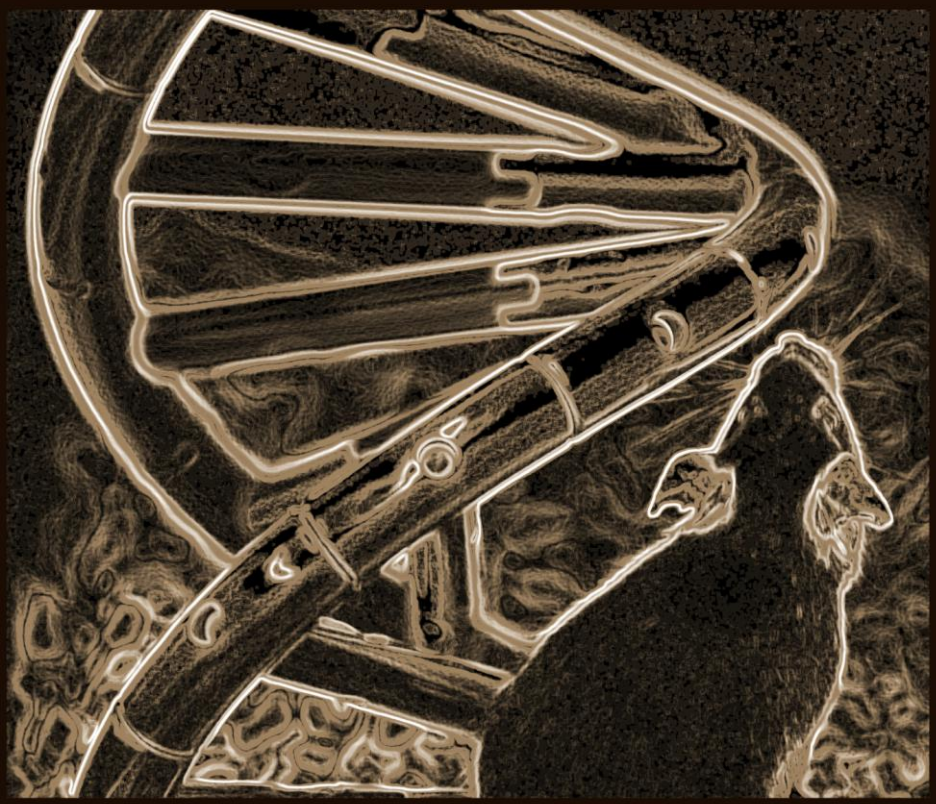
# On the Genetics of Intracranial Aneurysms and on Growth Factor Induced Angiogenesis in the Murine Brain

Emília Ilona Gaál



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**Emília Ilona Gaál**

## ACADEMIC DISSERTATION

*To be publicly discussed, with the permission of the Faculty of Medicine of the University of Helsinki, in the Lecture Hall 1 of Töölö Hospital on the 30th of November, 2012 at 12 o´clock noon.*

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*"I think that only daring speculation can lead us further and not accumulation of facts."*

*Albert Einstein*

*"Judah Folkman (who was both a surgeon and a basic scientist) once said that the difference between surgeons and basic scientists is that when someone can't reproduce the results of a basic scientist, the scientist becomes alarmed. When someone can't reproduce the results of a surgeon, the surgeon takes it as a compliment to their superior skills."*

*Brent R. Stockwell: The Quest for the Cure*

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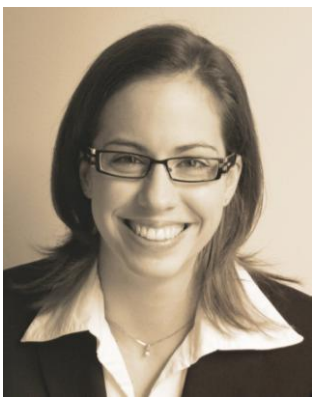

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II Yasuno K, Bilguvar K, Bijlenga P, Low SK, Krischek B, Auburger G, Simon M, Krex D, Arlier Z, Nayak N, Ruigrok YM, Niemelä M, Tajima A, von und zu Fraunberg M, Dóczi T, Wirjatijasa F, Hata A, Blasco J, Oszvald A, Kasuya H, Zilani G, Schoch B, Singh P, Stüer C, Risselada R, Beck J, Sola T, Ricciardi F, Aromaa A, Illig T, Schreiber S, van Duijn CM, van den Berg LH, Perret C, Proust C, Roder C, Ozturk AK, **Gaál E**, Berg D, Geisen C, Friedrich CM, Summers P, Frangi AF, State MW, Wichmann HE, Breteler MM, Wijmenga C, Mane S, Peltonen L, Elio V, Sturkenboom MC, Lawford P, Byrne J, Macho J, Sandalcioglu EI, Meyer B, Raabe A, Steinmetz H, Rüfenacht D, Jääskeläinen JE, Hernesniemi J, Rinkel GJ, Zembutsu H, Inoue I, Palotie A, Cambien F, Nakamura Y, Lifton RP, Günel M: Genome-wide association study of intracranial aneurysm identifies three new risk loci. **Nature Genetics** 2010 May;42(5):420-5.

III **Gaál EI**, Salo P, Kristiansson K, Rehnström K, Kettunen J, Sarin A-P, Niemelä M, Jula A, Raitakari OT, Lehtimäki T, Eriksson JG, Widen E, Günel M, Kurki M, von und zu Fraunberg M, Jääskeläinen JE, Hernesniemi J, Järvelin M-R, Pouta A, The International Consortium for Blood Pressure Genome-Wide Association Studies, Newton-Cheh C, Salomaa V, Palotie A, Perola M: Intracranial aneurysm risk locus 5q23.2 is associated with elevated systolic blood pressure. **PLoS Genetics** 2012 Mar;8(3):e1002563. Epub 2012 Mar 15.

IV **Gaál EI**, Tammela T, Anisimov A, Marbacher S, Honkanen P, Tatlisumak T, Hernesniemi J, Niemelä M, Alitalo K: Comparison of vascular growth factors in the murine brain reveals placenta growth factor as prime candidate for CNS revascularization. *Submitted.*

Additional unpublished data is included.

*The publications are referred to by their Roman numerals in the text.*

# Abstract

Cerebrovascular diseases continue to challenge us by robbing lives and leaving many disabled still in their prime working age. Some cerebrovascular diseases are more acute in nature, and some erode the quality of life over a long period of time.

A life-threatening form of acute cerebrovascular disease is brought on by the rupture of an intracranial aneurysm (IA). Most IAs are berry-shaped pouches at the forking site of cerebral arteries. Since according to autopsy results, 2-5% of the population harbours IA, it is a common disease. Most IA go unnoticed during one's lifetime, however, often the first symptom they give is their deadly rupture. Likely, both environmental factors and a compound genetic susceptibility, contribute to the risk of IA, making it a complex disease. The aim of studies I-III was to test whether in humans common genetic variants contribute to the susceptibility to IA (I,II), and to seek genetic evidence for their pathomechanism (III). In multinational genome-wide association studies (I,II) we identified 5 loci with strong statistical evidence of association with IA, and a further 14 loci with suggestive evidence. Further, we found that suggestive IA risk locus at 5q26 is strongly associated with high systolic blood pressure in over 210 000 individuals of European descent, highlighting the connection between hypertension and IA (III).

To gain further insight into cerebral vasculopathies and to facilitate the development of novel therapies, in study (IV) we turned our attention to vascular growth factor induced angiogenesis in a model organism. We tested by viral gene transfer the known vascular growth factors in the murine central nervous system and characterised extensively the angiogenesis upon treatment. The aim of the study was to identify the best candidate vascular growth factor(s) for therapeutic brain angiogenesis. We identified placenta growth factor as the most safe and efficient candidate for therapeutic revascularisation of the central nervous system. We envision a placenta growth factor enhanced multiple bur hole indirect extracranial-intracranial bypass as a novel therapeutic approach in the brain, possibly aiding the treatment of diseases such as chronic cerebral hypoperfusion, complex IAs and stroke.

# Abbreviations

| | |
|---|---|
| AAA | Abdominal Aortic Aneurysm |
| Ab | Antibody |
| AGU | Aspartylglucosaminuria |
| AMD | Age-Related Macular Degeneration |
| Ang | Angiopoietin |
| AAV | Adeno-Associated Virus |
| ACH | Acute Cerebral Hypoperfusion |
| AD | Alzheimer Disease |
| ADPKD | Autosomal Dominant Polycystic Kidney Disease |
| ASA | Acetylsalicylic Acid |
| AVM | Arteriovenous Malformation |
| BBB | Blood-Brain barrier |
| BMI | Body Mass Index |
| bp | Base Pair |
| BP | Blood Pressure |
| CAD | Coronary Artery Disease |
| CBV | Cerebral Blood Volume |
| CFH | Complement Factor H |
| CH | Cerebral Hypoperfusion |
| CCH | Chronic Cerebral Hypoperfusion |
| CNS | Central Nervous System |
| CNV | Copy Number Variation |
| CSF | Cerebrospinal Fluid |
| CT | Computed Tomography |
| CTA | Computed Tomography Angiography |
| DAVF | Dural Arteriovenous Fistula |
| DBP | Diastolic Blood Pressure |

| | |
|---|---|
| DGV | Database of Genomic Variants |
| DIND | Delayed Ischaemic Neurological Deficit |
| DSA | Digital Subtraction Angiography |
| EC | Endothelial Cell |
| EC-IC | Extracranial-Intracranial |
| EDAS | Encephalo-Duro-Arterio-Synangiosis |
| EDAMS | Encephalo-Duro-Arterio-Myo-Synangiosis |
| EDS-IV | Ehlers-Danlos Syndrome, type IV |
| ELANA | Excimer Laser-Assisted Nonocclusive Anastomosis |
| FDA | US Food and Drug Administration |
| HSA | Human Serum Albumin |
| HUGO | Human Genome Organisation |
| IA | Intracranial Aneurysm |
| ICA | Internal Carotid Artery |
| ICBP-GWAS | The International Consortium for Blood Pressure Genome-Wide Association Studies |
| IC-IC | Intracranial-Intracranial |
| kb | Kilobase Pair |
| LD | Linkage Disequilibrium |
| MAF | Minor Allele Frequency |
| MAP | Mean Arterial Pressure |
| Mb | Megabase Pair |
| MMD | Moyamoya Disease |
| MMP | Matrix Metalloproteinase |
| MRI | Magnetic Resonance Imaging |
| MRA | Magnetic Resonance Angiography |
| NP | Neuropilin |
| OPT | Optical Projection Tomography |
| PPA | Posterior Probability of Association |
| PlGF | Placenta Growth Factor |
| PP | Pulse Pressure |

| | |
|---|---|
| QC | Quality Control |
| qPCR | Quantitative Real Time Polymerase Chain Reaction |
| RR | Relative Risk |
| SBP | Systolic Blood Pressure |
| SD | Standard Deviation |
| SMA | Smooth Muscle Actin |
| SMC | Smooth Muscle Cell |
| SNP | Single Nucleotide Polymorphism |
| STA-MCA | Superficial Temporal Artery to Middle Cerebral Artery |
| T2D | Type 2 Diabetes |
| TAF | Tumor Angiogenic Factor (i.e. VEGF) |
| TIA | Transient Ischaemic Attack |
| Tie | Tyrosine Kinase with Immunoglobulin and EGF homology domains |
| TIMP | Tissue Inhibitor of Metalloproteinase |
| VEGF | Vascular Endothelial Growth Factor |
| VEGFR | VEGF-Receptor |
| VD | Vascular Dementia |
| vLINCL | Finnish variant of Late Infantile Neuronal Ceroid Lipofuscinosis |
| VPF | Vascular Permeability Factor (i.e. VEGF) |

Gene symbols are not shown here, all symbols used follow the Human Genome Organisation (HUGO) guidelines (Wain et al., 2002).

# Introduction

Cerebral vasculature is one of the densest vascular networks in the human body (Picture 1) (Zlokovic et al., 1998). Cerebral arteries are distinct from their extracranial counterparts in multiple ways: their walls are weakened by the lack of the external elastic lamina (Nyström, 1963) and they are armoured by strong autoregulation in order to keep cerebral blood flow stable (Astrup et al., 1981) within the confined space defined by the skull. Sadly, a variety of diseases plague cerebral arteries.

Medical research aspires to decipher pathobiology for the sake of improving prevention and patient care. Our understanding of the pathobiology and of possible therapies may be advanced by a variety of approaches; such as dissecting genetic susceptibility, understanding the functional effect(s) of genetic variants, and by studying the healthy or the pathological process in a model organism. The studies summarised here represent different sections of this spectrum of approaches. They aim to provide further insight into cerebrovascular biology and pathobiology.

