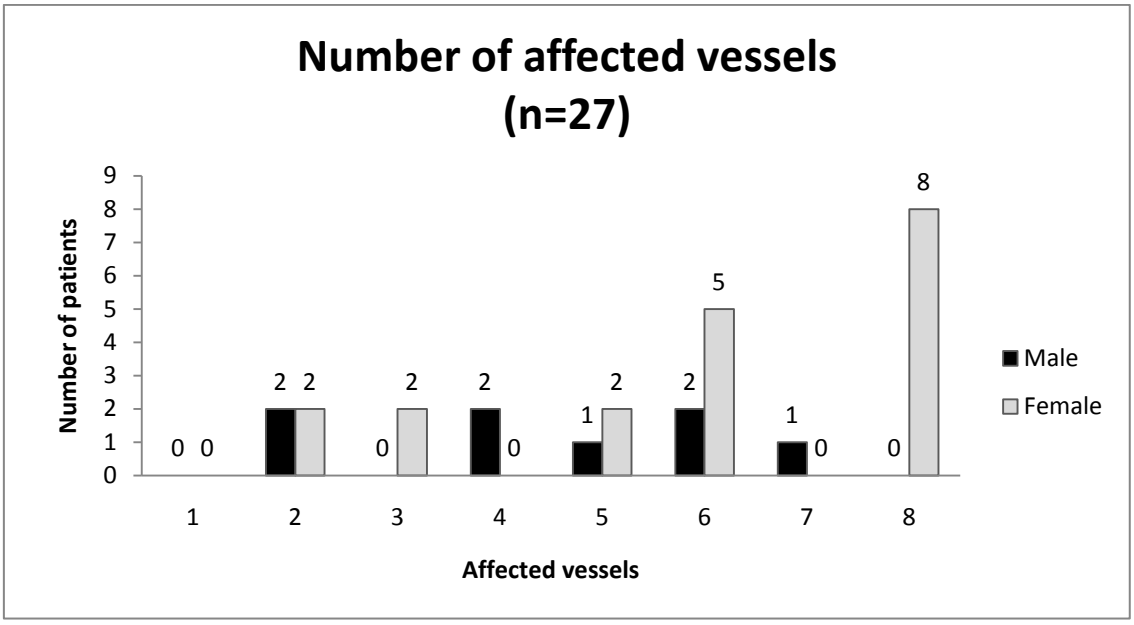


Figure 13: Number of affected vessels

Earlier studies showed genetic differences between patients of familial and non-familial MMD [54]. None of the patients examined in our study had a family history for MMD.

2.3 Controls

The control group consisted of sixty-eight participants, who provided us with a DNA sample. All participants lived in Germany and were of German descent. In addition to the DNA sample, all participants were asked to complete a questionnaire concerning their past and present health status as well as their families'. None of the participants showed distinctive features (i.e. familial accumulation of strokes, former cerebral hemorrhage, etc.) that would lead to exclusion. The range of age at examination was from fifteen to sixty-five years with a mean-age of twenty-eight years. Forty-three participants were female, twenty-five male, resulting in a female to male ratio of 1,72:1.

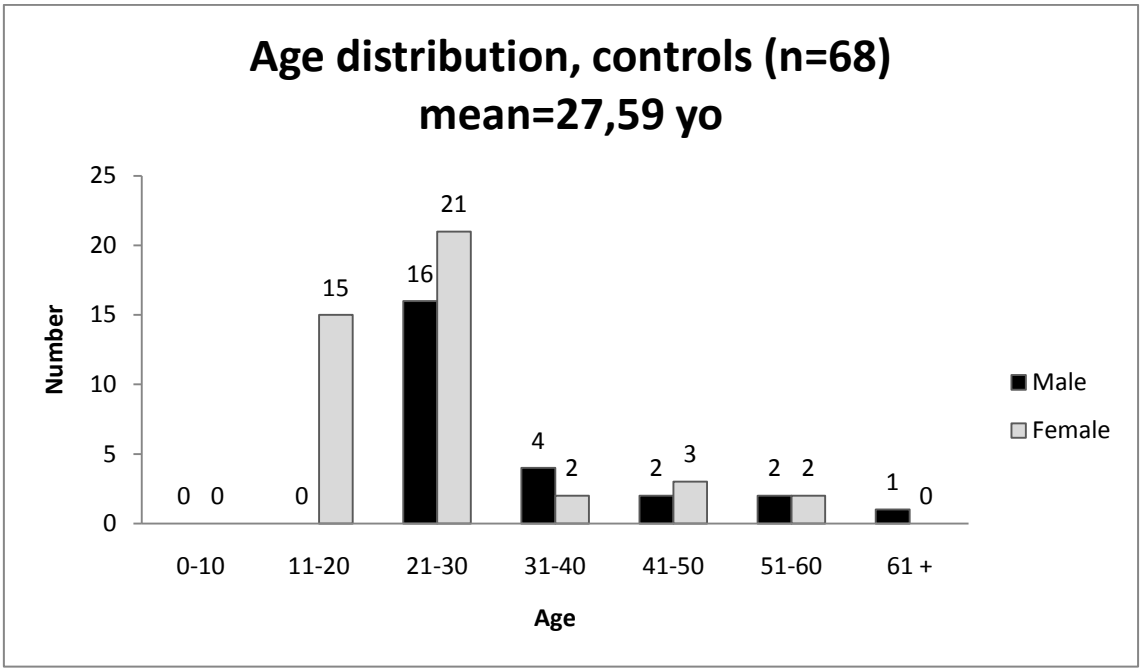


Figure 14: Age distribution, controls

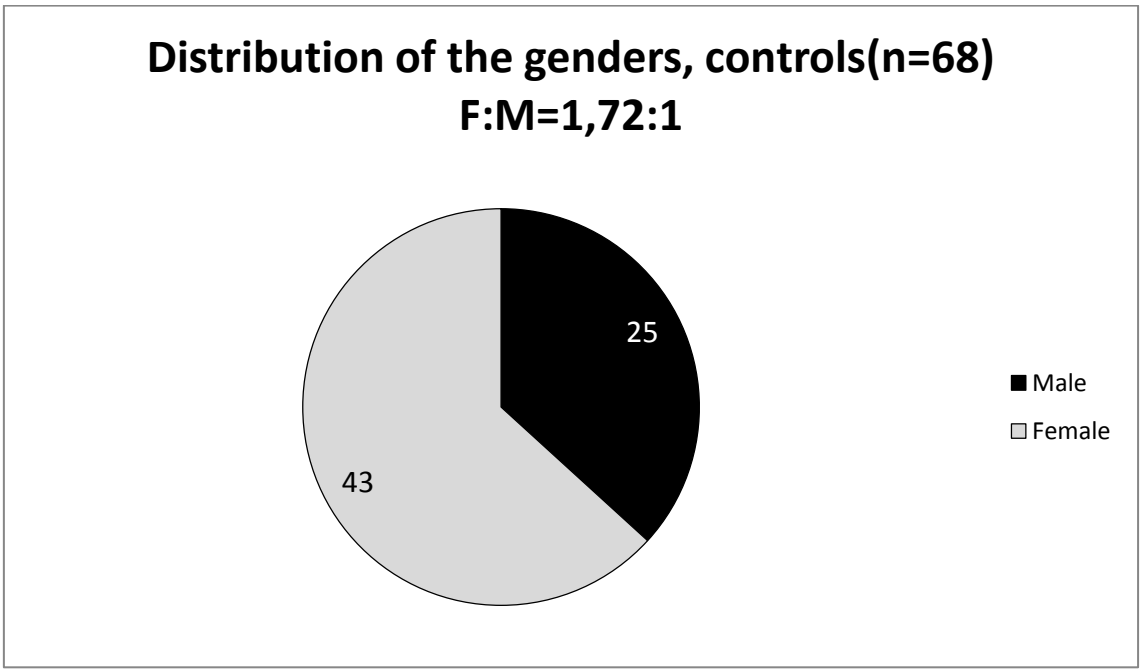


Figure 15: Distribution of the genders - controls

All data was collected and processed in Microsoft Excel charts.

2.4 Retrieval of the blood sample

18 ml of venous blood were withdrawn into two 9ml-EDTA tubes. The samples were frozen at -80° Celsius until the DNA was isolated. All samples were marked with a unique code consisting of letters and numbers, enabling a clear identification and pseudonymization.

2.5 DNA preparation

DNA was isolated using the Flexigene DNA Kit (Qiagen, Hilden; Germany). 7ml whole blood was mixed with 17,5ml FG1 buffer in a 50ml Falcon tube to lysate the red blood cells. After inverting the tubes five times, they were centrifuged for 6 minutes at 3000xG. While the next step the supernatant was discarded and the tubes were left inverted on a clean piece of absorbent paper for 2 minutes with attention to the remaining of the pellet in the tubes. Then 3,5ml of the premixed Protease/FG2 buffer (3,5ml FG2 and 35µl Protease) were added for lyses and denaturing of the white blood cells. The samples were vortexed until the pellet was homogenized and incubated in a water bath at 65°Celsius for 10 minutes. Correct digestion was indicated by a change in color of the sample-solution to olive green. After incubation 3,5ml isopropanol (100%) were added and the tubes were inverted until the precipitated DNA became visible. The tubes were centrifuged for 4 minutes at 3000xG. The supernatant was discarded and the pellets were dried up side down in the tubes. As the next step 3,5ml ethanol (70%) were added and the tubes were vortexed until the pellet solved from the bottom, in order to wash the DNA pellet. To get compact and clean pellets, the tubes were centrifuged for 6 minutes at 3000xG. After discarding the supernatant, the pellets were taken from the bottom with a pipette tip and were put into a 1,5ml Eppendorf tube for 5 minutes for drying. After the drying, 700µl of FG3 Hydration buffer were added into the Eppendorf tubes, they were vortexed and then put into a water bath for incubation for 1 hour at 65°Celsius. The DNA concentrations were measured on the Nanodrop ND-1000 (Peqlab, Erlangen; Germany) photometer.

The DNA was stored at -20°Celsius until the needed amounts were pipetted onto 96 well plates.

2.6 DNA analysis

Further analysis was performed in cooperation with the International Research and Educational Institute for Integrated Medical Sciences at the Tokyo Women's Medical University which was supported by the Program for Promoting the Establishment of Strategic Research Centers, Special Coordination Funds for Promoting Science and Technology, Ministry of Education, Culture, Sports, Science and Technology (Japan).

For shipping the samples were stored in a box with dry ice in order to provide accurate cooling.

2.6.1 DNA amplification with PCR

For sequencing it is necessary to produce high copy numbers of small regions of 200-500bp. PCR (Polymerase chain reaction) is the method of choice. The main principle of PCR consists of two synthetic oligonucleotide primers (forward and antisense reverse) annealing to denaturated DNA strands and a thermo-stable polymerase synthesizing a new DNA strand complementary to the DNA template between the primers. The primers are designed for binding a unique sequence at a melting temperature around 60°C. The melting temperature relies on several factors, like salt concentrations and primer length. It can be calculated according to the Wallace rule, which basically concerns about the differences in binding strengths between Pyrimidin and Purin base pairs. Therefore the melting temperature can be calculated with the following formula: T_m=2*([A]+[T])+4*([G]+[C]). For synthesizing high copy numbers of the template DNA, 30-50 repeats of temperature cycles is needed:

- Initialization step: This step consists of heating the reaction to a temperature of 94–96°C for heat-activation of the polymerase. This step is only performed once in the beginning.
- Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA.

- Annealing step: The reaction temperature is lowered to 50–65°C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

- Extension/elongation step: At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dideoxynuclotides (dNTPs) that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand.