Our understanding of the genetic background of diseases has increased considerably during the last few decades. The first boom of modern genetics was between the end of the 1980s and early 2000s. During this period, the genetic background of monogenic, highly penetrant (i.e. Mendelian) diseases was dissected in large numbers (Peltonen et al., 1999). Hence, the era may be referred to as the "Mendelian disease boom". The initial success made some believe for a while that understanding of the genetic background will provide a shortcut to cures. However, it turned out that only some rare disorders or rare manifestations of common disorders obey Mendelian rules (Luft, 2003). Genetic susceptibility of most traits (like height or longevity) and of most common diseases (such as hypertension, diabetes or intracranial aneurysm) remained elusive at a population level (Schork, 1997). We have witnessed the second boom of modern genetics in the last seven years. In this new "era of genome-wide association studies (GWAS)", cohort sizes peaked to a new, previously unheard of, level (study participants up to 200 000 or more) (Ehret et al., 2011). We gradually learned that in complex diseases, each variant contributes only a small individual effect (Hindorff et al., 2009), leaving their cumulative effect comparable with that of traditional risk factors, such as

environmental and lifestyle related risks (Visscher et al., 2012). Furthermore, we came to appreciate the importance of the intergenic regions, once referred to as genetic deserts (Hindorff et al., 2009). Currently, we are on the verge of a new era, the "post GWAS era", where we search for the contributing variants by sequencing the whole exome, or the whole genome of individuals. Publications I-III dissect the genetic susceptibility to intracranial aneurysm (IA), revealed by the "GWAS era".

Understanding the mechanism of genetic contribution to disease risk may facilitate future prevention. Although studies I-III greatly improved that of IA risk, yet, the most efficient currently known preventions are smoking cessation and treatment of high blood pressure. When IAs form, however, they often necessitate invasive treatment. Most IAs can be surgically directly clipped, or coiled by endovascular means. Complex IAs occasionally may require bypass surgery prior to the closure of the vessel segment harbouring the aneurysm (Hopkins et al., 1979; Spetzler et al., 1980; Sanai et al., 2009). Cerebral bypass surgeries remain technically challenging, involving significant risks, and are confined to subspecialised centres (Uno et al., 1998; Mesiwala et al., 2008).

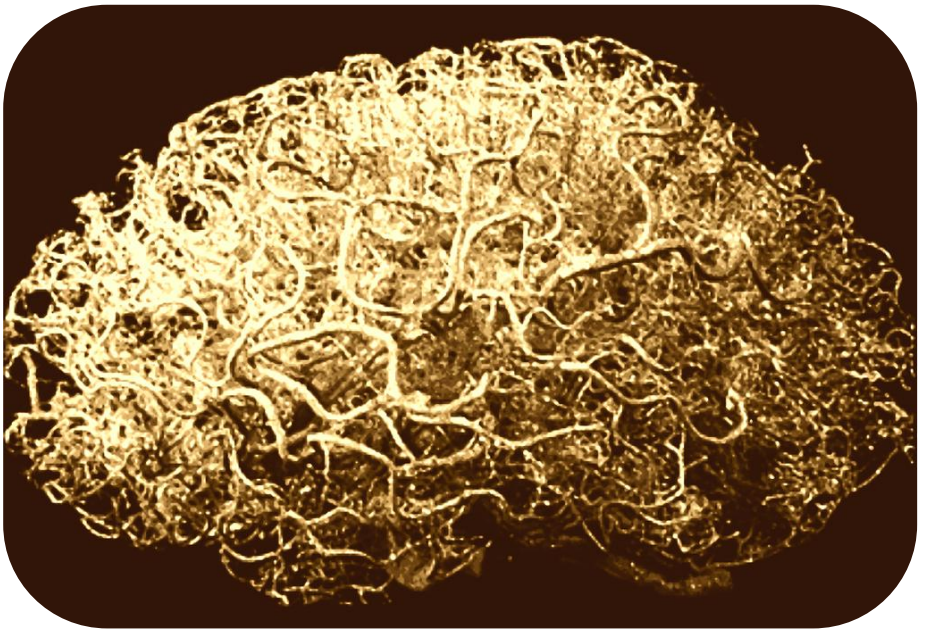
In order to facilitate the development of a technically less challenging cerebral bypass, we studied brain angiogenesis in a model animal. Angiogenesis is the vascular growth factor promoted formation of new blood vessels from the pre-existing vasculature. Publication IV presents an unfolding translational project aiming to harvest decades of vascular research in order to utilise vascular growth factors in the central nervous system (CNS). Vascular growth factors may boost cerebral bypasses, hence providing a new treatment approach for revascularisation. It may be utilised in conditions such as chronic cerebral hypoperfusion, seen in diseases like vascular dementia and moyamoya disease, or it may be involved in the treatment strategy of complex aneurysms or subarachnoid haemorrhage.

In this thesis, I review and present the results of modern genetics in deciphering the genetic contribution to IA. Further, I review the role of angiogenesis in biological and pathological processes and show how we identified the vascular growth factor most suitable for revascularisation of the brain.

# Review of Literature

## *1 Special Features of Cerebral Circulation*

### 1.1 General Features



**Picture 1.** Vasculature of the brain (modified from (Zlokovic et al., 1998)).

The average adult human brain is composed of 80-120 billion neurons (Azevedo et al., 2009; Herculano-Houzel, 2009) with an actual mass of approximately 1.5 kg (Carpenter, 1996). Typically 750 ml of blood rushes through the brain per minute (Ito et al., 2003). Hence, the brain, weighing less than 2% of the body's weight, receives 15% of the cardiac output and demands around 20% of the oxygen consumed by the whole body (Kumar et al., 2003). The cerebral blood volume, i.e., the volume of blood within the cerebral vasculature at a given time, is approximately 50 ml, or 3-5% of the total intracranial volume, with significant temporospatial differ-

ences to it (Ito et al., 2003; Sourbron et al., 2009; Krieger et al., 2012). The brain and its vasculature is suspended in the cerebrospinal fluid (CSF) which has a net weight of around 25 grams (Noback, 2005).

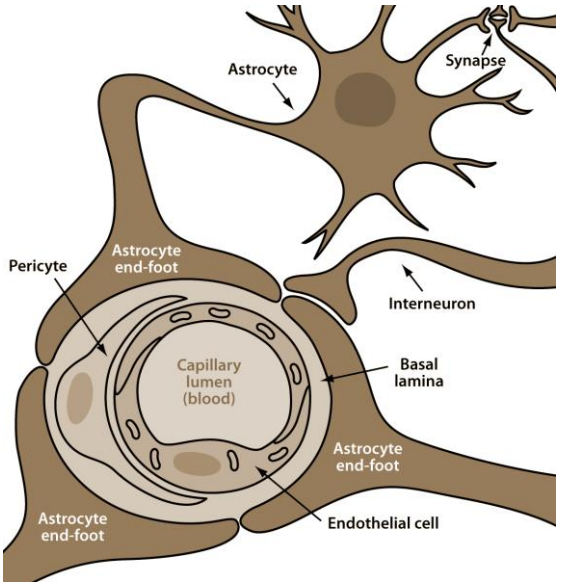
## 1.2 Cerebral Blood Supply

The brain derives its blood supply for its dense capillary network (Picture 1) from the internal carotid- and vertebral arteries. The internal carotids and their branches are referred to as the anterior circulation. The two vertebral arteries unite at the caudal border of the pons to form the basilar artery. The vertebrobasilar arterial system and its branches are called the posterior circulation of the brain. The anterior and the posterior circulations are joined by the anterior and posterior communicating arteries in front of the brainstem in the circle of Willis (Willis, 1664).

Global disruption of blood supply to the brain leads to irreversible damage within minutes (Astrup et al., 1981; Sakoh et al., 2001; Sobesky et al., 2004). Due to this vulnerability, the brain is protected by its autoregulation of blood vessels that provide a steady blood flow in a wide range of perfusion pressures (Astrup et al., 1981). Local interruption of blood flow commonly leads to cerebral ischaemia or stroke (Astrup et al., 1981; Sakoh et al., 2001; Sobesky et al., 2004). Though branches of the cerebral arteries form anastomoses with each other (Liebeskind, 2003, 2007), this is mainly restricted to the surface of the brain in the form of leptomeningeal collaterals (Brozici et al., 2003; Kucinski et al., 2003; Alastruey et al., 2007; Krishnaswamy et al., 2010). These collaterals have only a limited capability to correct for a sudden blockage of cerebral arteries (Liebeskind, 2005a; Miteff et al., 2009). However, if a vessel closes over a long period of time, further collaterals may form that may be sufficient to prevent stroke (Schaper et al., 2003; Schaper, 2009; Shuaib et al., 2011). There are many physiological variants of the cerebral circulation, leading to different levels of compensatory ability in the case of adverse events (Liebeskind, 2005a, b; Miteff et al., 2009; Liebeskind et al., 2010; Shuaib et al., 2011).

## 1.3 The Blood-Brain Barrier

**Figure 1.** The schematic ultrastructure of the blood-brain barrier (modified from (Tam et al., 2010)).




The CNS is separated from the systemic immune system, hence it is an immune-privileged organ, with some restrictions to it. Though there is a lack of conventional lymphatic system, immune cells are present in the CNS (Carson et al., 2006) and antigens are drained via the CSF to the cervical lymph nodes (Hatterer et al., 2006). Furthermore, recent results suggest a critical role for astroglial cells in the clearance of interstitial solutes (Iliff et al., 2012). The most important component of the barrier between the CNS and the systemic immune system is the blood-brain barrier (BBB). The BBB is a highly selective physiological barrier that occurs along the capillaries of the CNS. It consists of multiple layers; such as a single layer of endothelial cells joined by tight junctions (Risau, 1998), pericytes and astrocyte foot processes (Figure 1). The complex of the BBB is regulated by the pericytes (Armulik et al., 2010). The functional importance of pericytes is emphasised by their exceptionally high density in the CNS (Sims, 1991; Bergers et al., 2005). Disruption of the BBB leads to vasogenic oedema, when fluid escapes from the vasculature to the interstitial space (Kumar et al., 2003). Vasogenic oedema is seen, for example, with abnormally permeable vessels of malignant brain tumours, such as high-grade gliomas.

## 1.4 The Cerebral Artery Wall

Walls of cerebral arteries, like the walls of extracranial ones, consist of three histological layers (Figure 2). However, cerebral arteries lack an external elastic lamina, and their adventitia is only weakly developed (Nyström, 1963; Stehbens, 1972; Ostergaard et al., 1987), leading to an anatomically less firm structure. Furthermore, vasa vasori, the small capillaries supplying the blood vessel walls, are rare and confined to the proximal segments of the internal carotid- and vertebral arteries (Aydin, 1998).

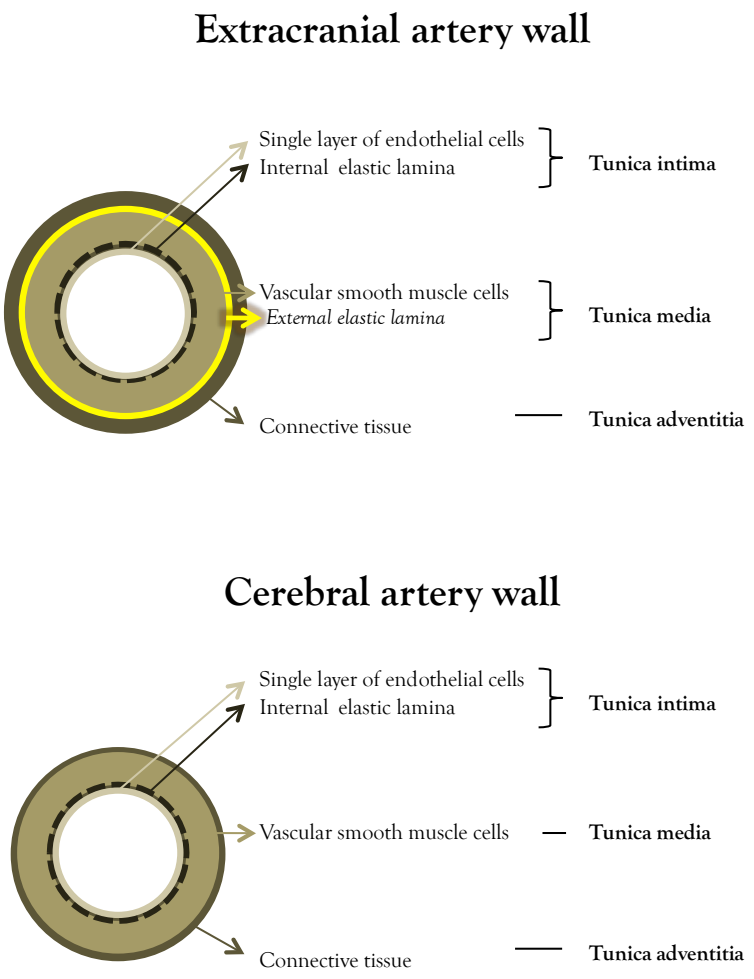



**Figure 2.** Comparing anatomy of the extracranial and the cerebral arterial walls.

# 2 Common Pathologies of the Cerebral Vasculature

## 2.1 General Classification of Cerebrovascular Disorders

Cerebrovascular disorders are a heterogeneous group of diseases affecting the blood vessels supplying the brain. Cerebrovascular disorders may be categorised as (1) cerebral hypoperfusion (CH), (2) intracranial bleeds and (3) vascular malformations.