To check whether the PCR generated the anticipated DNA fragment, agarose gel electrophoresis is employed for size separation of the PCR products.

2.6.2 PCR protocol

For the PCR the following reagents were used:

- 10xAccuPrime PCR buffer II*+ 5.0µl
- Sense/forward primer (10µM) 0.5µl
- Antisense/reverse primer (10µM) 0.5µl
- AccuPrime Taq High Fidelity Polymerase* 0.1µl(0.5unit)
- Template DNA (10ng/µl) 1.0µl
- Ultra pure water 12.9µl

* Invitrogen Cooperation, USA

+ Ingredients: 600 mM Tris-SO4 (pH 8.9), 180 mM (NH4)2SO4, 20 mM MgSO4, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime™ protein, 10% glycerol.

The PCR protocol consisted of the following cycles:

PCR	Temperature	Time	Cycles
Initial denaturation	94°C	30sec.	1x
Denaturation	94°C	20sec.	35x
Annealing	57°C	20sec.	
Elongation	68°C	30sec.	
Final Elongation	68°C	6min.	1x

Table 9: PCR protocol

Before sequencing, PCR products were diluted. All PCR products with concentrations under 10ng/µl were diluted two times, all products above 10ng/µl were diluted three times.

2.6.3 Cycle sequencing

Sequencing describes the process of determining the exact base composition of certain DNA strands. The main principle of this technique consists of enzymatic DNA elongation at the ends of primers flanking the region of interest (sequencing PCR) with regular dNTPs and with fluorescently labeled dideoxynuclotides (ddNTPs). ddNTPs are similar molecules like dNTPs, which are used for regular PCRs, but do not have a 3'-hydroxyl-group. Therefore dNTPs function as chain terminators because they are not capable of building a phosphodiester bond at the 3' end. The incorporation of fluorescently labeled ddNTPs (with a specific fluorochrome for each base) is a random process leading to DNA strands of various lengths with a specific base at the point of determination and the same 5'-sequence up to the primer. After a purification step, electrophoresis in a polymer matrix column was performed with the products of the sequencing PCR. This separates the differently sized DNA fragments according to their length and the specific fluorochromes at the last base of each strand can be detected by a laser in the genetic analyzer. These fluorescence signals are translated into A, T, G, or C (or their heterozygous combinations) according to their wavelength and signal intensity.

2.6.4 Cycle sequencing protocol

For the cycle sequencing the following reagents were used:

- Big Dye Terminator ver. 3.1 (Applied Biosystems, USA) 1µl
- 5x Big Dye Sequencing buffer (Applied Biosystems, USA) 1.5µl
- Diluted template DNA/PCR product (5-10ng/µl) 1µl
- Primer (1.6µM) 2µl
- Ultra pure water 4.5µl

Cycle sequencing	Temperature	Time	Cycles
Initial denaturation	96°C	2min.	1x
Denaturation	96°C	10sec.	25x
Annealing	57°C	10sec.	
Elongation	60°C	2min.	

Table 10: Cycle sequencing protocol

2.6.5 Purification of the cycle sequencing products

To improve the quality of the genetic analysis result, the templates must be purified and all proteins, dNTPs and ddNTPs must be removed. This goal was achieved by using Agencourt CleanSEQ dye terminator removal kits (Beckman Coulter, USA) according to the manufacturer's protocol: 2.5 µl of CleanSEQ and 30 µl of 85%-ethanol were added to the sequencing reaction mixture and mixed vigorously. As the next step, the samples were placed onto an Agencourt SPRI (Solid Phase Reversible Immobilization) magnetic plate for four minutes, to immobilize the magnetic beads with the DNA on their surface to the walls of the reaction tube. After aspirating and discarding the supernatant, a washing step with 100µl of 85%-ethanol was repeated twice by adding and carefully aspirating the ethanol. Afterwards the samples were air-dried for 10 minutes at room-temperature. The dried samples were resuspended in 40µl ultra pure water and incubated at room temperature for 5 minutes. 35µl of the magnetically-cleared bead-free supernatant was pipetted on a new plate for loading the genetic analyzer.

2.6.6 Genetic analysis

Before starting the analysis, the purified and cleaned samples were centrifuged at 1200rpm for five minutes. For analysis, the Genetic Analyzer 3130xl (Applied Biosystems, USA) was used. The detection was performed with the 3130 POP-7 Polymer on the 50 cm capillary-array (Applied Biosystems, USA). The analyzer ran with the following protocols:

Instrument Protocol: FastSeq50_POP7_(15,1600)
Analytical Protocol: 3130KB_POP7_v3

2.6.7 Analysis software

For analysis, the GENETYX-MAC ver.15.0.1 (GENETYX Corporation, Japan) software was used.

2.7 Statistics

Genotype and allele frequencies were calculated for all SNPs separately for cases and controls. The frequencies were compared to the latest reference-data available at http://www.pubmed.gov, http://www.hapmap.org, and http://www.appliedbiosystems.com, to detect possible irregularities. The Hardy-Weinberg Equilibrium (HWE) was calculated for each SNP in each of the groups (cases and controls) to ensure that the genotype and allele frequencies in the examined population were in equilibrium. The equilibrium was accepted for all HWE p value aberrations above 0.05. To determine differences between cases and controls, the χ^2 test was used. Results with p-values below 0.05 were accepted as statistically significant. The χ^2 test was performed for the distribution of genotypes, the different alleles and for the different alleles under the assumption of dominance of certain alleles. To compare the probabilities of MMD between the groups, the odds ratio (OR) and the 95% confidence interval (CI) was calculated for all SNPs, also under the assumption of dominant alleles. An OR greater than 1 indicates a higher risk for the respective group. HWE, χ^2 and OR with CI were calculated using a preprogrammed excel chart (Tim Becker, Bonn).

As statistical characteristics we calculated the following values:

- P-value for the genotypes
- P-value for the alleles
- OR with CI for the alleles
- P-value for the alleles under the assumption of dominance of allele 1
- OR with CI for the alleles under the assumption of dominance of allele 1
- P-value for the alleles under the assumption of dominance of allele2
- OR with CI for the alleles under the assumption of dominance of allele 2
- Hardy-Weinberg-Equilibrium for cases and for controls

2.8 Single Nucleotide Polymorphisms

SNP selection was performed by literature analysis as described earlier.

SNP #1: rs8326

SNP alleles: [C/G]

Ancestral allele: C

Location: 7q11.2

Chromosome position (NCBI build 130): Chr. 7 - 73121625

Sequence (NCBI build 129):

```
GGGGAACCCC CTACCTGGGG CTCCTCTAAA GATGGTGCAG ACACTTCCTG GGCAGTCCCA
GCTCCCCCTG CCCACCAGGA CCCACCGTTG GCTGCCATCC AGTTGGTACC CAAGCACCTG
AAGCCTCAAA GCTGGATTCG CTCTAGCATC CCTCCTCTCC TGGGTCCACT TGGCCGTCTC
CTCCCCACCG ATCGCTGTTC CCCACATCTG GGGCGCTTTT GGGTTGGAAA ACCACCCCAC
ACTGGGAATA GCCACCTTGC CCTTGTAGAA TCCATCCGCC CATCCGTCCA TTCATCCATC
[C/G]
GTCCGTCCAT CCATGTCCCC AGTTGACCGC CCGGCACCAC TAGCTGGCTG GGTGCACCCA
CCATCAACCT GGTTGACCTG TCATGGCCGC CTGTGCCCTG CCTCCACCCC CATCCTACAC
TCCCCCAGGG CGTGCGGGGC TGTGCAGACT GGGGTGCCAG GCATCTCCTC CCCACCCGGG
GTGTCCCCAC ATGCAGTACT GTATACCCCC CATCCCTCCC TCGGTCCACT GAACTTCAGA
GCAGTTCCCA TTCCTGCCCC GCCCATCTTT TTGTGTCTCG CTGTGATAGA TCAATAAATA
```

Primer: 5’ GGTCCACTTGGCCGTCTCC (T_m=61.5°C)

5’ TATCACAGCGAGACACAAAAAGATGG (T_m=60.6°C)

Adjacent genes: Elastin (*ELN*), LIM Domain Kinase 1 (*LIMK1*)

Genotype and allele frequencies (Pubmed CEPH-Panel):

Genotype			Allele frequencies	
NA	NA	NA	C	G
NA	NA	NA	0.090	0.910

Publications: Akagawa et al. [2]

Assumed associations: Intracranial aneurysms

SNP #2: rs34208922

SNP alleles: [-/A]

Ancestral allele: unknown

Location: 7q11.2

Chromosome position (NCBI build 130): Chr. 7 - 73121467:73121468

Sequence (NCBI build 129):

```
CTTCAGAGGC AAGGGCCATG TGGTCCTGGC CCCCCACCCC ATCCCTTCCC ACCTAGGAGC
TCCCCCTCCA CACAGCCTCC ATCTCCAGGG GAACTTGGTG CTACACGCTG GTGCTCTTAT
CTTCCTGGGG GGAGGGAGGA GGGAAGGGTG GCCCCTCGGG GAACCCCCTA CCTGGGGCTC
CTCTAAAGAT GGTGCAGACA CTTCCTGGGC AGTCCCAGCT CCCCCTGCCC ACCAGGACCC
ACCGTTGGCT GCCATCCAGT TGGTACCCAA GCACCTGAAG CCTCAAAGCT GGATTCGCTC
[-/A]
TAGCATCCCT CCTCTCCTGG GTCCACTTGG CCGTCTCCTC CCCACCGATC GCTGTTCCCC
ACATCTGGGG CGCTTTTGGG TTGGAAAACC ACCCCACACT GGGAATAGCC ACCTTGCCCT
TGTAGAATCC ATCCGCCCAT CCGTCCATTC ATCCATCGGT CCGTCCATCC ATGTCCCCAG
TTGACCGCCC GGCACCACTA GCTGGCTGGG TGCACCCACC ATCAACCTGG TTGACCTGTC
ATGGCCGCCT GTGCCCTGCC TCCACCCCCA TCCTACACTC CCCCAGGGCG TGCGGGGCTG
```

Primer: 5’ GGGCTCCTCTAAAGATGGTGC (T_m=59.4°C)

5’ GTGTGGGGTGGTTTTCCAACC (T_m=60.9°C)

Adjacent Genes: Elastin (*ELN*), LIM Domain Kinase 1 (*LIMK1*)

Genotype and allele frequencies (Pubmed CEPH-Panel):

Genotype			Allele frequencies	
NA	NA	NA	-	A
NA	NA	NA	0.762	0.238

Publications: Akagawa et al. [2]

Assumed associations: Intracranial aneurysms

SNP #3: rs710968

SNP alleles: [C/T]

Ancestral allele: T

Location: 7q11.2

Chromosome position (NCBI build 130): Chr. 7 - 73135664

Sequence (NCBI build 130):

GCCCCTTCCG CCCTTCATTC ATTCTTCTTT CTCCCAGCGA CAGGCCTCGG CTGGGAGCTG
CGAGGCCCAG CTGTCTCGGA GGGCCGCTAG CTCTGCTGGG GCAGGCCAGG AGGATGTAGG
AGCCTAGAGA AGGGGCCTCC GCTTCCCTGC GCGAGGCCCC CACAGACCCA AACCAGCTGC
AGCCGGGAAC TCGGATCAGA GC
[C/T]
GGAGAGGCCC TGTGGACCCC CAGGACACTG GTGGTCGCCT TGGGGAGGGG AGGGAGGCGG
GCCGGGTTTT GTCCCTCTCT CTGAGGCCTG CATTGAGGCC AGGCTGGGCC CGGGGAAGGT
GACGGTCCGC GTCTCAGGCC TCGCGAATGA GCAGCACCAG AGAAAGGAGC GAGCCCCTGC
CAGGCGGTGA CAGGTGTACT CTTCTCAGTG CCGATTACCT GGAGCTGGGG CTGCCCTGCC
CAGGGTCCTG GAGGGAGCAC GGGTTTGGGC TGAGTCAAGC GTCCAAAGGA ATTGGGTGGT
CCAGTCCAGC ACTGATGGTG GAACGCCCTG CCTAGATGTG GACAATTCTA GAATGCTGGA
CATGGGCAGG AAGCAACCCT GTCCCCCCTA GGTCTACCTT CCACGTCATC CCTCTGCCCA

Primer: 5’ CGAGGCCCAGCTGTCTCG (T_m=61.2°C)

5’ TGGACCACCCAATTCCTTTGG (T_m=59.6°C)

Adjacent genes: Elastin (*ELN*), LIM Domain Kinase 1 (*LIMK1*)

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele frequencies	
NA	NA	NA	NA	NA
NA	NA	NA	NA	NA

Publications: Akagawa et al. [2]

Assumed associations: Intracranial aneurysms

SNP #4: rs6460071

SNP alleles: [A/G]

Ancestral allele: A

Location: 7q11.2

Chromosome position (NCBI build 130): Chr. 7 - 73135132

Sequence (NCBI build 129):

```
AGGTCAGGAG TTCAGGACCA GCCTGGTCAA CATGGTGAAA CCTCATCTCT ACTAAAAATA
CAAAAATTAG TCGGTCATGG TGGTGCACGC CTGTAATATC AGCTACTCGG GAGGCTAAGG
TGGGAGAATT GCTTGAACCT GGGAGGTGGA GACTGCGGTG AGCCGAGATC CCGCCACTGC
ACTCCAGCCT AGGCAACAGA GCGAGACCGA ATCTAAAAAA AGAAAAAAAA AATCTAGTAT
TTTCTTCCCA CCCCACGTAA TTGTTTTGCA CCCAGGGGTG AGTGCCCCCC ACTTTGCAGA
C [A/G]
TGCCTAGACC TCTCCCCACA AACATGATGA CTAGAGTTGG AGCTGGGCTA GCGCTGCCTC
TTGTTCACTG GGTTCACGTG GGCACGTGGC TCCACTCCGG TCACTGCTTC CTGGGCAGAG
GGATGACGTG GAAGGTAGAC CTAGGGGGGA CAGGGTTGCT TCCTGCCCAT GTCCAGCATT
CTAGAATTGT CCACATCTAG
```

Primer: 5’ GCAACAGAGCGAGACCGAA (T_m=59.0°C)

5’ TAGGTCTACCTTCCACGTCATCC (T_m=60.0°C)

Adjacent genes: Elastin (*ELN*), LIM Domain Kinase 1 (*LIMK1*)

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele frequencies	
A/A	A/G	G/G	A	G
0.034	0.241	0.724	0.155	0.845

Publications: Akagawa et al. [2]

Assumed associations: Intracranial aneurysms

SNP #5: rs10757278

SNP alleles: [A/G]

Ancestral allele: [A]

Location: 9p21.3

Chromosome position (NCBI build 130): Chr. 9 - 22114477

Sequence (NCBI build 130):

```
TTCTGAGGTC GCAACTAAAA GCCAAGATTT TAATCCATTT CTATTTGATG TAAAGTCTGG
TCTTTTTTCA GCAAACCACA ATCCCACATT TTAAGGGCAT TAAGAAAGGG ATGGGTAGAC
AAAATGTAGA GGTAGTAGGT ACAGAATACA AAGTTTCAAG AAATTAAAAG CTTCTAAACT
AACAAACAGC CAATTTGTGG AGTGTCACTG GAAAGTGACA AAGAGGACAG TTAAGTTAGT
TGGAACTGAA CTGAGGCCAG ACAGGGCTGT GGGACAAGTC AGGGTGTGGT CATTCCGGTA
[A/G]
GCAGCGATGC AGAATCAAGA CAGAGTAGTT TCTCCTTCTC TCTCTCTCTT TAATTGTAAC
GCCTTTTATA ACAAACAAAT ATTATGCTTA TTTCTGTCTT TAAATTTTTT GTAGTAATTT
CTCATCACTT AACCTCTATT TTTTAAAAAA CTAACTTTTC TCTTGTTTTT CTAGTTGAGC
TATCATTCAT ATTTATTATG TGGAACTAGA GGTAGTCCTG GCTACTTGGG AACAGCGTGG
AGTCTAGCCA TGTCAGGGCC AGAAGTCGTC TCAGCTAAGT TAGAATGTGA TACCATTGTT
```

Primer: 5' CCAATTTGTGGAGTGTCACTGGA (T_m=60.0°C)

5' CTTAGCTGAGACGACTTCTGGC (T_m=59.4°C)

Adjacent genes: Cyclin Dependend Kinase 2 A and B (*CDKN2A* and *CDKN2B*)

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele frequencies	
A/A	A/G	G/G	A	G
0.236	0.527	0.236	0.500	0.500

Publications: Helgadottir et al. [31-32]

Assumed associations: Myocardial infarction (Risk Allele G); Abdominal aortic aneurysm; Intracranial aneurysms

SNP #6: rs2383206

SNP alleles: [A/G]

Ancestral allele: A

Location: 9p21

Chromosome position (NCBI build 130): Chr. 9 - 22105026

Sequence (NCBI build 130):

```
TGCTGTGCTT AGTAATTGGC TGGGAGCAGC CATAGAAAGC ATGGACTCAG TACAAACTTG
GTGATGAATT TCAGAGGCAG CGGCTAAGAC CATCAGTCTA TTATTTTCCT TGCAATAGCT
GTCATAATTA CTATCAGAGC AAACAATTTT TTGTGTGTGA ATTCATGAGA TAATGGTCTT
TTCTTTCCTG CTTTCAAGAG CCATGTCATT GAAAGAGCTA ATCATTCAAA TTTATGCTGC
ATTACTGACT AAATACTTTC ATTTATCATA CTTTATTTTA AAATACTTTA ACTCATGGCC
CGATGATTTT CAGTTAACCA AATTCTCCCT TACTATCCTG GTTGCCCCTT CTGTCTTTTC
CTTAGAAATG TTATTGTAGT
[A/G]
TTTGCAAGAT GGCCTGAATC CTGAACCCCC CATCTTCAAT GAGCACCAAA TGGTAATTAT
AGATTCCCAG CTGTAGAGCT ATGTCAGACA AAGGAAACTT CATTAGTATG TACCAATGTT
TGGACTCCAA ATGCTTTTGT GTCTGAGTCA CAAGGACTCC TCTTCCTTGG TGGCTTAAAG
TTAGGCTGAA GAAGATTTAC ATTATGTTGT GCATGACCTC TTTAGTTTGG TTCTACTTAT
```

Primer: 5’ AGAGGCAGCGGCTAAGACC (T_m=60.7°C)

5’ CCAAGGAAGAGGAGTCCTTGTG (T_m=59.2°C)

Adjacent genes: Cyclin Dependent Kinase 2 A and B (*CDKN2A*, *CDKN2B*)

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele frequencies	
A/A	A/G	G/G	A	G
0.183	0.583	0.233	0.475	0.525

Publications: McPherson et al. [83], Helgadottir et al. [31-32], Shen at al. [126]

Assumed associations: Myocardial infarction (risk allele GG); Abdominal aortic aneurysm; Intracranial aneurysms

SNP #7: rs10757274

SNP alleles: [A/G]

Ancestral allele: A

Location: 9p21

Chromosome position (NCBI build 130): Chr. 9 - 22086055

Sequence (NCBI build 130):

```
CTTTTGATTT TGTTGGTTTA AAGTCTGTTT TATCAGAGAC TAGGATTGCA ACCCCTGCCT
TTTTTTGTTT TCCATTTGCT TGGTAGATCT TCCTCCATCC CTTTATTTTG AGCCTATGTG
TGTTTCTGCA CATGGTGATG GGAGGTACTG GTATTACAAA AAGCTTCTCC CCCGTGGGTC
AAATCTAAGC TGAGTGTTG
[A/G]
GACATAATTG AAATTCACTA GATAGATAGG AGATAGGGGT AGGGAATTCT AATCAGAGGG
AATAGCACAT GTAAGGCAAA CAATACAGTG CATCTGGGAA AGCTATACAA TTTTATTGTT
ATAGGACAAA TGTTGGGGAA TGTTGAGAGA TGGAACTGGA GAGTGAGGCA GAAGTTAGCA
TTTATTCATT TATTCAGCAG
```

Primer: 5’ TCCTCCATCCCTTTATTTTGAGCC (59.4°C)

5’ TGCTAACTTCTGCCTCACTCTCC (60.9°C)

Adjacent genes: Cyclin Dependent Kinase 2 A and B (*CDKN2A*, *CDKN2B*)

Genotype and allele frequencies (HapMap HCB-Asian):

Genotype			Allele frequencies	
A/A	A/G	G/G	A	G
0.289	0.511	0.200	0.544	0.456

Publications: McPherson et al.[83], Helgadottir et al. [31-32], Shen at al. [126]

Assumed associations: Myocardial infarction (risk allele GG); Abdominal aortic aneurysm; Intracranial aneurysms

SNP #8: rs501120

SNP alleles: [A/G]

Ancestral allele: G

Location: 10q11.2

Chromosome position (NCBI build 130): Chr. 10 – 44073873

Sequence (NCBI build 130):

```
TGATTGGACA CATGATAAAA TTGCATGGAA CCAAACACAC ACACACAAAT GAGTGCATGT
GAGCCTGATG AAATTTGAAT AAAGTCGATC CTTGGATCAA TGTGAATTTC TGGTTATGAC
AGCTATTCTT ATGTAAGATG TTACCACTAG GGGAAACTGG AGAATCTCTG TAATATTTCT
TACCACTGCA TGTGAACCTA CAACTCTCTA AAAATATTTT TTAATTGAAA AAAATTAATT
CTCACACTCC
[A/G]
AAGTGCATTT AATTTAAGCT ACTTTAGTAA ATAGGCAGCT GAAGCCCAGG AGGGGCTCCT
CCGTCTTTGC TGCTGTCCCC TCCTGGCACC TGTGGCTGCA GGTGAGTGCC AGGAGGAGCA
TTTCAACAGG GGGAGCCCCG GGGAGAGGGC TGGGCTGGGA CTTGCTAATG CTGGTCTTCA
AGGAGAACCT AGGAGTGAGT CAGGGCTGGG AGTGAGTCTG CAGGAGGAGC CCTCTGGAGG
CCCAGGGGTA
```

Primer: 5’ CAAATGAGTGCATGTGAGCC (T_m=56.4°C)

5’ GAAGACCAGCATTAGCAAGTCCC (T_m=60.4°C)

Adjacent genes: N/A

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele frequencies	
A/A	A/G	G/G	A	G
0.667	0.333	0.000	0.833	0.367

Publications: Samani et al. [113]

Assumed associations: Coronary artery disease

SNP #9: rs1333049

SNP Alleles: [C/G]