Lack of sufficient cerebral blood supply (1), i.e. CH, acutely may lead to ischaemic stroke, whereas chronically it is suspected to cause cognitive impairment, such as vascular dementia (VD) (Sarti et al., 2002; Ruitenberg et al., 2005). Intracranial bleeds (2) may be intracerebral in nature, often due to the harmful effect of sustained hypertension, or the bleeding may be confined to the subarachnoid space, referred to as subarachnoid haemorrhage (SAH). The most common cause of SAH is the rupture of an IA (van Gijn et al., 2007). The more common forms of intracranial vascular malformations (3) are arteriovenous malformations (AVM) (Weber et al., 2006) and cavernomas (Weber et al., 2006; Vernooij et al., 2007), whereas dural arteriovenous fistulae (DAVF) are a more rare pathology (Newton et al., 1969).

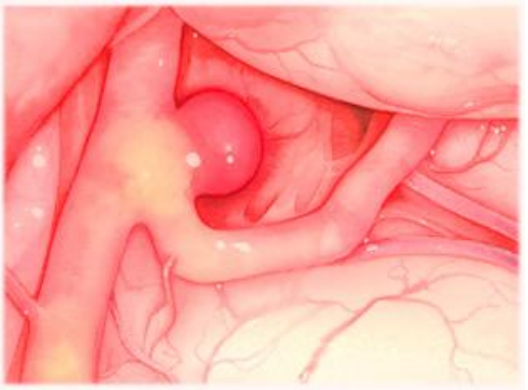
From the common cerebrovascular disorders, this thesis focuses on IA and hypoperfusion.

## 2.2 Intracranial Aneurysms

The majority (97%) of IAs are berry-shaped pouches at the branching sites of cerebral arteries, referred to as saccular IAs (Picture 2). A minority (3%) of IAs are fusiform, indicating a dilated arterial segment. Most research, including that presented in this thesis, focuses on saccular IA.

### 2.2.1 Epidemiology of IA

IAs are acquired lesions (Rinkel et al., 1998). The prevalence of IA is 2-5% in most studied populations (Rinkel et al., 1998; Ronkainen et al., 1998; Vernooij et al., 2007; Vlak et al., 2011) and will likely never become symptomatic (Brisman et al., 2006). Up to one third of patients may carry multiple IAs (Inagawa, 1990a; Rinne et al., 1994; Ellamushi et al., 2001). The majority of IAs are sporadic, with less than 10% of patients showing familial aggregation (Ruigrok et al., 2005). True familial IA, with Mendelian-like pedigree, is very rare (Verlaan et al., 2006; Kim et al., 2011).



**Picture 2.** Saccular IA (modified from: www.swedish.org).

### 2.2.2 Rupture of IA causes SAH

The rupture of an IA is the most common (85%) cause of SAH (van Gijn et al., 2007). SAH is a devastating form of stroke, the cumulative mortality at six months is 50% (Fogelholm et al., 1993; Hop et al., 1997; Pobereskin, 2001; Sivenius et al., 2004; Stegmayr et al., 2004) and SAH survivors are plagued by a 12% excess mortality even up to 15 years after the event (Huttunen et al., 2011). Recently, the fatality rate of SAH has shown a gradual improvement (Hop et al., 1997; Stegmayr et al., 2004; de Rooij et al., 2007; van Gijn et al., 2007), still, 10-15% of SAH patients die before reaching medical attention (Pakarinen, 1967; Pobereskin, 2001; Huang et al., 2002). The rupture risk of an IA depends on, among other factors, its size and location (Morita et al., 2012).

### 2.2.3 Epidemiology of SAH

Though the lifetime prevalence of IA is comparable worldwide (Vlak et al., 2011), their rupture rates differ. In most populations the incidence of SAH is 6–10 cases per 100 000 person-years (Linn et al., 1996; ACROSS, 2000). However, in Finland,

Japan and Northern Sweden the incidence is much higher, being 16–20 cases per 100 000 person-years (Ohkuma et al., 2002; Sivenius et al., 2004; Stegmayr et al., 2004). In Finland more people die annually because of SAH than due to traffic accidents. In 2010, there were 295 deaths due to SAH compared to 251 deaths from traffic accidents (Finland-Statistics, 2010). The reason for this higher-than-average incidence is unknown, though, it seems to be predominantly due to environmental factors (Ruigrok et al., 2001; van Gijn et al., 2007; Korja et al., 2010). SAH affects the working age population, with a median age of 55 (Hop et al., 1997). The lost years of potential life are significant and similar to those lost to ischaemic stroke and intracerebral haemorrhage (Johnston et al., 1998; Rivero-Arias et al., 2010). Aneurysmal SAH, as a sudden, often deadly disease, places a heavy burden on families and the whole society both emotionally and financially (Johnston et al., 1998).

### 2.2.4 Risk Factors of IA and SAH

The risk factors of IA and SAH are overlapping, although appear not to be the same. Separating them would be useful, but it is rather difficult, among other factors, due to the confounding effect of actively treating unruptured IA. Positive family history of IA (Ronkainen et al., 1997), older age and female sex are all non-modifiable risk factors of developing IA (Rinkel et al., 1998; Vlak et al., 2011). Of the modifiable risk factors, smoking has been shown to increase the risk of IA formation (Juvela, 2002b), and high blood pressure has long been speculated to do so (Inci et al., 2000). As for SAH, the strongest non-modifiable risk factor is positive family history. Modifiable risk factors of SAH are hypertension, smoking and excessive alcohol consumption, with the latter two showing additive effect (Juvela, 2002a, 2003; Feigin et al., 2005; Lindekleiv et al., 2012).

### 2.2.5 Histopathology of IA

Studying the walls of IAs revealed multiple degenerative changes. The wall of IA is often characterised by a damaged endothelium (Scanarini et al., 1978; Kataoka et al., 1999; Frösen et al., 2004), by the loss of the internal elastic lamina (Nyström, 1963; Scanarini et al., 1978; Frösen et al., 2004), and by a disorganised, rather rigid

medial layer. The elasticity of the medial layer is lost both due to the decreased number of vascular smooth muscle cells (Scanarini et al., 1978; Sakaki et al., 1997; Frösen et al., 2004; Rajesh et al., 2004) and due to the phenotypic switch in them; from contractile towards proliferating type (Nakajima et al., 2000).

Not surprisingly, the walls of ruptured IAs differ greatly from those that are unruptured. Some differences are the consequences of the rupture, however, significant differences such as inflammation (Tulamo et al., 2006) and remodelling of the wall (Frösen et al., 2004, 2006; Laaksamo et al., 2008), exist prior to the rupture. These differences facilitate the classification of IAs according to their tendency to rupture (Frösen et al., 2004). Taken together, histopathological and epidemiological (Huttunen et al., 2010) evidence suggests that rupture-prone IAs form a distinct subgroup. Whether an IA can transform from non-rupture-prone subgroup to the rupture-prone one, or *vice versa*, is therapeutically an important question and is not yet well understood.

### 2.2.6 Diagnosis and Treatment of IA and SAH

Most IAs present with rupture, causing predominantly SAH. Common clinical symptoms of SAH are sudden onset of headache with altered level of consciousness or focal neurological deficit (Menghini et al., 2001; van Gijn et al., 2001). The diagnosis of SAH is primarily achieved with CT-scan (Adams et al., 1983), however, a minor SAH may not be detectable with CT and a lumbar puncture is needed for confirmation (Vermeulen et al., 1989). In patients with SAH, IA should be suspected and sought by cerebral angiography (Pedersen et al., 2001). Ruptured, untreated IAs carry a high risk of rebleeding, with the peak incidence in the first 24 hours (Pakarinen, 1967; Kassell et al., 1983; Inagawa et al., 1987; Juvela, 1989). Up to 60% of patients die after rebleeding (Pakarinen, 1967; Kassell et al., 1983; Inagawa et al., 1987; Juvela, 1989), hence, the primary aim of SAH treatment is to prevent it. IAs can be excluded from the circulation by microneurosurgical or endovascular approaches (Mayberg et al., 1994; Molyneux et al., 2002). Early microneurosurgical treatment of ruptured IA has been shown to both decrease mortality and to improve quality of life in the survivors (Öhman et al., 1989; Fogelholm et al., 1993). Treatment in specialised centres significantly improves the outcome

(Meretoja et al., 2011), however, the strongest predictor of outcome is the patient's neurological grade on admission (Kassell et al., 1990; Saveland et al., 1992; Hernesniemi et al., 1993; Osawa et al., 2001; Bracard et al., 2002; Laidlaw et al., 2003; Weir et al., 2003; Mocco et al., 2006), followed by the patient's age (Rosengart et al., 2007). In Helsinki, with a long and vast experience in vascular neurosurgery, microneurosurgical clipping is favoured over endovascular coiling. Furthermore, clipping shows better long-term outcomes (Molyneux et al., 2005; Pyysalo et al., 2010) and factors concerning aneurysm location favour an open vascular approach in the Finnish population (Dashti et al., 2007). Additionally, the surgical treatment of complex IAs might necessitate a bypass procedure prior to clipping of the IA sac (Hopkins et al., 1979; Spetzler et al., 1980; Yeh et al., 1997; Sanai et al., 2009).

#### *2.2.6.1 Cerebral Vasospasm after SAH*

Cerebral vasospasm refers to the delayed arterial narrowing commonly occurring between the fourth and ninth day after SAH (Weir et al., 1978). Angiographic vasospasm is common (50-75%) among SAH patients (Kassell et al., 1985; Inagawa, 1990b), however, it may be clinically silent. Symptomatic vasospasm, on the other hand, is associated with delayed ischaemic neurological deficit (DIND) (Haley et al., 1992; Longstreth et al., 1993), a likely multifactorial, possibly fatal complication. Although most treatment options have only limited value, reversal of the arterial narrowing, by means such as angioplasty, and hence correcting hypoperfusion can prevent ischaemia and reverse neurological deficit (Pluta et al., 2009). The pathophysiology of cerebral vasospasm and DIND are poorly understood, though, a mounting body of evidence support the role of inflammation in the chain of events (Dumont et al., 2003; Suarez et al., 2006; Pradilla et al., 2010). The strongest predictor of vasospasm is the amount of blood on the initial CT scan (Fisher et al., 1980; Hijdra et al., 1988; Claassen et al., 2001).

### 2.2.7 Prevention of IA and SAH

Currently no specific prevention is known for IA formation, and the best preventive actions are limited to cessation of smoking (Huttunen et al., 2012) and treatment of high blood pressure. In the case of familial IA, i.e. if two or more first degree rela-

tives are diagnosed with IA (Ronkainen et al., 1997), adult family members are offered screening, what is shown to be cost effective (Takao et al., 2008). MRA is the preferred imaging modality in screening to avoid excess radiation, however, smaller IAs may be misdiagnosed, possibly necessitating a CTA for definite diagnoses (Numminen et al., 2011). Prevention of the complications of SAH may include in the future preventive reperfusion treatment for those at high risk for vasospasm.

## 2.3 Cerebral Hypoperfusion

CH refers to the state when the cerebral blood flow is insufficient to meet the needs of the brain parenchyma.

In its nature, CH may be:

- severe or mild,
- global or localised,
- permanent or transient,
- acute or chronic.

### 2.3.1 Acute Cerebral Hypoperfusion

In the case of acute cerebral hypoperfusion (ACH), since the insult happens suddenly, there is no time for the brain vasculature to adjust and compensate for the loss of blood supply. Hence, abrupt occlusion or severe narrowing of a vessel commonly leads to ischaemia. Acute, severe decrease of blood flow to the entire brain, if sustained over a few minutes, leads to global hypoxic-ischaemic encephalopathy, resulting in death. In contrast, acute, localised decrease of blood flow, if persistent, leads to stroke. Vasospasm after SAH often leads to acute localised decrease of blood flow and DIND, furthermore, rarely to globally decreased blood flow. If the occlusion resolves and the decreased blood flow occurs only briefly, it leads, by definition, to transient ischaemic attack.