Ancestral allele: C

Location: 9p21

Chromosome position (NCBI build 130): Chr. 9 - 22115503

Sequence (NCBI build 130):

```
ACTGAAGAAG TAAAAAAAGA ATGGGCTGCT GACTCTGAAG ATCATACCCG AAGTAGAGCT
GCAAAGATAT TTGGAATATT GGTAATATCC AATAAAGAAT GACCTTCATG CTATTTTGAG
GAGATGTTTA AATGTCGAAT TATTGAAATA TTTATAAAAT ACAAATAAAC TAACTCTGCT
TCATATTCCA ACTTGTGTAT GACACTTCTT AGGCTATCAT TTCATTCCAA ATTTATGGTC
ACTACCCTAC TGTCATTCCT CATACTAACC ATATGATCAA CAGTT
[C/G]
AAAAGCAGCC ACTCGCAGAG GTAAGCAAGA TATATGGTAA ATACTGTGTT GACAAAAGTA
TGCAGAAGCA GTCACATTTA TACAGTAGTG AAGGAAATGT AAATTGGACA AACTTTTTGG
AAGATAAGTT GAGAATGTCA AAAATCAAAA CACACTTTCT GTTTTATTCA GCAATTATGA
GCCCTTTGTT TTACAGCTAT GCTCACAAAT ATATACAAAC ATGTATGCAC AATTATGTTC
ACTGTGGTAT TGCGCTAGAA AAATACTAAA AACAAACCAA ATGTTCATCA ATAGGGAAAT
TGTTCAACAA ATTACAGTAT ATCTAAAAAA AGAATAATAT ATAACAACTG AAAAAATAAA
```

Primer: 5’ CCTTCATGCTATTTTGAGGAGATG (T_m=56.5°C)

5’ ATACTGTAATTTGTTGAACAATTTCCC (T_m=56.1°C)

Adjacent genes: Cyclin Dependent Kinase 2 A and B (*CDKN2A*, *CDKN2B*)

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele Frequencies	
C/C	C/G	G/G	C	G
0.217	0.550	0.233	0.492	0.508

Publications: Samani et al. [113], Hinohara et al. [36]

Assumed associations: Coronary artery disease (risk allele C)

SNP #10: rs2943634

SNP alleles: [A/C]

Ancestral allele: A

Location: 2q36

Chromosome position (NCBI build 130): Chr. 2 - 226776324

Sequence (NCBI build 130):

```
TTGTTGAAAT TGTAGCATCA TTTGCTTCAT TTTAAATAAA AGGTAAAACT GAATATCCTG
AGCATGCTGA AACTGCTTTG AAATTTCTTC TTTAGTTTTT GTCACATATT CTGGAAAACT
GGTTTTTCTA TTATGAATGT AATTTTTTAA AAAGATAGAA ACATTTTAGA TATACATCTT
TCCCTAGAAT ACTGTTGTAA TCAGTGCAAC CTAGAATGGA TACATTAACA AGCAAAAAGC
AAGCACATCT GTGGCATTAC
[A/C]
AACATTAAAT ATTTATATAC ATAGTGTTCA TTTCATTCTA AATACAGATG TTTAAAATAA
GGGATTACTA TTTCAGTAAT AACTGTAAGT TACAAATGTG GAGCAACTAC AATTTTCAAG
TGTAATAACC AGGAAGTGTT ATGTTTCCAG CTTTGTTCTT TCTTAAGATA GCTTTGACTA
CTCTGGGCCT TTTGTGATTC CACCATATGA ATTTTAGAAT TGTTTTTTTC TATTTCTGTG
```

Primer: 5’ TCCTGAGCATGCTGAAACTGC (T_m=60.1°C)

5’ AAAAGGCCCAGAGTAGTCAAAGC (T_m=60.1°C)

Adjacent genes: Pseudogene (*ENSG00000197218*)

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele Frequencies	
A/A	A/C	C/C	A	C
0.183	0.350	0.467	0.358	0.642

Publications: Samani et al. [113]

Assumed associations: Coronary artery disease (risk allele C)

SNP #11: rs599839

SNP Alleles: [A/G]

Ancestral allele: G

Location: 1p13

Chromosome position (NCBI build 130): Chr. 1 - 109623689

Sequence (NCBI build 130):

```
AAAGAGAAAT TGGAGCAGGG GCTGGGTGCG GTGGCTCACA CCTGTAATCC CAGCACTTTG
GGAGGCTGAG GCGGGTGGAT CACCTGAGGT TAGGAGTTTG AGACCAGCCT GGCCAACATG
GTGAAACCCC GTCTCTACTA GAAATACAAA AAATTAGCCG GACGTGGGTG GCAGGCGCCT
GTAATCTCAG CTACTCGGGA GGCTGAGAAA GGAGAATTGC TTGAACCCAG AAGGTGGAAG
TTGCAGTGAG CCCAGATCGC GCCATTAAAC TCCAGCCTGG GTGACAGAGC AAGATTCTGT
CTAAAAAAAG AGAAAGAAAT AGGAGCAGGA TC
[A/G]
ACTTCCAGAT ATACAGAGAA TATAAAAATA CATTCACTTT ATTTTAGAAA AATGAAGACT
CATAGAGTAA GCTTATCACA AACTGGCCTA TTAGGAGTCA CAGAATTCAC AGGAAACAAT
TTCTGAAGAC CAGGTGCCTG CTGCCACCTC TCCAAGCAGG CCAGAGTCCA GTAGAGAATG
CGATTCAGGA AGATGGCTCC
```

Primer: 5’ TGGCTCACACCTGTAATCC (T_m=55.7°C)

5’ CTGGTCTTCAGAAATTGTTTCCTGTG (T_m=59.6°C)

Adjacent genes: Proline/Serine-rich coiled-coil 1 (*PSRC1*)

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele frequencies	
A/A	A/G	G/G	A	G
0.475	0.390	0.136	0.669	0.331

Publications: Samani et al. [113], Sandhu et al. [114]

Assumed associations: Coronary artery disease, LDL Cholesterol (Risk allele G)

SNP #12: rs6922269

SNP alleles: [A/G]

Ancestral allele: A

Location: 6q25

Chromosome position (NCBI build 130): Chr. 6 - 151294678

Sequence (NCBI build 130):

```
TTGGAGTGCA TGGTGAGGCC CATGGCTAAT GGCAGTGGTG CTATTCAACA AGATTCTGGG
TCTTTAGAAT AGTTCAAAGT TTTACCACTT CTCTCTGGAT AAGCCATCTT TTTGACCTTT
GAGTAAATTA TAAACTTCTT TTCAGATTGT GTCCAAATGG TCACAGGTAC TTTGACAGTT
TTTACTGTAA CTGCCAATAA
[A/G]
TAATACTCAT CTTTAAAAAG ACATCATTCT GGCAGGGCAT GGTGGCTCAC GCCTGTAATG
CCAGCCCTTT GGGAGGCCGA GGCGGGCGGA TCATTTGAGG TCAGGAGTTC GAGACCAGCC
TGGCCAACAT GGTGAAACCC TGTTTCTATT AAAAATACAA AAATTAGCTG GGCATGGTGA
TGGGTGCCTG TAATCCCAGC TACTCAAGAG GGTGAGGCAA GAGAATTGCT TGAGCCCGGG
AGGTGGAGGT TGCAATGAGC AGAGATCACA CCATTGCAGT CCAGCCTGGG CAACAGAGTG
```

Primer: 5’ GGTGCTATTCAACAAGATTCTGGG (T_m=58.8°C)

5’ TGCAATGGTGTGATCTCTGC (T_m=57.3°C)

Adjacent genes: Methylenetetrahydrofolate dehydrogenase-1-like protein (*MTHFD1L*)

Genotype and Allele frequencies (HapMap CEU):

Genotype			Allele frequencies	
A/A	A/G	G/G	A	G
0.100	0.400	0.500	0.300	0.700

Publications: Samani et al. [113]

Assumed associations: Coronary artery disease (risk allele A)

SNP #13: rs17228212

SNP alleles: [C/T]

Ancestral allele: T

Location: 15q22.33

Chromosome position (NCBI build 130): Chr. 15 - 65245693

Sequence (NCBI build 130):

```
TAATTTGGAC GTGACGTTCA TCCTGGGTTA GTTTACTGGG CTGAGATTGG CTACAGGCCT
GTGTGCTTGG GTCAGAGTGT TAAGGAGGAG ACCCACTGTC CAACCTTCTC AGATCCTTTG
CGGGTAGCCC TGGCGTCCCG CGGGTAAGTC AACTACTCCC TGTTCAAAGA GCAAATCTTG
GAGGGCTTCA GTCAGGGTCT GGGGGAGGTT TCAGAGAAAT AGATCACACT GTCTTTGCCG
TCATTGAACT
[C/T]
GCAACCTAAC TGCTGAGTGA GGACACGTCC CTTAGAGACA ACAAATAATA ACACCTGGTC
TGCACAGAAG AAGATAGTAG GGACCAAAGG GTGGGTCTGA GCCTGCAGAT CTTCAGAGGA
CAGAAAGGGG TGAGGCGGAG GCAAGTCTGG ACGCCTCCCT GGAGGGGGTG GGGCTTAACC
CTCACCTTGA AGGACCAGGA TAAGTTAGCC TGGCAGAGGC TCTAGGAGTA CACATTTCAG
ACTTGGGAGT
```

Primer: 5’ GGGTAAGTCAACTACTCCCTGTTC (T_m=59.3°C)

5’ TGTACTCCTAGAGCCTCTGCC (T_m=59.5°C)

Adjacent genes: *SMAD 3*

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele frequencies	
C/C	C/T	T/T	C	T
0.083	0.433	0.483	0.300	0.700

Publications: Samani et al. [113]

Assumed associations: Coronary artery disease

SNP #14: rs17465637

SNP alleles: [A/C]

Ancestral allele: NA

Location: 1q41

Chromosome position (NCBI build 130): Chr. 1 - 220890152

Sequence (NCBI build 130):

```
ACAGCAGAAG GCAAAGCGAG AGCAGGCCAT CTTACATGGT GGGAGCAGGA GCAAGAGAGA
GTGAGCGGGG AGGTGCTGCA CACTTTTAAA ACCAGATTCC ACAAGAACTC AGTCACCATC
ACAGAATAGC ACCACAGGGA TGGTGATAAA CCACTCATGA GAAATCTACC CTTATGATCC
AATCACCTTC CACCAGGCCC CACCTGAACA CTGGGTACTA TAATTTGTTA TGAGATTTGA
TGGAGACACA GAACCAAACC ATATCACTTT TTAAAACCAT AATAGTTATG CTGAGAAGTT
[A/C]
TTTTTTGTCA TAGTGCAAGA TAACATGTCT TTGCTGCTGA TACATTGGGT AATTCAATTT
CATTTAGTGA TCATAATAAC CTTATTATTT TTTTCTTTTT TCAGTCACGG AACAGCAAAT
TTCTGAGAAG TTGAAGACTA TCATGAAAGA AAATACAGAA CTTGTACAAA AATTGTCAAA
TTATGAACAG AAGGTATGAT TATTCTTTGT TTTGTTTTTT TTTCATGGTC CCAGCTTGAA
TTATTTGTCA CCTATCTTAT AGTTCAGTTA CTATGGTATT TTTAAATAGT ATTTATTATT
```

Primer: 5’ CCACCTGAACACTGGGTAC (T_m=56.2°C)

5’ TACCATAGTAACTGAACTATAAGATAGG (T_m=54.7°C)

Adjacent genes: Melanoma inhibitory activity family, member 3 (*MIA 3*)

Genotype and allele frequencies (HapMap CEU):

Genotype			Allele frequencies	
A/A	A/C	G/C	A	C
0.500	0.500	0.000	0.750	0.250

Publications: Samani et al. [113]

Assumed associations: Coronary artery disease

SNP #15: rs1800817

SNP alleles: [A/C]

Ancestral allele: N/A

Location: 22q13

Chromosome position (NCBI build 130): Chr. 22 - 37969799

Sequence (NCBI build 130):

```
GCCGGGGCCG GGGGCGGCGG CGCCCGGGGG CCATGCGGGT GAGCCGCGGC TGCAGAGGCC
TGAGCGCCTG ATCGCCGCGG ACCCGAGCCG AGCCCACCCC CCTCCCCAGC CCCCCACCCT
GGCCGCGGGG GCGGCGCGCT CGATCTACGC GTCCGGGGCC CCGCGGGGCC GGGCCCGGAG
TCGGCATGAA TCGCTGCTGG GCGCTCTTCC TGTCTCTCTG CTGCTACCTG CGTCTGGTCA
GCGCCGAGGT GAGTTGCCAC GGCGGCTGGG GCTGGTTCTT CATTCATTAC CTTCGCCCCC
[A/C]
CCTTCTGACC GCCCCCTCCT CTCCCTGCAG TGAACTTTGG ACCCTTGCAG CCCGCGAGCC
TGACGCCGGG CGCTGGGTGA CCTCTTCGGG CTGGGAGCGA GGTCCGGGGG TGACAGGCTC
TAAGGGAAGG CAACAGCGGT GGCTTTCTTT CCAACCGGCG GGCGAATCTG GCTCCCTAAG
CCGTTCCGTG TCGGGGGAGG GTGTGTGTGG CCCTGTCCCC CACCCTTTGG GAACCCGAGA
ACAAGCCCCT TCCCGGCCGG GGGAGAGGGG GTGGGGTGGT GCCCAGGGTG CAGAAGGCAG
```

Primer: 5’ GGAGTCGGCATGAATCGCTG (T_m=60.1°C)

5’ CCTTCCCTTAGAGCCTGTCACC (T_m=61.1°C)

Adjacent genes: Platelet derived growth factor B (*PDGFB*)

Genotype and allele frequencies:

Genotype			Allele frequencies	
NA	NA	NA	NA	NA
NA	NA	NA	NA	NA

Publications: Tambur et al. [142]

Assumed associations: Cardiac allograft vasculopathy (risk allele: CC)

SNP #16: rs1800818

SNP Alleles: [A/G]

Ancestral allele: A

Location: 22q13

Chromosome position (NCBI build 130): Chr. 22 - 37970649

Sequence (NCBI build 130):

```
CTCCCTGCGC ACCCGCAGCC TCCCCCGCTG CCTCCCTAGG GCTCCCCTCC GGCCGCCAGC
GCCCATTTTT CATTCCCTAG ATAGAGATAC TTTGCGCGCA CACACATACA TACGCGCGCA
AAAAGGAAAA AAAAAAAAAA AAGCCCACCC TCCAGCCTCG CTGCAAAGAG AAAACCGGAG
CAGCCGCAGC TCGCAGCTCG CAGCTCGCAG CCCGCAGCCC GCAGAGGACG CCCAGAGCGG
CGAG
[A/G]
GGCGGGCAGA CGGACCGACG GACTCGCGCC GCGTCCACCT GTCGGCCGGG CCCAGCCGAG
CGCGCAGCGG GCACGCCGCG CGCGCGGAGC AGCCGTGCCC GCCGCCCGGG CCCCGCGCCA
GGGCGCACAC GCTCCCGCCC CCCTACCCGG CCCGGGCGGG AGTTTGCACC TCTCCCTGCC
CGGGTGCTCG AGCTGCCGTT GCAAAGCCAA CTTTGGAAAA AGTTTTTTGG GGGAGACTTG
GGCCTTGAGG TGCCCAGCTC CGCGCTTTCC GATTTTG
```

Primer: 5’ CGCTGCAAAGAGAAAACCGGA (T_m=61.9°C)

5’ GCACCTCAAGGCCCAAGTCTCCC (T_m=66.5°C)

Adjacent genes: Platelet Derived Growth Factor B (*PDGFB*)

Genotype and allele frequencies (PubMed CEPH):

Genotype			Allele frequencies	
A/A	A/G	G/G	A	G
NA	NA	NA	0.420	0.580

Publications: Tambur et al. [142]

Assumed associations: Cardiac allograft vasculopathy (risk allele AA)

SNP #17: rs8179090 (TIMP2 Promoter -418)

SNP alleles: [G/C]

Ancestral allele: G

Location: 17q25

Chromosome position (NCBI build 130): 74433484

Sequence (NCBI build 130):

```
                                                                    C
CCCCAACTAA ACTGGCCAGG CGCACTTAAA ATTCTAAGGC CTCCATTTGA AAAAGGGATC
CTGTCAGTTT CTCAATAGGC CACCCGCCCA CAGAAACGGG GAGGTGGCGA CAGGGAACGG
CCCCTGCTCC AAAGGACACC CCTTGGCTCG CCCCGA
[G/C]
GCTGGGCTCG AAGGGACCCC GGGGTGGCGG GGGACGGAGC AGCGTAGCCC TCCAGAGTCG
AGCTGAAGGG GAAAGGGTAG CGGGTGGGTC GCCTGGTGCC CTGGAAGAAC GGGCGCGAGT
CCCACGCGCT GAGTCAGGGA CCCCGGGCGC AGAAGGCCAC GCAGCGGGGA CCGGGGTCGG
GGGGCTGGGG GCGTCCGGGG ACCCCCGCGC GGGTGCGGGT CGCGGGCGCC AGGTGGTGCG
GGAAGCCCCC GACGTGCCAG GCCGGGCACA ACAAAAGCGC GGGCTG
```

Primer: 5’ CCCCCAACTAAACTGGCCAGG (T_m=62.1°C)

5’ AGCCCGCGCTTTTGTTGTG (T_m=61.1°C)

Adjacent genes: Tissue Inhibitor of Metalloproteinase (*TIMP2*)

Genotype and allele frequencies (PubMed Asian):

Genotype			Allele frequencies	
C/C	C/G	GG	C	G
0.200	0.400	0.400	0.400	0.600

Publications: Kang et al. [54]

Assumed associations: MMD

3. Results

Sixty-eight DNA samples of the healthy control group and forty of the MMD case group were collected and analyzed. Sporadic variations of maximum +/-2 in the number of samples, was caused by insufficient signals in the sequencing procedure.

All p-values for the HWE were above 0.05.

rs501120 showed a slight deviation with a p-value of 0.024, which may be explained by the limited number of patients to make an equilibrium, gene flow, genetic drift, nonrandom mating, or natural selection [54].

Concerning the genotype and allele frequencies, p-values below 0.05 were accepted as statistically significant.

Out of the 17 SNPs analyzed in our study, one SNP (rs599839 [A/G]) reached statistical significance. The SNP rs501120 [A/G] showed strong tendencies towards significance. The complete analysis results are listed in tables 11-13.

Sequencing results (absolute numbers)								
Name	**Controls**				**Cases**			
Elastin								
rs8326 [C/G]	n=68				n=40			
	CC	0	0%	Allele Frequency:	CC	0	0%	Allele Frequency:
	CG	20	29.41%	C=0.147	CG	7	17.5%	C=0.085
	GG	48	70.59%	G=0.853	GG	33	82.50%	G=0.915
rs34208922 [-/A]	n=68				n=40			
	-/-	38	55.88%	Allele Frequency:	-/-	27	67.50%	Allele Frequency:
	-/A	24	35.29%	-=0.735	-/A	11	27.50%	-=0.813
	A/A	6	8.82%	A=0.265	A/A	2	5.00%	A=0.187
LIMK1								
rs710968 [C/T]	n= 67				n=39			
	CC	43	64.18%	Allele Frequency:	CC	28	71.79%	Allele Frequency:
	CT	22	32.84%	C=0.806	CT	11	28.21%	C=0.85
	TT	2	2.99%	T=0.194	TT	0	0%	T=0.15
rs6460071 [A/G]	n=68				n=39			
	AA	0	0%	Allele Frequency:	AA	0	0%	Allele Frequency:
	AG	20	29.41%	A=0.147	AG	8	20.51%	A=0.103
	GG	48	70.59%	G=0.853	GG	31	79.49%	G=0.897
Helgadottir								
rs10757278 [A/G]	n=68				n=40			
	AA	15	22,06%	Allele Frequency:	AA	11	27.50%	Allele Frequency:
	AG	34	50.00%	A=0.471	AG	19	47.50%	A=0.513
	GG	19	27.94%	G=0.529	GG	10	25.00%	G=0.487
McPherson								
rs2383206 [A/G]	n=68				n=40			
	AA	12	17.65%	Allele Frequency:	AA	9	22.50%	Allele Frequency:
	AG	36	52.94%	A=0.441	AG	20	50.00%	A=0.475
	GG	20	29.41%	G=0.559	GG	11	27.50%	G=0.525
rs10757274 [A/G]	n=68				n=40			
	AA	15	22.06%	Allele Frequency:	AA	10	25.00%	Allele Frequency:
	AG	33	48.53%	A=0.463	AG	20	50.00%	A=0.500
	GG	20	29.41%	G=0.537	GG	10	25.00%	G=0.500

Name	Controls				Cases			
Samani								
rs501120 [A/G]	n=68				n=40			
	AA	56	82.35%	Allele Frequency:	AA	30	75.00%	Allele Frequency:
	AG	12	17.65%	A=0.912	AG	7	17.50%	A=0.838
	GG	0	0%	G=0.088	GG	3	7.50%	G=0.162
rs1333049 [C/G]	n=68				n=40			
	CC	19	27.94%	Allele Frequency:	CC	10	25.00%	Allele Frequency:
	CG	34	50.00%	C=0.529	CG	19	47.50%	C=0.488
	GG	15	22.06%	G=0.471	GG	11	27.50%	G=0.512
rs2943634 [A/C]	n=68				n=40			
	AA	6	8.82%	Allele Frequency:	AA	5	12.50%	Allele Frequency:
	AC	33	48.53%	A=0.331	AC	13	32.50%	A=0.288
	CC	29	42.65%	C=0.669	CC	22	55.00%	C=0.712
rs599839 [A/G]	n=68				n=40			
	AA	45	66.18%	Allele Frequency:	AA	17	42.50%	Allele Frequency:
	AG	19	27.94%	A=0.801	AG	18	45.00%	A=0.650
	GG	4	5.88%	G=0.199	GG	5	12.50%	G=0.350
rs6922269 [A/G]	n=68				n=40			
	AA	3	4.41%	Allele Frequency:	AA	2	5.00%	Allele Frequency:
	AG	26	38.24%	A=0.235	AG	13	32.50%	A=0.213
	GG	39	57.35%	G=0.765	GG	25	62.50%	G=0.787
rs17228212 [C/T]	n=68				n=40			
	CC	3	4.41%	Allele Frequency:	CC	1	2.50%	Allele Frequency:
	CT	34	50.00%	C=0.294	CT	17	42.50%	C=0.238
	TT	31	45.59%	T=0.706	TT	22	55.00%	T=0.762
rs17465637 [A/C]	n=68				n=40			
	AA	5	7.35%	Allele Frequency:	AA	3	7.50%	Allele Frequency:
	AC	29	42.65%	A=0.287	AC	17	42.50%	A=0.288
	CC	34	50.00%	C=0.713	CC	20	50.00%	C=0.712