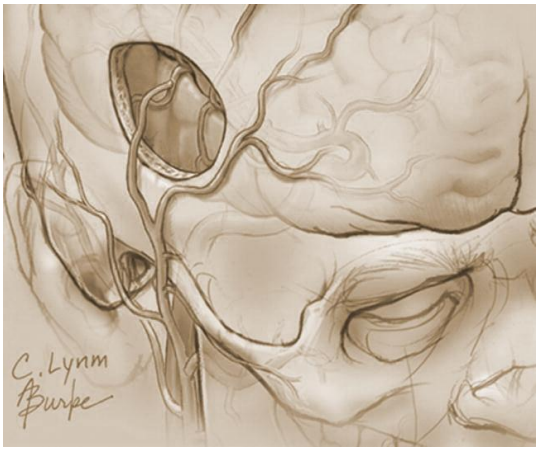
#### *2.3.1.1 Treatment of ACH*

ACH treatment should be prompt and should focus on reopening the occluded vessel. The first-line reperfusion therapy in industrialised countries is attempting

thrombolysis by recombinant tissue plasminogen activator (Wardlaw et al., 2003), followed by endovascular means such as intra-arterial prourokinase treatment, mechanical clot retrieval and angioplasty (Adams et al., 2005; Goldstein, 2007). In stroke, "time is brain" - in a typical large vessel stroke 120 million neurons are lost each hour when untreated (!) (Saver, 2006).

### 2.3.2 Chronic Cerebral Hypoperfusion

In chronic cerebral hypoperfusion (CCH), the narrowing or occlusion of the vessel occurs gradually, over a long period of time. Hence, corrective mechanisms, such as formation of compensatory collaterals may occur, possibly totally offsetting the perfusion deficit, and the event of vessel occlusion may remain silent (Schaper et al., 2003; Schaper, 2009; Shuaib et al., 2011). Unfortunately, this does not always happen, and CCH may eventually lead to neuronal injury in the area affected (Bennett et al., 1998). CCH is commonly seen in the elderly when atherosclerotic plaques compromise the cerebral blood flow. Over a long period of time, CCH leads to cognitive decline and likely contributes to VD (Ruitenberg et al., 2005) and possibly to sporadic Alzheimer disease (AD) (de la Torre, 2000, 2002). These hypotheses are confirmed by studies in model animals (de la Torre et al., 1993; Pappas et al., 1996; Pappas et al., 1997; Yamada et al., 2011). However, CCH sometimes affects children and young adults, typically in the form of the rare moyamoya disease (MMD) or syndrome (Scott et al., 2009). In MMD progressive intimal hypertrophy leads to the gradual occlusion of the ICA and its proximal branches (Scott et al., 2009). CCH, by exhausting the brain's compensating capacity, often precedes and brings on ACH.




**Picture 3.** Schematic drawing of an STA-MCA bypass (modified from (Powers et al., 2011)).

**The types of cerebral bypass surgeries**

- **Direct extracranial-intracranial (EC-IC) bypass.** The most commonly utilised form of it is the superficial temporal artery-middle cerebral artery (STA-MCA) bypass (Picture 3). The technique was first described by Yašargil and co-authors in 1969 (Crowell et al., 1969).

- **Indirect EC-IC bypass** is when highly vascularised tissue is brought into straight contact with the hypoperfused brain, however, without performing vessel anastomosis. Different methods include EDAS (Matsushima et al., 1990), EDAMS (Ishikawa et al., 1997) and multiple bur hole indirect EC-IC bypass (Endo et al., 1989).

- **Direct intracranial-intracranial (IC-IC) bypass** is most commonly used when reconstructing an ill vessel segment during surgery of complex IA (Sanai et al., 2009).

- **Excimer laser-assisted nonocclusive anastomosis (ELANA)** (Streefkerk et al., 2003) is currently the only technique when temporary occlusion of parent vessels is not necessary. It is performed in only a few subspecialised centres, since it is technically highly challenging (Vajkoczy et al., 2012).

**Text box**

*2.3.2.1 Treatment of CCH*

Non-invasive treatment focuses on secondary prevention by correcting life-style related risk factors and by decreasing general cardiovascular risk with pharmaceuticals, such as ASA and statins. When CCH is due to the atherosclerotic narrowing of the extracranial segment of the carotid arteries, the plaques may be removed by carotid endarterectomy (Barnett et al., 1998), or alternatively, the ill vessel segment may be dilated by stenting (Brott et al., 2010).

If the ill vessel segment resides intracranially, surgical options are predominantly confined to bypass surgery (Text box). In athero-occlusive disease, though tempting as it sounds, EC-IC bypass repeatedly failed to prevent stroke more efficiently than non-invasive treatments (The-EC/IC-Bypass-Study-Group, 1985; Powers et al., 2011; Rodriguez-Hernandez et al., 2011; Komotar et al., 2012).

In the pediatric MMD, indirect EC-IC bypass is sufficient to restore perfusion (Ishikawa et al., 1997; Scott et al., 2009). In the case of the adult MMD, indirect bypass procedures are inefficient (Houkin et al., 1996). Direct EC-IC bypass procedures may relieve symptoms, though they carry significant risks, such as the hyperperfusion syndrome (Uno et al., 1998; Ogasawara et al., 2005; Fujimura et al., 2007; Jin et al., 2011).

### 2.3.3 Risk Factors of Cerebral Hypoperfusion

Modifiable risk factors of ACH and CCH are the common modifiable cardiovascular risk factors such as hypertension, smoking, hyperlipidaemia, diabetes mellitus and excessive alcohol consumption (Wolf et al., 1991b; Wannamethee et al., 1995; Hankey, 1999; Meyer et al., 2000; Vermeer et al., 2002; Reynolds et al., 2003; Patra et al., 2010). Furthermore, atrial fibrillation is an important independent risk factor for ACH (Wolf et al., 1987, 1991a). Non-modifiable risks are male gender and age (D'Agostino et al., 1994; Meyer et al., 2000). Furthermore, when ACH is seen with delayed cerebral vasospasm after SAH, the strongest risk factor is thick layers of subarachnoid blood on the admission CT scan (Fisher et al., 1980; Hijdra et al., 1988; Claassen et al., 2001).

# 3 Genetics of Intracranial Aneurysm

## 3.1 The Human Genome

Genome refers to the entire hereditary material of an organism.

### 3.1.1 The Structure of the Human Genome

In most organisms, the genetic code is written by the four nitrogenous bases of the deoxyribonucleic acid (DNA); adenine, cytosine, guanine and thymine. Two DNA molecules join to form a double helix (Watson et al., 1953) which is wound around histone beads and superstructured into chromosomes (Figure 3). Most human cells, with the exception of germ cells, are diploid, carrying two copies of the 22 autosomal chromosomes and a pair of sex chromosomes in their nuclei. The function of DNA is to carry the information needed to build and maintain an organism.

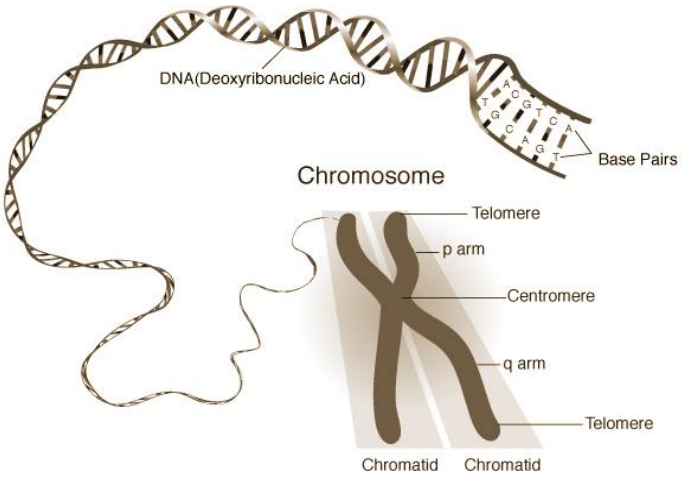



**Figure 3.** Two DNA molecules are held together by hydrogen bonds forming the DNA duplex. The DNA double helix is superstructured into chromosomes (modified from: www.genome.gov).

According to the central dogma of molecular biology; DNA is transcribed into ribonucleic acid (RNA), which is translated into proteins. Hence providing us with over 20 000 different proteins, such as structural proteins, enzymes or cell signalling molecules. Our genome consists of over 3 billion base pairs, however, only 1.5% of it encodes proteins (Lander et al., 2001). The protein coding part of the genome is called the exome. The function of the rest of the genome is yet largely unknown, although it has been suggested lately to play a regulatory role.

### 3.1.2 Common and Rare Variations in the Genome

During DNA replication, every billionth base is copied with a mistake (Nachman et al., 2000; Loeb et al., 2003; Kunkel, 2004). If left uncorrected, mistakes lead to genetic variations, referred to as mutations. If the variation contributes to a fitter organism it is a progression in the evolution and the mutation will thrive, becoming more common with every generation. However, if the "mutant" organism's survival or reproduction is weaker, the mutation's frequency will be low or it will be purged at a population level. When the variation is functionally neutral, it may be carried on and randomly distributed among the population. If over 5% of the population carries a variation, it is called common. If it is seen with a frequency of 0.5-5%, it is referred to as a low frequency variant. If seen in less than 0.5% of the population, it is called rare (The-1000-Genomes-Project-Consortium, 2010). When a variant is extremely rare, seen maybe in only one family, it is referred to as a private variation. Variants with an allele frequency of 1% or higher are commonly referred to as polymorphisms (The-1000-Genomes-Project-Consortium, 2010). The most common types of polymorphisms in our genomes are single nucleotide polymorphisms (SNPs) and small structural variations (Table 1). Common variations are not distributed equally throughout the genome, but show a reduced frequency in the vicinity of genes (Cai et al., 2009; The-1000-Genomes-Project-Consortium, 2010).

#### *3.1.2.1 Single Nucleotide Polymorphisms (SNPs)*

The nucleotide sequence of the somatic genome differs on average with 0.1% between humans (Reich et al., 2002). One in every 100 to 300 base pairs may be variable (The-1000-Genomes-Project-Consortium, 2010). Single nucleotide variations are referred to as single nucleotide polymorphism (SNP, pronounced "snip") if over

1% of the population carries them. The function of SNPs is not fully understood as yet, although they appear to regulate gene expression (Stranger et al., 2007). SNPs, by giving close to accurate genome-wide information without the need for sequencing the whole genome, facilitated the development of the human genome's haplotype map (The-International-HapMap-Consortium, 2003, 2005; Frazer et al., 2007; Altshuler et al., 2010). Furthermore, GWAS that seek common genetic susceptibility to common diseases, utilise SNPs as genetic markers.

**Table 1. Overview of most common genetic variations.**

| | | STRUCTURAL VARIATIONS | | |
|---|---|---|---|---|
| | **SNP** | **Indels** | **CNV** | **Chr. Abn.** |
| *Size* | 1 bp | 1 bp-1 kb | 1 kb- 3 Mb | >3 Mb |
| *Number/genome** | millions | 100 000-500 000 | 5-50 | 0-few |
| *Phenotypes/Diseases examples* | numerous traits /diseases | less well described | neurodevelopmental disorders, cancer | syndromic disorders |

* Numbers are averaged estimates based on multiple publications. Chr. Abn.= chromosome abnormalities.

*3.1.2.2 Structural Variations*

Variations often encompass more than a single polymorphic base pair (Table 1). These are referred to as structural variations (Iafrate et al., 2004; Conrad et al., 2006; Feuk et al., 2006; Redon et al., 2006). Classically structural variations are defined to be between 1kb to 3Mb in length and are called copy-number variations (CNVs). However, some are smaller than 1 kb, these are typically small insertions/deletions, i.e. indels. Most structural variations and all indels are submicroscopic. The variations greater in length than 3 Mb are the classical microscopic chromosomal abnormalities. Structural variations may cover 13% of the human genome (Stankiewicz et al., 2010) and account for most interindividual differences of the genome (Conrad et al., 2010), that are on average 1.5% (Pang et al., 2010).

By their nature, structural variations may be balanced or unbalanced. Balanced variations, where no DNA segment is lost or added, include inversions and some

translocations (Figure 4). On the other hand, the most common form of unbalanced structural variation is CNVs, where a DNA segment is either lost (deletions), or multiplied (duplications, triplications etc.) (Figure 4). Smaller variations, such as short indels and small CNVs, are significantly more common than larger structural variants (Table 1) (The-1000-Genomes-Project-Consortium, 2010). CNVs tend to affect the expression levels of nearby genes, and they are suspected to shape the tissue transcriptome (Stranger et al., 2007; Henrichsen et al., 2009). CNVs most often form as a replication mistake when highly similar but non-related regions are joined (Hurles, 2005; Conrad et al., 2010). CNVs can be *de novo*, however, an overwhelming majority are inherited, hence, CNVs are plausible candidates for explaining heritability (McCarroll, 2008; Craddock et al., 2010). Nevertheless, common CNVs were found to have only a small effect on complex disease risk (Conrad et al., 2010; Anney et al., 2012).

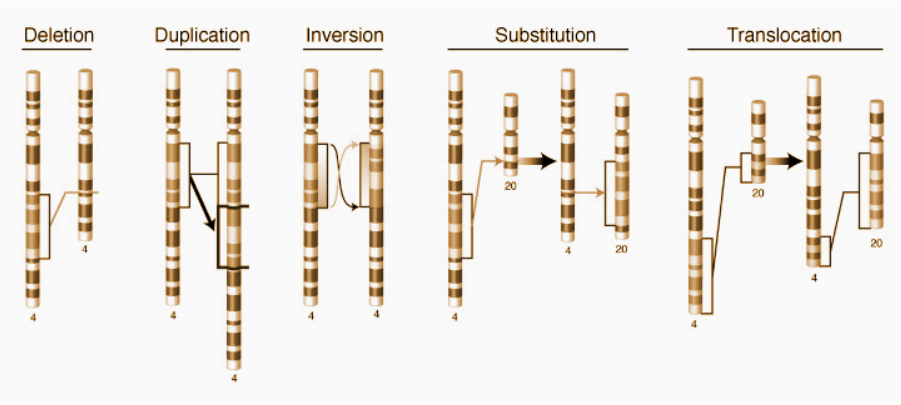



**Figure 4.** Examples of structural variations in the human genome (modified from: www.genome.gov).

### 3.1.3 Heritability

Nature (genes) and nurture (environmental and life-style related factors) together determine most traits. Commonly, a trait is referred to as a "genetic" one, when there is a significant genetic contribution to its risk. Nevertheless, one should bear in mind that most of our traits and diseases are shaped by genetic contribution, to different extents.

The heritability of a trait or a disease is the proportion of the total variation that can be attributed to genetic factors (Visscher et al., 2008). Given its definition as the proportion of the variation, the value of heritability always lies between 0 and 1 (Wray et al., 2008). For instance, the heritability of height in humans is estimated to be 0.8 (Macgregor et al., 2006), however, for most traits associated with fitness, heritability is typically 0.1-0.2 (Figure 5) (Visscher et al., 2008). If one could eliminate all contributing environmental and life-style related factors, heritability would be 1, meaning that the trait would only be expressed by those that have a strong genetic susceptibility to it. Since environmental and life-style habits may change over time and are different between populations, heritability tends to be time- and population specific (Visscher et al., 2008).

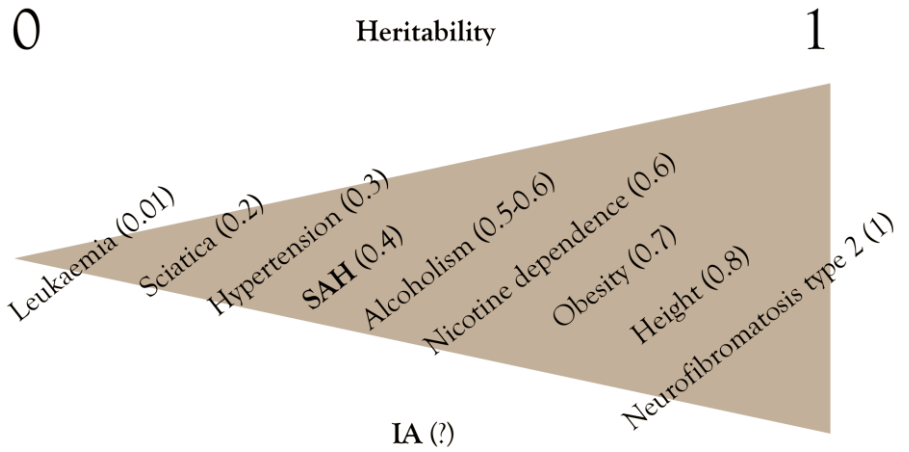



**Figure 5.** Comparing the heritability of traits and diseases (Heikkila et al., 1989; Czene et al., 2002; Agarwal et al., 2005; Walley et al., 2006; Lessov-Schlaggar et al., 2008; Silventoinen et al., 2008; Asthagiri et al., 2009; Stacey et al., 2009; Korja et al., 2010). As of today, the heritability of IA is unknown.

Heritability of a trait can be estimated by the help of family- and twin studies, and by measuring inter-ethnical differences. If genetic factors confer risk for the disease, closest relatives should display increased risk, which decreases toward the population prevalence for more distant relatives. Familial aggregation of a disease makes genetic contribution likely, but since most family members share environment as well, the two cannot be easily separated. Traditionally, heritability was estimated from study designs such as measuring the difference in phenotypic correlation of

monozygotic and dizygotic twin pairs (Wray et al., 2008). However, new methods have emerged estimating heritability with the help of genetic markers, by defining the proportion of the genome shared identical-by-descent (Visscher et al., 2006). When aiming to define the heritability of a trait or disease, factors such as the accessibility of it (eg. height is easily accessible in contrast with IAs that are "hidden" by the skull) or whether affected-unaffected status may change over time, can be crucial. As of today, we do not know the heritability of IA since the phenotype is difficult to access and affected-unaffected status may be time dependent. Currently there are no large twin- or migrant cohorts characterised for IA. The heritability of IA rupture leading to SAH is rather low (Korja et al., 2010).

## 3.2 Mendelian Genetics

Gregor Johann Mendel, the founder of genetics, was an Austrian scientist and an Augustinian monk in the XIX$^{th}$ century (Picture 4).

**Picture 4.** Gregor Johann Mendel (modified from: www.wikipedia.org).

### 3.2.1 Mendelian Diseases

Mendel studied the inheritance of certain traits in pea plants and based on those results, formulated the "Law of Segregation" (aka. First Law of Mendel), and the Law of Independent Assortment (aka. the Second Law of Mendel). The traits he studied were monogenic with perfect penetrance, meaning that a particular geno-

type at one locus is both necessary and sufficient to create the trait. Such traits are referred to as Mendelian, since they obey the laws of Mendel, and are examples of the rare genetic determination.

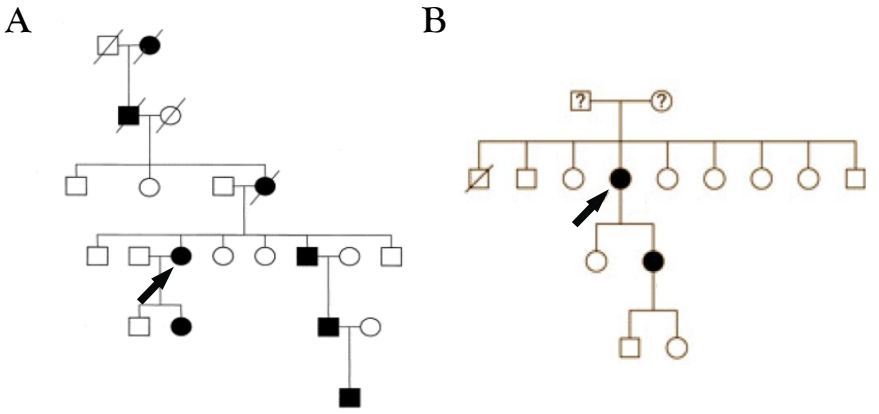



**Figure 6.** An example of an autosomal dominant Mendelian disease pedigree (A) (modified from (Fournier et al., 2001)) and a typical IA pedigree (B) (modified from: (Ruigrok et al., 2004b)). Arrows mark "index" patient (who brought the attention to the family), deceased individuals are marked with a diagonal line, question marks refer to unknown disease status.

The online catalogue of Mendelian diseases in humans (OMIM) currently lists approximately 4000 diseases. Mendelian diseases can be recognised from their characteristic pedigree patterns (Figure 6A). Extended pedigrees showing Mendelian inheritance in IA are extremely rare (Verlaan et al., 2006; Kim et al., 2011). Most IA pedigrees include just a few affected individuals in one, or possibly two generations (Figure 6B), where lack of diagnostic tools in the earlier times, or very young age in the newest generation, limit identification of affected individuals.

### 3.2.2 Linkage and Linkage Disequilibrium

Meiotic recombination during chromosomal crossing over is the exchange of genetic material between homologous chromosomes (Creighton et al., 1931). Since the number of recombination events in a generation is rather small (Visscher et al., 2012), offsprings inherit long stretches of DNA from the parental chromosomes.

Despite the fact that DNA segments tend to break up over many generations, even apparently unrelated individuals share recognisable segments of their genome. The regions of the chromosome that have not been broken up by recombination events are called haplotypes.

Linkage describes the genetic relationship between two loci (Morton, 1956). It refers to two loci being inherited together more commonly than expected by chance, i.e. recombination rarely separates them. Physical proximity is the most common cause of linkage, however, far apart loci might be linked as well. If two loci are in linkage, we observe linkage disequilibrium (LD) between them, i.e. they are co-inherited in a non-random way. LD occurs as haplotype blocks that are regions of the genome with little recombination events. The extent of LD depends on population history, hence varying from population to population (Reich et al., 2001a; The-International-HapMap-Consortium, 2005). The range of LD is shorter in older populations, typically among the Africans, and more extensive among younger populations characterised by multiple bottlenecks and rapid expansion, such as the Finns (Varilo et al., 2003; Service et al., 2006).

Genetic linkage studies in families rely on the co-inheritance, i.e. LD between the putative causative variant and genotyped markers. Due to the limited number of recombination events, haplotype blocks are large and often it is sufficient to genotype a relatively small number of markers per chromosome. The disadvantage of the small number of recombination events is that the resolution of these studies is often low. Nevertheless, linkage studies have been highly successful in mapping variants affecting Mendelian, i.e. single-gene disorders (Botstein et al., 2003). A supreme example of the success of linkage studies is the identification of the genes underlying rare, Mendelian disorders that are enriched in the Finnish population isolate.

#### *3.2.2.1 The Finnish Population Isolate*

Nearby populations have often mixed throughout history, decreasing their genetic differences. There are a few special cases in which populations remained isolated due to cultural, historical or geographical reasons. The Finns, French Canadians and the Ashkenazi Jews are examples of population isolates, which have proven to be invaluable in dissecting genetic background of rare diseases.

Currently, it is believed that Finland was first settled some 4000 years ago by eastern Uralic speakers, followed by approximately 2000 years ago by Indo-European speakers (Nevanlinna, 1972; de la Chapelle, 1993; Peltonen et al., 1995). For long centuries only a narrow strip was inhabited on the coastal south and southwest by a small number of settlers (Sajantila et al., 1996). The population was likely only around 50 000 in the XII$^{th}$ century carrying only a limited genetic pool, reaching 250 000 in the XVI$^{th}$ century (Peltonen et al., 1999). Internal migration from the coastal regions towards the north and inner lands started in the XVI$^{th}$ century, both to gain land for cultivation and to escape taxation from the Swedish Crown (Peltonen et al., 1999) (Figure 7).

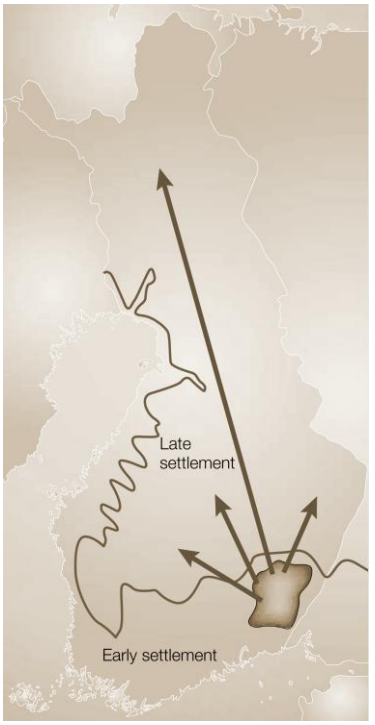



**Figure 7.** Inner migration from the early coastal settlements towards the inland in the XVI$^{th}$ century (modified from: (Peltonen et al., 2000)).

Both paternally inherited Y chromosome haplotypes and maternally inherited mitochondrial DNA show an exceptionally low genetic diversity in the Finns (Sajantila et al., 1996). Genealogical records kept by the Lutheran church since the XVII$^{th}$ century facilitated genetic research (Norio, 2003b). Researching genetic diseases in a population isolate does not only benefit the isolate, since results may highlight the pathological pathway(s) involved worldwide (Lifton et al., 2001). This was well demonstrated by the discovery of the breast cancer gene mutations (*BRCA1* and *BRCA2*), predominantly seen in the Ashkenazi Jews (Rubinstein, 2004). Furthermore, complex disease may have a more simple genetic background in population isolates that went through multiple bottlenecks, due to the lower amount of variations available. Hence, population isolates are believed to facilitate the mapping of complex disease loci or pathways (Peltonen et al., 2000).