Name	Controls				Cases			
Tambur/*PDGFB*								
rs1800817 [A/C]	n=68				n=38			
	AA	27	39.71%	Allele Frequency:	AA	20	52.63%	Allele Frequency:
	AC	33	48.53%	A=0.640	AC	13	34.21%	A=0.697
	CC	8	11.77%	C=0.360	CC	5	13.16%	C=0.303
rs1800818 [A/G]	n=68				n=40			
	AA	21	30.88%	Allele Frequency:	AA	18	45.00%	Allele Frequency:
	AG	36	52.94%	A=0.574	AG	15	37.50%	A=0.638
	GG	11	16.18%	G=0.426	GG	7	17.50%	G=0.362
TIMP2								
rs8179090 [C/G]	n=68				n=39			
	GG	68	100.00%	Allele Frequency:	GG	39	100.00%	Allele Frequency:
	CG	0	0%	G=1.000	CG	0	0%	G=1.000
	CC	0	0%	C=0	CC	0	0%	C=0

Table 11: Sequencing results in absolute numbers

Sequencing results (P values and odds ratios)			
Name	**P value genotypes**	**P value allele-test**	**Odds ratio allele-test**
Elastin			
rs8326 [C/G]	N/A	0.2012	1.80(0.72;4.46)
rs34208922 [-/A]	0.4644	0.1970	1.56(0.79;3.07)
LIMK1			
rs710968 [C/T]	0.4613	0.3268	1.47(0.68;3.16)
rs6460071 [A/G]	N/A	0.3529	1.51(0.63;3.61
Helgadottir			
rs10757278 [A/G]	0.8094	0.5517	1.18(0.68;2.06)
McPherson			
rs2383206 [A/G]	0.8273	0.6297	1.15(0.66;1.99)
rs10757274 [A/G]	0.8686	0.6014	1.16(0.67;2.01)

Name	P value genotypes	P value allele-test	Odds ratio allele-test
Samani			
rs501120 [A/G]	0.0717	0.0994	2.00(0.87;4.64)
rs1333049 [C/G]	0.8094	0.5517	1.18(0.68;2.06)
rs2943634 [A/C]	0.2635	0.5074	1.23(0.67;2.24)
rs599839 [A/G]	0.0518	0.0136	2.17(1.17;4.05)
rs6922269 [A/G]	0.8349	0.6993	1.14(0.59;2.22)
rs17228212 [C/T]	0.6056	0.3672	1.34(0.71;2.52)
rs17465637 [A/C]	0.9996	0.9908	1.00(0.55;1.85)

Name	P value genotypes	P value allele-test	Odds ratio allele-test
Tambur/*PDGFB*			
rs1800817 [A/C]	0.3483	0.3952	1.30(0.71;2.37)
rs1800818 [A/G]	0.2608	0.3546	1.31(0.74;2.31)
TIMP2			
rs8179090 [C/G]	N/A	N/A	N/A

Table 12: Sequencing results of p values and odds ratios

Sequencing results (Dominance testing)				
Name	**Genotype dominance-test 1**	**Odds ratio genotype dominance test 1**	**Genotype dominance-test 2**	**Odds ratio genotype dominance test 2**
Elastin				
rs8326 [C/G]	0.1674 (C)	1.96(0.746;5.17)	N/A	N/A
rs34208922 [-/A]	0.4638 (-)	1.84(0.353;9.58)	0.2336 (A)	1.64(0.725;3.71)
LIMK1				
rs710968 [C/T]	0.2760 (C)	N/A	0.4214 (T)	1.42(0.603;3.35)
rs6460071 [A/G]	0.3135(A)	1.61(0.633;4.12)	N/A	N/A
Helgadottir				
rs10757278 [A/G]	0.7391 (A)	1.16(0.478;2.83)	0.5230(G)	1.34(0.545;3.30)
McPherson				
rs2383206 [A/G]	0.8320 (A)	1.10(0.461;2.62)	0.5383(G)	1.35(0.514;3.57)
rs10757274 [A/G]	0.6211 (A)	1.25(0.516;3.03)	0.7264(G)	1.18(0.471;2.95)

Name	Genotype dominance-test 1	Odds ratio genotype dominance test 1	Genotype dominance-test 2	Odds ratio genotype dominance test 2
Samani				
rs501120 [A/G]	0.0220(A)	N/A	0.3596(G)	1.56(0.602;4.02)
rs1333049 [C/G]	0.5230(C)	1.34(0.545;3.30)	0.7391(G)	1.16(0.478;2.83)
rs2943634 [A/C]	0.2143(A)	1.64(0.748;3.61)	0.5418(C)	1.48(0.420;5.19)
rs599839 [A/G]	0.2295(A)	2.29(0.576;9.07)	0.0163(G)	2.65(1.185;5.91)
rs6922269 [A/G]	0.5991(A)	1.24(0.557;2.76)	0.8883(G)	1.14(0.182;7.13)
rs17228212 [C/T]	0.3448(C)	1.46(0.666;3.20)	0.6114(T)	1.80(0.181;17.91)
rs17465637 [A/C]	1.000(A)	1.00(0.458;2.18)	0.9775(C)	1.02(0.231;4.52)

Name	Genotype dominance-test 1	Odds ratio genotype dominance test 1	Genotype dominance-test 2	Odds ratio genotype dominance test 2
Tambur/ *PDGFB*				
rs1800817 [A/C]	0.8339(A)	1.14(0.344;3.76)	0.1989(C)	1.69(0.757;3.76)
rs1800818 [A/G]	0.8585(A)	1.10(0.388;3.11)	0.1402(G)	1.83(0.816;4.11)
TIMP2				
rs8179090 [C/G]	N/A	N/A	N/A	N/A

Table 13: Sequencing results of the dominance testing

3.1 SNPs statistically associated with MMD

- rs501120 [A/G] showed tendency towards a risk allele G with a p-value of 0.0994 and an OR of 2.00, CI 95% = 0.87-4.64 for the allele-test. Assuming dominance of the A allele, the p-value equaled 0.0220. 82.4%(56) of the controls were homozygous for A, in comparison to only 75.0%(30) of the cases. None of the controls were homozygous for the G allele, 7.5% (3) of the cases were. The heterozygous AG alleles were distributed equally with 17.6% (12) in the controls and 17.5% (7) in the cases.

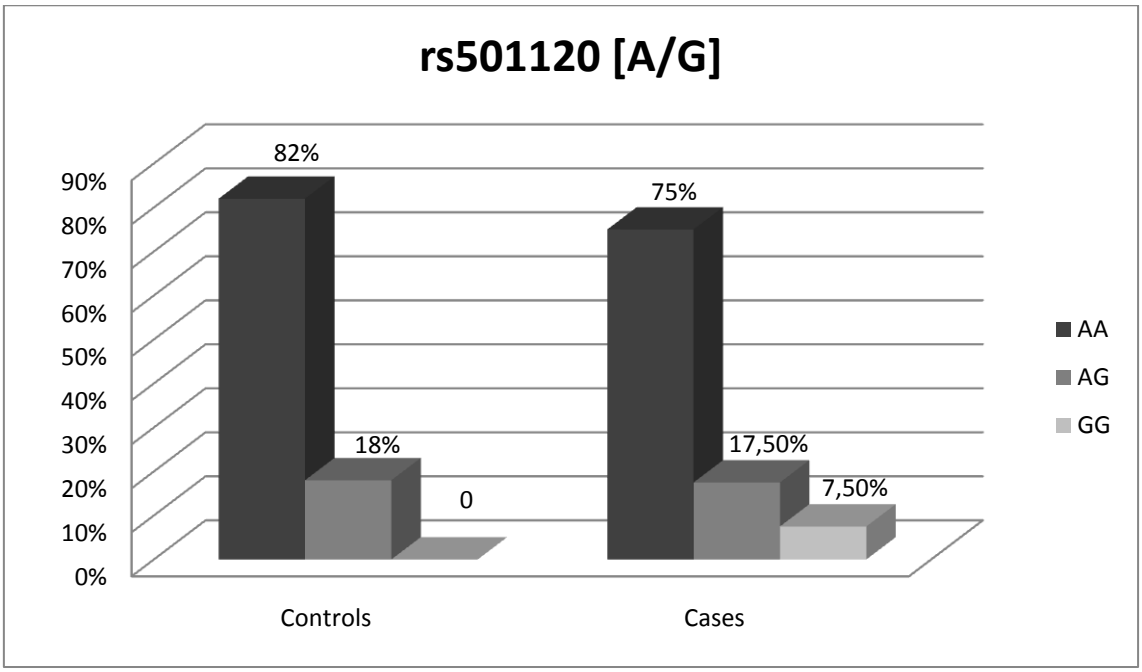


Figure 16: Results rs501120[A/G]

- rs599839 [A/G] has a risk allele G with a p-value of 0.0136 and an OR of 2.17, CI95% = 1.17-4.05 for the allele test. Assuming the dominance of the G allele, the p-value accounted for 0.0163 with an OR of 2.65, CI95% = 1.185-5.91. 66.2%(45) of the controls were homozygous for A, in comparison to only 42.5%(17) of the cases. Concerning the G allele, 5.9%(4) of the controls were homozygous, whereas 12.5%(5) of the cases showed this genotype. The heterozygous AG alleles were much

more frequent in the case group, than in the control group with 45.0%(18) vs. 27.9%(19).

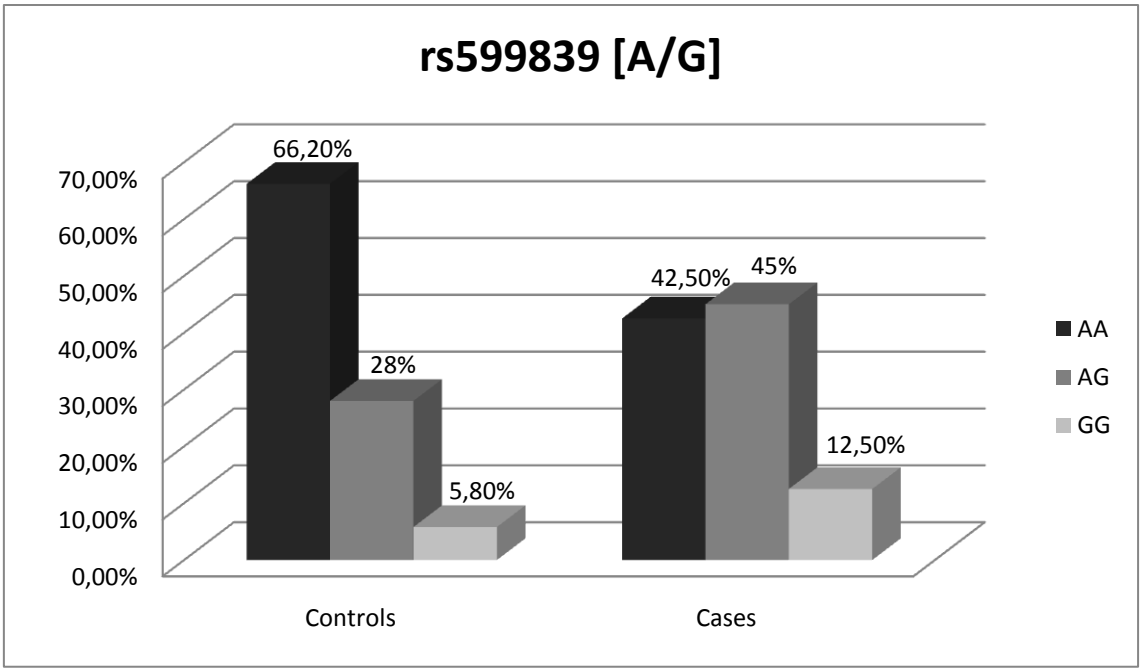


Figure 17: Results rs599839[A/G]

3.2 SNPs statistically not associated with MMD

- rs8326 [C/G] had a p-value of 0.2012 with an OR of 1.80, CI95%=0.72-4.46 for the allele test. Assuming the dominance of the C allele, the p-value is 1.674 with an OR of 1.96, CI95%=0.746-5.17. 70.59% (48) of the controls were homozygous for the G alleles, whereas only 82.50% (33) of the cases shared this genotype. 29.41%(20) of the controls were heterozygous C/G, whereas only 17.5% (7) of the cases had both alleles. No participant of both groups was homozygous for the C alleles.
- rs34208922 [-/A] had a p-value of 0.1970 with an OR of 1.56, CI95= 0.79-3.07 for the allele test. Assuming the dominance of the A allele, the p-value was 0.2336 with an OR of 1.64, CI95%=0.725-3.71. 55.88% (38) of the controls were homozygous for the missing insertion SNP as well as 67.50% (27) of the cases. 35.29% (24) of the controls

were heterozygous, but only 27.50% (11) of the cases. The homozygous A alleles accounted for 8.82% (6) of the persons in the control group, but only for 5.00% (2) in the group of cases.