*3.2.2.2 Finnish Disease Heritage*

One of the best-known reflections of the bottleneck effect is the Finnish Disease Heritage (FDH), dominated by recessive Mendelian disorders due to rare alleles. If the limited number of founders of Finland happened to carry mutations for certain genetic diseases, the disease will be enriched in the population. In the genetically well-studied Finnish population, this unique group of diseases is called the FDH. Conversely, there are conditions like cystic fibrosis or phenylketonuria, that are commonly seen worldwide, but rarely in Finland (Peltonen et al., 1999). FDH consists of almost 40 hereditary diseases (Norio, 2003a; Finnish-Disease-Database, 2012). The first disease of the FDH, the congenital nephrosis of the Finnish type, was described already in the 1940s and characterised in the 1960s. Carrier frequencies and geographical distribution of FDH mutations vary, among other factors, depending on the time of their introduction to the Finnish population (Pastinen et al., 2001). Common ones, with assumed early introduction, such as the mutation responsible for aspartylglucosaminuria (AGU), show rather even distribution throughout the country with high carrier frequency (1:65) (Peltonen et al., 1999). Whereas the mutation causing the Finnish variant of late infantile neuronal ceroid lipofuscinosis (vLINCL) is assumed to be a young mutation, with its carriers clustering in southern Botnia. The carrier frequency of the vLINCL mutation for the whole country is 1:120 (Peltonen et al., 1999). One in three Finns is estimated to carry an FDH mutation (Pastinen et al., 2001). Most FDH diseases were characterised in the 1990s (Figure 8) by the late Academician of Science, Professor Leena Peltonen-Palotie, utilising the extended LD characteristics of the Finnish population.

*Finland, Finland, Finland*
*The country where I want to be...*
*You're so near to Russia*
*So far from Japan...*
*You're so sadly neglected*
*And often ignored...*
*Finland, Finland, Finland*
*Finland has it all.*
*-Monty Python, "Finland"*

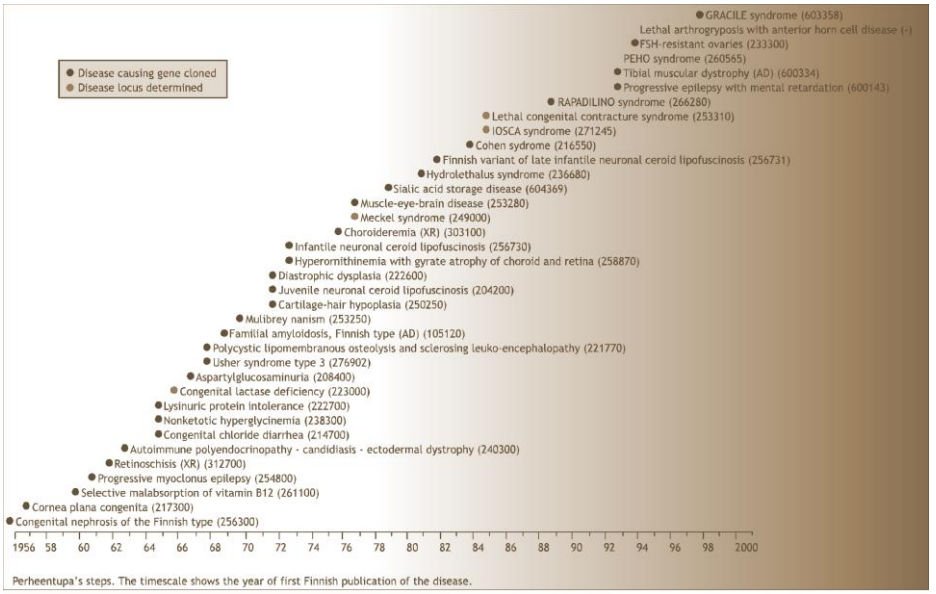



**Figure 8.** Perheentupa's steps, showing the year of the first Finnish publication of the FDH diseases (modified from: Finnish Disease Database; www.findis.org).

## 3.3 Genetics of Complex Diseases

### 3.3.1 Complex Diseases

Monogenic traits proved too fragile from an evolutionary point of view, and as an insurance, information about most traits is divided into hundreds of loci with small individual effect. Hence, most human traits and diseases are not Mendelian in nature, and their manifestation is likely affected by a large number of genes and modified by multiple environmental factors (Table 2). In complex traits, individual genotypic variants are neither necessary nor sufficient to create the trait (Visscher et al., 2012). No matter how many genetic or environmental factors contribute to it, the trait or disease may be dichotomous in its nature, such as IA (one either has or does not have an IA), or continuous, such as blood pressure (it may take any value on a continuous scale). Genes or genetic loci underlying dichotomous characters are called susceptibility loci. In the case of continuous characters, they are referred to as quantitative trait loci. The scientific community has put great effort into identifying these loci with significant success (Hindorff et al., 2009). These loci, obvious by the

nature of their discovery, do not usually contribute to disease risk significantly at an individual level, but their significance lies in increasing disease risk at a population level and by enlightening disease related pathways (Huang et al., 2001; Hirschhorn et al., 2002; Hirschhorn, 2009).

**Table 2. Comparison of Mendelian and complex diseases.**

| | Diseases | |
|---|---|---|
| | **Mendelian** | **Complex** |
| *Prevalence* | low | low-high |
| *Penetrance* | high | medium-low |
| *Familial aggregation* | enigmatic | less evident |
| *Twin concordance* | high | medium-low |
| *Number of genes involved* | one-few | multiple |
| *Effect of environmental factors* | small | large |
| *Clinical manifestation* | uniform | varying |

### 3.3.2 Susceptibility

Influenced by both genetic and environmental factors, susceptibility refers to an individual's increased likelihood to develop a particular disease.

### 3.3.3 Association and GWAS

Linkage studies and candidate gene studies failed to yield results in complex diseases, due to the likely small effect of individual genes/variants (Hirschhorn et al., 2002). However, it was still possible that the magnitude of the variants' cumulative effect would be significant. In the mid 1990s Risch and Merikangas proposed that association scan of a million variants over the genome, in a cohort of unrelated individuals, could be a more powerful tool than linkage analysis (Risch et al., 1996). Association is not a specifically genetic phenomenon, it is a statistical statement, referring in genetics to the co-occurrence of alleles and phenotypes. It took approximately 10 years for the idea of Risch and Merikangas to materialise. GWASs

utilise LD between SNPs at the population level (Visscher et al., 2012). The differences of linkage and association analyses are summarised in Table 3.

The discovery of SNPs was accelerated by the sequencing of the human genome (Lander et al., 2001; Venter et al., 2001), and their LD was quantified by the HapMap project (The-International-HapMap-Consortium, 2005). At the same time, technological advances and biobanks facilitated the ability to conduct GWASs. GWAS is a hypothesis generating approach, since it is unbiased with respect to the genetic location of the putative causal variant, unlike, for example, candidate gene studies. Nevertheless, GWAS is not free of *a priori* assumptions. By the design of the study, GWAS is only powered to detect relatively common causal variants (Visscher et al., 2012).

**Table 3. Comparing linkage and association analyses** (modified from (Molyneux et al., 2002).

| *Property of mapping approach* | **Linkage analysis** | **Association analysis** |
|---|---|---|
| *Data type studied* | Relatives | Unrelated or related individuals |
| *Biological basis of approach* | Observe (or infer) recombination in pedgree data | Exploit unobserved recombination events in past generations |
| *Relevant parameter* | Recombination fraction | Association statistics |
| *Range of effect detected* | Long (>5Mb) | Short (<100kb) |
| *Number of markers required for genome-wide coverage* | Moderate (500-1000) | Large (>100 000) |
| *Statistics used* | Requires tailor-made likelihood methods | Can use the range of classical statistical tools |
| *Genotyping errors* | Potentially detected | Often undetected |
| *Most suitable application* | Rare, dominant traits | Common traits |

*3.3.3.1 The Dawn of the "GWAS era"*

An early attempt to test the GWAS approach was made in 2002 (Ozaki et al., 2002). A few years later, the group led by Josephine Hoh at Yale University conducted the first highly successful GWAS (Klein et al., 2005). They identified the complement factor H (*CFH*) polymorphism behind age-related macular degeneration (AMD). Their sample size was minute compared to current studies (96 cases and 50 controls) and they "only" tested a little over 110 000 SNPs genome-wide. However, AMD happened to be a disease with a strong single locus genetic contribution to its risk. Their success fired up the scientific community and hence the "GWAS era" began. Manhattan plots, named after resembling the Manhattan skyline, became an enigma of the era (Figure 9). The first landmark study of the era was conducted by the Wellcome Trust Case Control Consortium, studying seven common diseases in 14 000 individuals, with shared controls (Wellcome-Trust-Case-Control-Consortium, 2007).

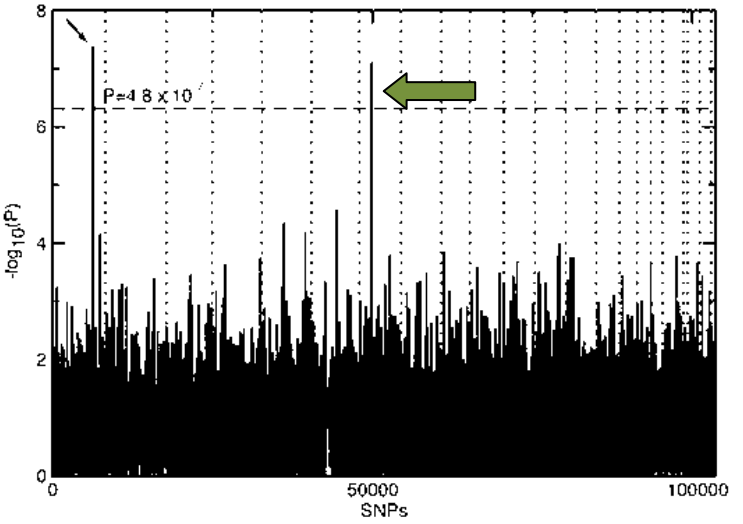



**Figure 9.** Manhattan plot of GWAS on AMD. Horizontal dashed line shows the cutoff for P = 0.05 after Bonferroni correction. The green arrow points out the sole significant association peak with the *CFH* gene (modified from (Klein et al., 2005)).

#### *3.3.3.2 The Fruits of the "GWAS era" in a Nutshell*

As of today, over a thousand GWASs have been conducted (Hindorff et al., 2012) and well over 2000 loci have been found to show significant and robust association with one or more complex traits (Visscher et al., 2012). Typically, the significantly associated SNPs together explain only a small proportion (<10%) of the genetic variation for most complex traits or diseases. For certain quantitative traits, however, GWAS identified loci account for up to 10-20% of their genetic component (Visscher et al., 2012).

Furthermore, GWASs have taught us important lessons about the likely nature of complex traits and diseases. These lessons are, among others, the following: (1) in complex traits the number of contributing loci is high, significantly higher than estimated earlier. (2) There are risk loci where multiple alleles associate with disease risk. (3) The same variant may associate with multiple traits in a pleiotropic way (Manolio, 2010; Sivakumaran et al., 2011). (4) Some risk variants confer risk in multiple ethnicities (Visscher et al., 2012).

GWASs are significant investments, Visscher and colleagues estimate the price of a discovered locus to be $125 000 (Visscher et al., 2012). Nonetheless, this investment will likely pay itself back in the form of more cost-efficient public health decisions, due to improved understanding of basic biological disease mechanisms.

#### *3.3.3.3 The Multiple Testing Problem*

The null hypothesis of every GWAS is that there is no association between the trait or disease of interest and any of the SNPs tested. Nowadays, up to 1 million SNPs are commonly tested for association genome-wide, tagging most common variations in non-African populations (The-International-HapMap-Consortium, 2005). This high number of tests ($10^6$), although not all independent, means that the likelihood of type I (false positive) error is significant. Hence, results have to be corrected for multiple testing. The most conservative method is the Bonferroni correction (Bonferroni, 1935). The Bonferroni correction sets the significance threshold of a 1 million SNP GWAS to $5x10^{-8}$, calculated as the commonly accepted significance threshold (0.05) divided by the number of tests performed ($10^6$). However, there are alternative methods that take into consideration that the tests in the GWAS are not

all independent, hence aiming to lower the rate of the type II (false negative) errors. Such methods include the computationally demanding permutation tests (Churchill et al., 1994), or measuring statistical significance based on false discovery rate (Storey et al., 2003) or with the help of the Bayes factor (Wakefield, 2007). Nevertheless, the stringent cut-off of $5x10^{-8}$ is still often applied to GWAS. Additional approaches to overcome the multiple testing problem include the replication of results in independent cohorts and functional studies (McCarthy et al., 2008).

#### *3.3.3.4 The Missing Heritability Problem*

GWASs have greatly improved our understanding of the genetic bases of disease risk, even though they tend to identify only a fraction of the specific causal loci, hence leaving a significant part of the heritability unexplained (Maher, 2008; Manolio et al., 2009; Gibson, 2011). This unexplained portion is often referred to as the "missing or hidden heritability". At the end of the 1990s and the beginning of the 2000s, many hypothesised that common diseases are likely to be caused by common variants (Lander, 1996; Reich et al., 2001b; Pritchard et al., 2002; Botstein et al., 2003). It became evident that a few dozen loci of moderate effect and intermediate frequency will not explain the genetic risk to most common diseases. Currently there are three main hypotheses explaining where the hidden heritability may lie: (1) there might be a large number of small-effect variants of different frequencies (Visscher et al., 2008; Lango Allen et al., 2010), (2) a large number of large-effect rare variants (Cirulli et al., 2010), supported by the fact that low-frequency and rare variants vastly outnumber common variants (The-1000-Genomes-Project-Consortium, 2010), or (3) the hidden heritability may lie in the combination of genotypic, environmental and epigenetic interactions (Feldman et al., 1975; Eichler et al., 2010). Possibly, the mixture of all three. Large-scale whole-exome and whole-genome sequencing studies may aid us in decreasing missing heritability.

## 3.4 Studies on the Genetics of IA Before the "GWAS Era"

Familial aggregation of a disease is suggestive of a genetic contribution to the risk (Norrgard et al., 1987). Although only a minority of IAs show familial aggregation, 10% of patients with aneurysmal SAH have first or second degree relatives with SAH or unruptured intracranial aneurysms (Ronkainen et al., 1993; Bromberg et

al., 1995; Schievink et al., 1995; Wang et al., 1995; De Braekeleer et al., 1996; Ronkainen et al., 1999; Wermer et al., 2003). This, and the fact that familial predisposition is the strongest risk factor for developing IA (Rinkel et al., 1998; Ruigrok et al., 2001), provoked a Mendelian hypothesis in search of IA genes. The tools were limited by the era to linkage analysis and candidate gene studies.

### 3.4.1 Linkage Studies

Familial aggregation of IA was noticed already in 1954 (Chambers et al., 1954), however, as mentioned above, true familiar IA is rare and likely it is rather different from the sporadic disease, as seen with other phenotypes (Lifton et al., 1992; Shimkets et al., 1994). Indeed, familial IAs seem to behave clinically distinctly. Familial IA patients more commonly carry multiple IAs (Ruigrok et al., 2004a) and their IAs tend to rupture at a younger age (Leblanc, 1996; Ohashi et al., 2004). Nevertheless, the search for IA kindreds has been very active since the 1990s (Schievink et al., 1994). Linkage studies were hampered by over-simplistic hypotheses assuming Mendelian inheritance (Wills et al., 2003) and by inaccurate phenotyping, both due to the relative novelty of diagnostic tools and due to change in case-control status over time (Roos et al., 2004).

Linkage scans identified multiple loci with strong or suggestive linkage but only a fraction of them replicated in other cohorts (Table 4). This might reflect true locus heterogeneity, since mutations might be family/region-specific, however, a fraction of the loci are likely to have arisen from false positive results. Unfortunately, linkage studies failed, thus far, to identify underlying mutations. Table 4 summarises the results of most genome-wide and replication linkage scans performed in IA families.

**Table 4. Genetic linkage studies identifying suggestive IA loci.**

| *Chr band* | *Mb* | *LOD/P* | *Series* | *Populations** |
|---|---|---|---|---|
| **1p36.1** | 18.6-24.9 | 3.18 | Ruigrok et al., 2008 | Dutch |
| **1p36.1–34.3** | 19.6–35.1 | **4.2** | Nahed et al., 2005 | US |
| **1p33** | 46.6 | 0.037 (P) | Onda et al., 2001 | Japanese |

| | | | | |
|---|---|---|---|---|
| **1p22.1** | 93 | 0.022 (P) | Onda et al., 2001 | Japanese |
| **2q13** | 112.7 | 0.044 (P) | Onda et al., 2001 | Japanese |
| **3q29** | 198.5 | 0.014 (P) | Onda et al., 2001 | Japanese |
| **4p16.1–15.3** | 6.7-13.3 | 1.27 | Olson et al., 2002 | Finnish |
| **4q34.1** | 174.8 | 0.04 (P) | Onda et al., 2001 | Japanese |
| **5p15.2–14.3** | 9.4-21.1 | 3.6 | Verlaan et al., 2006 | French Canadian |
| **5q14.3-5q15** | 85.4-95.8 | **0.001 (P)** | Onda et al., 2001 | Japanese |
| **5q22–31** | 118.9–140.9 | 2.24 | Onda et al., 2001 | Japanese |
| | 111.5-159.9 | 2.91 | Santiago-Sim et al., 2008 | French Canadian |
| **6q14.1** | 77.6 | 1.2 | Olson et al., 2002 | Finnish |
| **7p22.2** | 4.2 | 1.67 | Olson et al., 2002 | Finnish |
| **7p14.1** | 38.96 | 0.032 (P) | Onda et al., 2001 | Japanese |
| **7q11** | 71.1–92.3 | 3.22 | Onda et al., 2001 | Japanese |
| | 97-104 | 2.34 | Farnham et al., 2004 | US |
| **7q21.1** | 77.5-88.1 | 0.021 (P) | Onda et al., 2001 | Japanese |
| **7q22.1** | 101.3 | 0.014 (P) | Onda et al., 2001 | Japanese |
| **7q34** | 139.4 | 0.9 | Olson et al., 2002 | Finnish |
| **8p22.2** | 11.28 | 3.61 | Kim et al., 2011 | South-Korean |
| **9p24.2** | 3.9 | 0.011 (P) | Onda et al., 2001 | Japanese |
| **9p23** | 9-14.1 | 2.93 | Santiago-Sim et al., 2008 | French Canadian |
| **11p15** | 5.9 | ≈2 | Mineharu et al., 2007 | Japanese |
| **11q13.2** | 67.6 | 0.042 (P) | Onda et al., 2001 | Japanese |
| **11q25** | 125–131 | **4.3** | Ozturk et al., 2006 | US |
| | 131.2 | 0.023 (P) | Onda et al., 2001 | Japanese |
| **12p12** | 14.8-26.3 | 3.1 | Santiago-Sim et al., 2008 | French Canadian |
| **13q14.2** | 47.8 | 0.034 (P) | Onda et al., 2001 | Japanese |
| | 45.1-57.4 | **4.56** | Santiago-Sim et al., 2008 | French Canadian |
| **14q22** | 63.6-87.6 | 3 | Ozturk et al., 2006 | US |
| | 69.6–77.6 | 2.31 | Onda et al., 2001 | Japanese |
| **14q34.2** | 99.7 | 1.36 | Olson et al., 2002 | Finnish |

| | | | | |
|---|---|---|---|---|
| **17p12-q11.2** | 14.2–27.1 | 3 | Yamada et al., 2004 | Japanese |
| **17q11.2** | 24.3 | 0.027 (P) | Onda et al., 2001 | Japanese |
| **18p11** | 0.6 | 0.049 (P) | Onda et al., 2001 | Japanese |
| | pter-16.1 | 3.15 | Santiago-Sim et al., 2008 | French Canadian |
| **19q13.3** | 38.7–55.6 | 2.58 | Olson et al., 2002 | Finnish |
| | 39.8–57.4 | 3.16 | van der Voet et al., 2004 | Finnish |
| | 46.7–54 | 2.15 | Yamada et al., 2004 | Japanese |
| | 46.7-48.3 | **4.1** | Mineharu et al., 2007 | Japanese |
| **Xp22.2** | 14.4 | 2.08 | Olson et al., 2002 | Finnish |
| | 14.4–21.8 | 2.16 | Yamada et al., 2004 | Japanese |
| | 4.-13.1 | **4.54** | Ruigrok et al., 2008 | Dutch |

Mb, physical position is specified with the help of markers identified in the original publications, with relaxed criteria of linkage. LOD/P-values are the maximum values reported for the region by the study referenced. P-values are marked with a (P) after them. Regions showing LOD>4 or P<0.01 in at least one study, are marked with dark green and the corresponding LOD/P is in bold and underlined.
*Populations refer to the nationality of the studied families, or when not specified, the nationality of the study.

### 3.4.2 Candidate Gene Studies

Typical candidate genes of IA have a known or proposed function in the vessel wall, such as the collagens or elastin. Alternatively, candidate genes may have an indirect effect on vessel wall, such as extracellular matrix proteins. Some candidate genes are positional candidates, meaning that linkage studies highlighted the region from where the biologically most plausible gene was further studied as a candidate gene. Further hypotheses that supported the candidate gene theory are genetic diseases, such as type IV Ehlers Danhlos syndrome (EDS-IV) appearing to show a higher-than-average prevalence of IA. Unfortunately, regardless of the great numbers of studies performed (Kuivaniemi et al., 1993; Brega et al., 1996; Schievink et al., 1996; Takenaka et al., 1999; van den Berg et al., 1999; Yoon et al., 1999; Peters et al., 2001; Zhang et al., 2001; Hofer et al., 2003; Krex et al., 2003; Yoneyama et al., 2003; Hofer et al., 2004; Kassam et al., 2004; Krex et al., 2004a; Krex et al., 2004b; Ruigrok et al., 2004c; Yoneyama et al., 2004), candidate gene studies have also failed to identify a *bona fida* causative variant, similar to most candidate gene studies

of other disorders (Hirschhorn et al., 2002; Tabor et al., 2002). The most studied IA candidate genes are summarised in Table 5.

**Table 5.** **Most studied IA candidate genes** (modified from (Ruigrok et al., 2005)).

| *Candidate gene* | *Chromosomal band* | *Linkage studies* | *Populations** |
|---|---|---|---|
| **Elastin** | 7q11.2 | Onda et al., 2001 | Japanese<br>Dutch<br>German |
| **Collagen type 3 A2** | 2q31 | N/A | US<br>Dutch<br>mixed |
| **Collagen type 1 A2** | 7q22.1 | Onda et al., 2001 | Japanese |
| **Lysyl oxidase** | 5q22.3–q31.2 | Onda et al., 2001 | Japanese<br>German |
| **Fibrillin 2** | 5q23–q31 | Onda et al., 2001 | Japanese |
| **α1 antitrypsin** | 14q32.1 | N/A | US |
| **MMP-9** | 20q11.2–q13.1 | N/A | US<br>UK<br>Finnish<br>German |
| **MMP-1, MMP-3, MMP-12** | 11q22–q23, 11q23, 11q22.2–22.3 | N/A | UK<br>Finnish |
| **TIMP-1, TIMP-2, TIMP-3** | Xp11.3–p11.23, 17q25, 22q12.1–q13.2 | N/A | German |

If the candidate gene is a positional candidate the original study is identified in the Linkage study column.
*Populations refer to the nationality of the studied subjects in whom the candidate genes were tested, or when not specified, the nationality of the study. MMP: matrix metalloproteinase, TIMP: tissue inhibitor of MMP.

### 3.4.3 Associations with Other Disorders

Since the mid 1980s, familial IA was already noticed to sometimes associate with other disorders, such as Marfan's syndrome (ter Berg et al., 1986; van den Berg et al., 1996; Pfohman et al., 2001). Hence, further syndromes affecting the extracellular matrix proteins like EDS-IV (de Paepe et al., 1988), fibromuscular dysplasia (Cloft et al., 1998) and glucocorticoid remediable aldosteronism (Litchfield et al., 1998) were suspected to increase the risk of IA. Although EDS-IV may increase the risk of fusiform aneurysms (Pepin et al., 2000), currently, there is only evidence for autosomal dominant polycystic kidney disease (ADPKD) to associate with saccular IA (Chapman et al., 1992; Lozano et al., 1992; Schievink et al., 1992; Schievink, 1997; Belz et al., 2001; Torres et al., 2001).

Up to 10% of ADPKD patients harbour IA, however, it only accounts for less than 1% of all SAH (Ruigrok et al., 2001; Gieteling et al., 2003). The two genes mutated in ADPKD are *PKD-1* (Peral et al., 1997) accounting for around 85% of the cases, and *PKD-2* (Veldhuisen et al., 1997) accounting for the rest. IAs occur in patients with mutations in both genes and the precise mechanism is yet unknown, however, one can speculate that the effector is likely situated downstream in the common pathway. Intriguingly, *PKD-1* mutation carrier patients with IAs were significantly more likely to carry germ-line mutations in the 5′ half of the gene when compared to those without IAs (Rossetti et al., 2003; Gibbs et al., 2004; Ong, 2009).