- rs710968 [C/T] showed a p-value of 0.3628 with an OR of 1.47, CI95% = 0.68-3.18 for the allele test. No statistically significant results were achieved. The alleles were distributed almost equally between both cohorts with 64.18% (43) of the controls and 71.79% (28) of the cases being homozygous for the C alleles. 32.84% (22) of the controls and 28.21% (11) of the cases were heterozygous C/T, the homozygous C alleles were only present in the control group with 2.99% (2).
- rs6460071 [A/G] had a p-value of 0.3529 with an OR of 1.51, CI95% = 0.63-3.61 for the allele test. 70.59% (48) of the controls, but 79.49% (31) of the cases were homozygous for the G alleles. The heterozygous A/G alleles were shared by 29.41% (20) of the controls and by 20.51% (8) of the cases. No individual was homozygous for the A alleles.
- rs10757278 [A/G] had a p-value of 0.5517 with an OR of 1.18, CI95% = 0.69, 2.06 for the allele test. No statistically significant levels were reached. The distribution of the alleles, controls versus cases was 22.06% (15) versus 27.50% (11) for the homozygous A alleles, 50% (34) versus 47.50% (19) for the heterozygous A/G alleles and 27.94% (19) versus 25.00% (10) for the homozygous G alleles.
- rs2383206 [A/G] showed a p-value of 0.6297 with an OR of 1.15, CI95%=0.66-1.99 for the allele test in our cohorts. 17.65% (12) of the controls and 22.50% (9) of the cases were homozygous for the A alleles. The heterozygous A/G alleles were found in 52.94% (36) of the controls and in 50.00% (20) of the cases. Two G alleles were present in 29.41% (20) of the controls and in 27.50% (11) of the cases. No statistical significance was reached.
- rs10757274 [A/G] had a p-value of 0.6014 with an OR of 1.16, CI95% = 0.67-2.01 for the allele test. No statistically significant levels were reached. The allele combinations were shared in both cohorts quite

equally with the following distribution controls versus cases: AA 22.06% (15) versus 25.00% (10), AG 48.53% (33) versus 50% (20) and GG 29.41% (20) versus 25% (10).

- rs1333049 [C/G] had a p-value of 0.5517 with an OR of 2.18, CI95% = 0.68-2.06 for the allele test. Again, the distribution of the alleles was similar in both cohorts. 27.94% (19) of the controls and 25% (10) of the cases were homozygous for the A alleles. 50% (34) of the controls were heterozygous for C/G, as well as 47.50% (19) of the cases. The homozygous G alleles represent 22.06% (15) in the controls and 27.50% (11) in the cases. No statistical significance was reached.
- rs2943634 [A/C] showed a p-value of 0.5074 with an OR of 1.23, CI95% = 0.67-2.24 for the allele test. Assuming the dominance of the A allele, the p-value was 0.2134 with an OR of 1.64, CI95% = 0.748-3.61. 8.82% (6) of the controls and 12.50% (5) of the cases were homozygous AA. 48.53% (33) of the controls and 32.50% (13) of the cases were heterozygous AC. The homozygous CC alleles were shared by 42.65% (29) of the controls and 55% (22) of the cases.
- rs6922269 [A/G] had a p-value of 0.6993 with an OR of 1.14, CI95% = 0.59-2.22 for the allele test. No statistically significant numbers were reached. The control group shared the homozygous A alleles in 4.41% (3), the case group in 5.00% (2). Heterozygous A/G alleles were found in 38.24% (26) of the controls and 32.50% (13) of the cases. The homozygous GG alleles were present in 57.35% (39) of the controls and in 62.50% (25) of the cases.
- rs17228212 [C/T] had a p value of 0.3672 with an OR of 1.34, CI95% = 0.71-2.52 for the allele test. Concerning T as the risk allele, a p-value of 0.6114, OR=1.80, CI95% = 0.181-17.91 was calculated. 4.41% (3) of the controls were homozygous CC, whereat 2.50% (1) of the cases had this genotype. The heterozygous C/T genotype was distributed with 50% (34) in the control group and 42.50% (17) in the cases. The homozygous T alleles were shared by 45.59% (31) of the controls and by 55% (22) of the cases.

- rs17465637 [A/C] showed a p-value of 0.9908 with an OR of 1.00, CI95% = 0.55-1.85 for the allele test. The distribution of genotypes was equal. 7.35% (5) of the controls and 7.50% (3) of the cases were homozygous AA. The heterozygous A/C genotype was common in 42.65% (29) of the controls and 42.50% (17) of the cases. The homozygous C alleles were found in 50% (34) of the controls, as well as in 50% (20) of the cases. No statistical significance was reached.
- rs1800817 [A/C] had a p-value of 0.3952 with an OR of 1.30, CI95% = 0.71-2.37 for the allele test. No statistically significant levels were reached. The allele frequencies controls versus cases were: AA 39.71% (27) versus 52.63% (20), AC 48.53% (33) versus 34.21% (13) and CC 11.77% (8) versus 13.16% (5).
- rs1800818 [A/G] had a p value of 0.3546 with an OR of 1.31, CI95% = 0.74-2.31 for the allele test. 30.88% (21) of the controls were homozygous for AA, whereas 45.00% (18) of the cases shared this genotype. The heterozygous AG genotype was distributed with 52.94% (36) in the control group and 37.50% (15) in the cases. The homozygous G alleles were shared by 16.18% (11) of the controls and by 17.50% (7) of the cases.
- rs8179090 [C/G] was homozygous GG for 100% (68) of the controls and 100% (39) of the cases.

3.3 Conclusion

We found strong association of one SNP (rs599839 [A/G], OR=2.17, 95% CI=1.17, 4.05; p=0.01) with the risk allele G located in the 3' UTR region of the *PSRC-1* gene. Three further SNPs (rs8326, rs34208922, rs501120) in or adjacent to the genes *ELN* and *CXCL12* showed tendencies towards risk alleles with p-values between 0.1 and 0.2, but did not reach statistical significance in our cohort.

4. Discussion

This study was based on the assumption, that MMD and atherosclerotic diseases share common genetic mechanisms. This assumption was made by combining the results of different research analyses concerning the pathogenesis of Moyamoya. Several authors described and discussed histopathological findings in vessels of patients with MMD as being similar to those in patients with atherosclerosis, for example intimal thickening, a hypotrophic media, or the response to injury hypothesis [6, 42, 72, 79, 108, 132-133, 138-139, 160]. In addition, a SNP associated with MMD in the promoter region of *TIMP2* was included [54]. It is known to be a regulative element in repair processes after vascular injury [18, 29, 127] and therefore possibly related to atherogenesis. The four SNPs described by Akagawa et al.[2] close to the *Elastin* gene and associated with IAs were chosen for the fact that MMD is often associated with intracranial aneurysms [3, 62, 77, 89, 98] and that Karnik et al. [57] described Elastin as an important player in the signaling of vascular proliferative diseases. Abnormal proliferation and migration of vascular smooth muscle cells into the arterial lumen takes place in both diseases.

In conclusion, the aim of this study was to identify SNPs which were previously described to be associated with vascular abnormalities, which are possibly involved in the pathogenesis of MMD. The study was also based on the assumption, that common genetic variations between MMD and atherosclerosis are shared.
The SNPs analyzed can be summarized as follows:

- *Genetic Polymorphisms in the TIMP2 gene*: One SNP reported in the study by Kang et al. [54] in MMD patients.
- *Genetic Polymorphisms related to the Elastin gene*: SNPs reported by Akagawa et al. [2] concerning polymorphisms in the region of the Elastin gene in patients with Intracranial Aneurysms.

- *Genetic Polymorphisms related to Atherosclerotic Diseases:* SNPs reported by Samani et al.[113], Helgadottir et al. [32] and McPherson et al. [83].
- *Genetic Polymorphisms related to Atherosclerotic Cardiac Allograft Vasculopathy:* SNPs reported by Tambur et al. [142].

4.1 Genetic Polymorphisms related to the TIMP2 gene

TIMPs (Tissue Inhibitor of Metalloproteinase) are the physiological regulators of MMPs (Matrix Metalloproteinase), which are responsible for the mobilization of SMCs (smooth muscle cells) and the turnover of ECM (extracellular matrix) components. Besides their physiological function, it is known that MMPs are involved in the activation of SMC caused ECM component synthesis and in the induction of intimal hyperplasia after vascular injury within repair processes [18, 29, 127]. Continuing shear stress at the most frequent altered sites of MMD, the terminal portions of the ICAs, may lead to an increase in repair processes resulting in misdirected overgrowth caused by a defect in the regulating TIMPs. Kang et al. [54] investigated SNPs within the genes of *TIMP 2* and *4*, because of a known association between their genetic locations on Chromosomes 3p25 and 17q25 and familial MMD. The study showed significant association with familial MMD at the promoter region of *TIMP2* (promoter -418; rs8179090). Although Mineharu et al. [84] tried to reproduce this finding without success, we analyzed the SNP rs8179090 in our population. Our results resemble the findings of Mineharu et al. [84], also showing no polymorphism at this position. This may be explained by strong population diversity for this SNP.

4.2 Genetic Polymorphisms related to the Elastin gene

We genotyped the SNPs rs8326 [C/G], rs34208922 [-/A], rs710968 [C/T], rs6460071 [G/A], which had formerly been described by Akagawa et al. [2] as being associated with intracranial aneurysms in Japanese and Korean cohorts. Two of these four SNPs are located in the 3' UTR region of *ELN* (Elastin gene), two in the promoter region of *LIMK1*, which is located downstream of *ELN*. Elastin was described as being responsible for the VSMC proliferation and

migration from the media towards the intima and as an autocrine stimulator for different synthesis processes in SMCs in vasculoproliferative diseases [57, 133]. None of the four SNPs reached statistical significance in our population comparing the genotypes of healthy controls and MMD patients. But rs34208922 [-/A] and rs8326 [C/G] showed tendencies towards risk alleles. According to Akagawa et al. [2], [A] for rs34208922 and [C] for rs8326 are the risk alleles for IAs, leading to a decrease in Elastin synthesis. However, [–] for rs34208922 and [G] for rs8326 showed tendencies towards risk alleles for the development of MMD in our study. As the risk alleles [A] and [C] would decrease the amount of Elastin in vitro, our thesis that a lower amount of this protein in Moyamoya patients is responsible for the SMC migration towards the intima would be antithetic to the expected increased amount of Elastin following our genetic results ([–] for rs34208922 and [G] for rs8326). Because the SNPs in the *Elastin* 3' UTR region showed tendencies towards statistical significance, further research in different populations and larger sample groups is needed. It is possible, that genetic changes in the 3' UTR region may lead to irregularities in the posttranscriptional processing, resulting in the inability to work as an autocrine stimulator. Therefore these SNPs may play a role in the development of MMD, although our findings did not reach statistical significance and were contrary to the findings in the DNA of patients with intracranial aneurysms by Akagawa et al. [2].

4.3 Genetic Polymorphisms related to Atherosclerotic Diseases

As mentioned, many histopathological studies of MMD vessels showed similar changes in arteries from MMD patients and from patients with atherosclerosis [1, 80, 134, 159, 161]. The main changes consisted of a SMC-caused thinning of the tunica media, combined with the formation of a neointima [80] and development of "intimal pads" representing the "response to injury" hypothesis by Ross et al. [108]. Also an increased exposure to cytokine blood constituents because of extended repair processes was observed, which all together leads to stenotic changes [159]. Therefore a literature analysis on the genetic findings describing polymorphisms in patients with atherosclerotic diseases was performed. Three major studies describing the association between SNPs and

atherosclerotic disease were found, which were used for the analysis in MMD samples.

As result of a genome-wide association study on CAD (coronary artery disease), Samani et al. [113] identified seven strongly with CAD associated SNPs (rs1333049, rs6922269, rs2943634, rs599839, rs17465637, rs501120, rs17228212), which were genotyped in our cohort. One of these showed a significant disease association with MMD, another one strong tendencies towards significance.

rs599839 [A/G] showed a risk allele G with a p-value of 0.0136 and an OR of 2.17, CI95% = 1.17-4.05 for the allele test. This SNP is located on Chromosome 1p13.3 in the 3' UTR region of the *PSRC-1* (Prolin/Serin rich coiled coil 1) gene and close to the *CELSR2* (Cadherin, EGF LAG seven-pass G-type receptor 2) gene. This genetic locus is at the telomeric portion of a 150kbp segment, which has been described to be associated with increased LDL cholesterol levels in genome-wide association studies (GWAS) [59, 112, 114, 147, 149]. LDL Cholesterol is known to play an important role among the risk factors for the genesis of atherosclerotic lesions [107]. Furthermore, analysis of the human liver transcriptome and hepatic mRNA showed strong association between rs599839 and *PSRC1*, *CELSR2* and *SORT1* (Sortilin1). While the functional relevance of *PSRC1* and *CELSR2* in the lipid metabolism is still unclear, *SORT1* is known to limit lipoprotein-lipase uptake and degradation in liver cells leading to higher blood levels of these molecules [94, 112, 117]. In addition, Schadt et al. [117] proved that all three genes work together in a transcriptional subnetwork influencing the metabolic profile and atherosclerotic changes in humans and in a rodent model. To our knowledge, no data on cholesterol levels in patients with MMD is available. LDL Cholesterol may be involved in the development of the disease causing stenosis in patients with MMD. If this metabolic change plays a role in MMD, it needs to be evaluated in further functional and epidemiological studies. Also research on the exact role of rs599839 has to be done in large cohorts with vascular diseases to determine the risk allele. Our results showed a risk allele G for MMD, similar to studies by

Sandhu et al. [114] and Wallace et al. [147] who analyzed cardiovascular disease. In contrast to our results, Samani et al. [112] and Willer et al. [149] reported the A allele to be the variation with a higher risk of having increased LDL cholesterol levels .

rs501120 [A/G] showed a risk allele G with a p-value of 0.0994 and an OR of 2.00, CI 95% = 0.87-4.64 for the allele-test. This SNP is located on 10q11.21, upstream of the *CXCL12* gene (chemokine (C-X-C motif) ligand 12) and was described twice, by Samani et al. [113] and the Coronary Artery Disease Consortium [15], as being associated with CAD, risk allele T (reverse read). These findings are antithetic to our results in the group of MMD, in which the risk allele is G. Besides the evaluation of the risk allele, this SNP is located upstream of the chemokine *CXCL12*, also called stromal cell derived factor 1 (SDF-1) gene, which is known to play a key role in stem-cell homing and tissue regeneration in ischemic cardiomyopathy [7], ischemic tissues [11] and in promoting angiogenesis through recruitment of endothelial progenitor cells [15, 169]. Because of their ability to enhance the migratory capacity of endothelial cells and to support the homing of endothelial progenitor cells, CXC chemokines are known to be key molecules in the regulation of angiogenesis [17, 38]. In this group, CXCL12 is known to be one of the most potent regulators, inducing the tubulogenesis and migration of microvascular endothelial cells by enhancing the expression of VEGF and FGF [86]. Studies showed that repair processes in ischemic tissues induced by SDF-1 correlate inversely with the oxygen tension, similar to reactions with the hypoxia inducible factor (HIF-1) [11, 158], which is known to be over-expressed in MCAs (middle cerebral artery) of patients with MMD [132]. Our findings on rs501120, which is located upstream of the *CXCL12* gene, could bring attention to a new genetic region with a possible involvement in the genesis of MMD. On 10q11.21 there is a SNP (rs501120) not only associated with CAD [113], but also a gene (CXCL12) which plays a major role in the vasculogenesis of ischemic and therefore hypoxic regions. Approaching the genetic background of MMD with this region could possibly clear the two most important pathomechanisms of this disease: the stenotic change of the artery and the excessive collateralization.

Genetic and functional studies with larger cohorts are needed to elucidate the role of this SNP in the genesis of MMD and possibly also uncover the true risk allele.

The G allele of rs10757278 on 9p21.3 was described by Helgadottir et al. [32] in an association study with 4587 cases and 12.767 controls as being strongly associated with myocardial infarction. In our study, this SNP did not show any association with MMD.

McPherson et al. [83] performed a genome-wide association study on coronary heart disease with almost 2000 cases and 10.000 controls from three different groups (Ottawa heart study 1 and 2, Atherosclerosis Risk in Communities Study), finding two susceptible loci on 9p21.3, rs10757274 and rs2383206, that could be validated with around 2.300 cases and around 10.300 controls (Copenhagen City heart study, Dallas heart study and Ottawa heart study 3). These findings and two further SNPs were replicated by Shen at al. [126] in an Italian population with myocardial infarction. Therefore we genotyped rs10757274 and rs2383206, comparing the DNA of healthy controls and MMD cases. The results showed no significant differences between our two cohorts.

However the region 9p21.3 remains a region of strong interest for future studies because of several findings showing association with atherosclerotic disease, intracranial aneurysms and diabetes mellitus [10, 26, 113, 116, 119, 168]. As pointed out before, we suspect IAs, atherosclerotic disease and MMD to share common pathways in their pathogenesis. To underline this assumption, 9p21.3 has several interesting genes, of which two are described in the following.

ANRIL (antisense non-coding RNA in the INK4 locus) is a newly annotated gene for antisense non-coding RNA and although its cellular function is still not known, it seems to be an interesting candidate for genetic analysis concerning atherosclerotic disease. It has been shown that *ANRIL* is expressed in human atheromatous vessels of abdominal aortic aneurysm and endarterectomy samples, as well as in vascular endothelial cells, monocyte-derived macrophages, and coronary smooth muscle cells [26]. All of these are related to

atherosclerotic disease. To find out more about the role of *ANRIL* in atherosclerotic disease, Jarinova et al. [52] recently performed a functional analysis of conserved non-coding sequences within 9p21.3. As result it was demonstrated, that the risk variant significantly influenced the expression of different variants of *ANRIL*, which influenced the transcript levels of the cell cycle regulators *CDKN2A* and *B*, possibly leading to proliferation in smooth muscle cells.

CDKN2A and *B* encode for inhibitors of the Cyclin-dependent-kinase and for a regulatory element of p53, all regulators of senescence, cell aging and apoptosis in many cell types. The group around Murat Günel at YALE [10] hypothesized that *SOX17* and $p16^{INK4a}$, one of the expressed CDKN regulators, may play similar roles in vascular development and repair. SOX17, a member of the Sry-related HMG box transcription factor family, is located between two association peaks that they found in a GWAS on IAs. It may be part of the pathogenesis of aneurysms, because of its responsibility for endothelial formation and maintenance after injury with bone-marrow derived stem cells. On the other hand, $P16^{INKa}$ is also known to regulate progenitor cell populations of the vasculature. With this in mind and the fact that IAs are often located on branching points of vessels with high shear stress, they considered that the development of IAs may be caused by defective stem-cell repair mechanisms [10]. This hypothesis is similar to the assumption, that the stenosis in MMD patients is triggered by an inadequate response mechanism to microinjuries in the walls of the arteries [104, 106, 159].

4.4 Genetic polymorphisms related to Atherosclerotic Cardiac Allograft Vasculopathy

Cardiac allograft vasculopathy (CAV) is a frequent atherosclerotic change in coronary arteries after heart transplantation. Tambur et al. [142] performed SNP analyses in candidate genes (mainly cytokines and growth factors) and found an association between rs1800817 (CC as protective alleles) and rs1800818 (AA as risk alleles), both located in the *PDGF* gene, and CAV. Because of

possible parallels in the development of atherosclerotic disease and MMD and the fact that these SNPs are located within the *PDGF* gene, we decided to genotype the above mentioned SNPs. Our results did not show any significant association between MMD and rs1800817 and rs1800818.

Although our study on these two SNPs did not show significant results, we still think that there is a need to further investigate genetic differences in the *PDGF* gene between healthy controls and MMD patients. As explained in detail in the introduction, several authors mentioned and examined PDGF as a possible initiator of different processes in the genesis of MMD [4-5, 159]. It was described, that PDGF supposedly plays a key role in the migration and replication of SMCs, resulting in a thickening of the intima [106, 159]. Also different reactions on a PDGF stimulus between SMCs of healthy controls and MMD patients were proven. Aoyagi et al. [4-5] investigated reactions of VSMCs on PDGF and described a difference in DNA synthesis and proliferation of MMD VSMCs in comparison to the controls. It was also shown, that this could be due to a down-regulation of PDGF receptors on VSMCs of patients with MMD, showing a need for further genetic studies not only on the PDGF gene, but also on the genes for the receptors. Besides in studies with MMD samples, it is proven that PDGF plays a major role in the directioning of SMCs from the tunica media to the tunica intima and to sites of injury where their proliferation results in an intimal thickening [104]. Also the differentiation of progenitors to VSMCs is very likely regulated by PDGF [71], another incentive to take a closer look at this gene.

5. Summary

The Moyamoya Disease (MMD) is an uncommon cerebrovascular disease with a very poor outcome if untreated. The etiology of this disease is still unknown; however genetics seem to be the most promising approach to understand the reason for the pathologic changes. In our study we genotyped seventeen SNPs in 40 affected patients and 68 healthy controls from central Europe. The SNPs were chosen based on publications describing histopathological changes in the walls of vessels affected by MMD as being in some aspects similar with those in atherosclerosis. The results of our study revealed a significant association for the SNP rs599839 [A/G] (p=0.0136, OR = 2.17, CI95%=1.17-4.05, risk allele G), and a strong tendency towards significance for the SNP rs501120 [A/G] (p=0.0994, OR = 2.00, CI 95% = 0.87-4.64, risk allele G).

In conclusion we found a new association between MMD and rs599839 and a strong tendency towards significant association for rs501120 in a central European cohort. For both SNPs an association with coronary artery disease was proven by Samani et al. [113] in a European cohort, underlining our assumptions of common pathways in the genesis of MMD and atherosclerotic disease.

Because of the limited amount of MMD samples and strong genetic variability between different ethnicities, further studies with larger cohorts from different ethnical groups are needed to verify our findings for a better understanding of the Moyamoya disease.

6. Figures and tables

7. References

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