## 3.5 9p21, the First Common Susceptibility Locus for IA

Just on the dawn of the first GWAS on IA (I), the Icelandic deCODE group successfully identified 9p21 as the first common IA susceptibility locus in a multinational cohort, including Finnish patients (Helgadottir et al., 2008). In 2008, 9p21 was a susceptibility locus for coronary artery disease and type 2 diabetes (McPherson et al., 2007). Little did we know how greatly important this locus would prove to be (Figure 10) (Helgadottir et al., 2007; Cunnington et al., 2010; Laaksovirta et al., 2010; Visel et al., 2010; Harismendy et al., 2011; Shea et al., 2011). Interestingly, Helgadottir and colleagues tested and found association at 9p21 with both saccular IA and abdominal aortic aneurysm (AAA). Although sometimes co-existing clinically (Nahed et al., 2005), IA and AAA are likely results from different underlying

disease processes (Humphrey et al., 2008), though with possible overlapping genetic background (Shibamura et al., 2004).

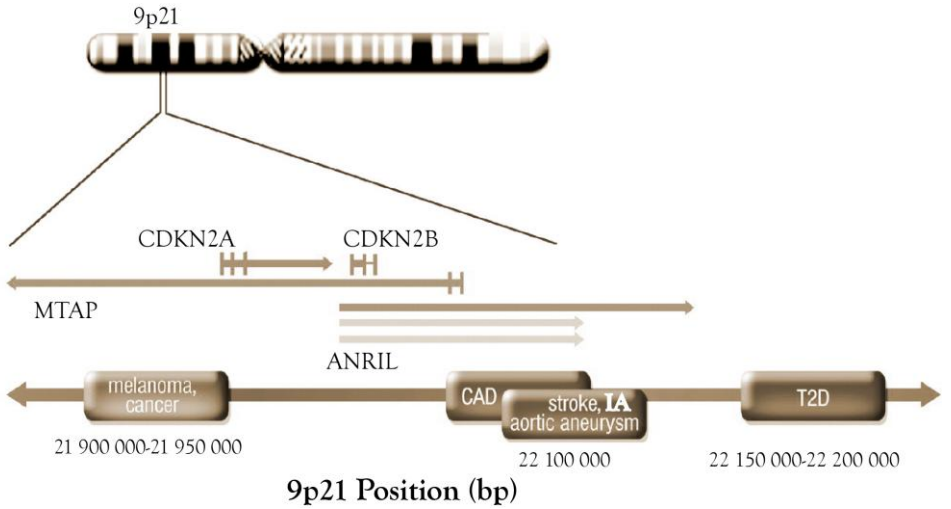



**Figure 10.** Association with multiple diseases at the 9p21 locus (modifeid from (Zeller et al., 2012)). CAD: coronary artery disease, T2D: type 2 diabetes. Genes are referred to by their HUGO symbols.

## 3.6 Genetics of SAH

Although SAH shows familial aggregation (Ronkainen et al., 1993; Ronkainen et al., 1999), the significance of the genetic component to SAH susceptibility appears to be low compared to acquired risk factors (Korja et al., 2010). Modifiable risk factors are estimated to account for six to seven, while genetic factors for only one of every ten SAHs (Ruigrok et al., 2001; van Gijn et al., 2007).

# 4 Aiming to Tame Angiogenesis for Therapy

Basic research tools, such as genetics, are invaluable in understanding essential disease risk. Furthermore, they provide a frame for corollary studies aiming to culminate in improved therapeutic approaches. Likewise, elucidation of physiological and pathophysiological processes advances therapy. The main focus of this thesis is cerebrovascular pathologies and this chapter focuses on vascular biology in the healthy and in the sick.

## 4.1 Functions of the Cardiovascular System

The circulatory system of blood vessels ensures the distribution of oxygen, nutrients, hormones and even cells in large multicellular organisms such as humans. Gases and nutrient exchange occurs at the level of capillaries, which are 10-20 μm in diameter. Blood vessel capillaries are found within 200 μm of any cell, with the exception of avascular tissues like cartilage, and the lens and cornea of the eye (Ambati et al., 2006).

The luminal surface of all blood vessels is lined by a monolayer of endothelial cells (ECs) (Figure 2- page 22) (Risau, 1998). The ECs have multiple functions; they regulate the blood flow via nitric oxide that acts on vascular smooth muscle cells (SMCs), they coordinate the traffic of cells and macromolecules between the blood and the interstitium, they alter their gene expression pattern to accommodate arterial or venous functions (Hirashima et al., 2006), and they react to growth factors that stimulate the formation of new blood vessels. Even though ECs may appear similar in their ultrastructure, they do acquire tissue-specific phenotype, hence promoting tissue-specific angiogenesis (Ruoslahti et al., 2000; Trepel et al., 2002). The basolateral side of ECs is lined with a basement membrane and a 50-100 nm thick extracellular matrix dominated by elastic fibers, forming the internal elastic lamina. They are further surrounded by mesenchymal mural cells, such as pericytes in capillaries and the smallest arterioles and vascular SMCs in arteries. Pericytes are in intimate contact with the ECs, promoting their survival and providing physical stability against haemodynamic stress (Armulik et al., 2005; von Tell et al., 2006). Vascu-

lar SMCs form a contractile ring in the tunica media of the arteries by which they regulate blood pressure and flow. In most arteries, with the exception of the cerebral ones (Nyström, 1963; Stehbens, 1972; Ostergaard et al., 1987), the vascular SMC layer is encircled by the external elastic membrane. The outer most layer of arteries is the tunica adventitia, a connective tissue layer.

## 4.2 Vasculogenesis and Angiogenesis

The cardiovascular system is the first organ system to develop during embryogenesis. Hemangioblasts are the common precursors of both the hematopoietic and EC lineages. The primitive vascular plexus of the mammalian embryo and yolk sac form via the aggregation of angioblasts that are of hemangioblast origin (Coultas et al., 2005). The formation of vascular network from *de novo* differentiated ECs is called vasculogenesis. The early vascular plexus expands by sprouting via migration and proliferation of ECs. This process, the formation of new blood vessels from pre-exsisting vasculature is referred to as angiogenesis.

The nascent vascular network remodels according to the tissues' needs by pruning excess vessels and the ECs adopt an arterial, venous, or capillary phenotype (Swift et al., 2009). The process of angiogenesis is closely linked to organogenesis (Lammert et al., 2001; Matsumoto et al., 2001; Lammert et al., 2003; LeCouter et al., 2003; Yoshitomi et al., 2004). After organogenesis the vessel network strives to meet the demands of the developing organ by sprouting, splitting, intussusception (longitudinal splitting- Figure 11), and circumferential enlargement (Djonov et al., 2000).

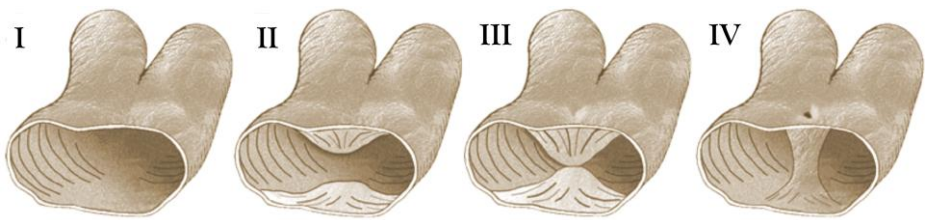



**Figure 11.** Basic steps of intussusception I-IV (modified from (Kurz et al., 2003)).

Furthermore, in pathological processes such as tumourigenesis, tumour cells may hijack the existing vasculature by growing alongside vessels, a phenomenon called cooption (Holash et al., 1999). Rarely tumours may gain their blood supply by growing into blood vessels and replacing their cells, what is referred to as "vascular mimicry" (Maniotis et al., 1999).

## 4.3 Biology of Angiogenesis

Physiological neovascularisation is uncommon in stable adult tissues, although hypertrophy such as seen in skeletal muscle growth or fat accumulation, or processes like wound healing, do stimulate the formation of new blood vessels (Zimmermann et al., 2003; Carmeliet, 2005). The primary mechanism of neovascularisation in adult tissues is angiogenesis, but vasculogenesis has been suggested to occur as well (Aicher et al., 2005; Kopp et al., 2006). Imbalances in the growth of blood vessels contribute to numerous disorders. Aberrant angiogenesis is associated with disorders such as proliferative retinal vasculopathies (Gariano et al., 2005), and insufficient angiogenesis can lead to tissue ischaemias (Schaper et al., 2003; Folkman, 2007). Furthermore, pathological angiogenesis is necessary for tumour growth and facilitates the haematogenous spread of cancer (Carmeliet et al., 2000; Hillen et al., 2007; Carmeliet et al., 2011; Weis et al., 2011). Hypoxic cells, inflammatory cells and tumour cells may release cytokines, such as vascular endothelial growth factors (VEGFs) or angiopoietin-2 (Ang2) that can induce angiogenesis (Carmeliet et al., 2011).

### 4.3.1 VEGFs and Their Receptors

The mammalian VEGF gene family is composed of five dimeric glycoproteins: vascular endothelial growth factor (VEGF or VEGF-A), VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF). VEGFs bind and activate their high-affinity VEGF-receptors (VEGFRs) tyrosine kinases and the neuropilins (NP) (Ferrara et al., 2003; Tammela et al., 2005). Binding to VEGFRs, VEGFs induce receptor dimerisation and autophosphorylation followed by activation of various intracellular downstream molecules. The intracellular signalling typically converges at mitogen

activated protein kinases and proteins activating the actin cytoskeleton, leading to cell proliferation, migration and survival (Olsson et al., 2006) .

#### *4.3.1.1 VEGF*

VEGF (also known as VEGF-A) was discovered as the first member of the VEGF gene family (Senger et al., 1983; Ferrara et al., 1989; Leung et al., 1989). By binding to and activating VEGFR-1, VEGFR-2 (de Vries et al., 1992; Quinn et al., 1993) or NP-1, NP-2 (Soker et al., 1998; Gluzman-Poltorak et al., 2000), VEGF induces EC proliferation, sprouting and migration (Ferrara et al., 2005; Olsson et al., 2006). Furthermore, by inducing anti-apoptotic proteins, VEGF contributes to EC survival (Benjamin et al., 1997; Gerber et al., 1998). Via VEGFR-2, VEGF leads to the disintegration of endothelial adherens junctions resulting in increased vascular permeability (Senger et al., 1983; Bazzoni et al., 2004; Gavard et al., 2006), hence its original name; vascular permeability factor (VPF). Additionally, VEGF can cause vasodilatation by inducing endothelial nitric oxide synthesis to decrease vascular smooth muscle tone (Hood et al., 1998; Kroll et al., 1999). In hypoxia, VEGF RNA and protein is strongly induced via the hypoxia-inducible factor–regulated elements of its promoter (Pugh et al., 2003). VEGF is ubiquitously expressed and indispensable during embryonic development; embryos lacking a single allele die *in utero* (Carmeliet et al., 1996; Ferrara et al., 1996; Weinstein, 1999).

#### *4.3.1.2 VEGF-B*

VEGF-B binds to VEGFR-1 and NP-1, but its biological role is poorly characterised. Homozygous deletion of *Vegfb* leads to a minimal phenotype in mice (Bellomo et al., 2000; Aase et al., 2001). Interestingly, in transgenic rats, VEGF-B appears to be a heart specific vascular growth factor inducing coronary vessel growth without increased permeability or inflammation (Bry et al., 2010).

#### *4.3.1.3 VEGF-C and VEGF-D*

VEGF-C and VEGF-D are very similar in their structure. Their proteolytically processed forms bind to VEGFR-2 and/or VEGFR-3 (Joukov et al., 1996; Achen et al., 1998). In mice, VEGF-C is expressed predominantly in the lymphatic vessels and in lymph nodes both during development (Kukk et al., 1996; Kärkkäinen et al., 2004) and in the adult organism (Lymboussaki et al., 1999). Via VEGFR-2, VEGF-C and VEGF-D can also increase vascular permeability (Veikkola et al., 2001; Saaristo et

al., 2002). Complete absence of lymphatic vasculature is seen in mouse embryos after homozygous deletion of *Vegfc* (Kärkkäinen et al., 2004), but not *Vegfd* (Baldwin et al., 2005).

#### *4.3.1.4 PlGF*

Similarly to VEGF-B, PlGF binds to VEGFR-1 and NP-1. PlGF is potently arteriogenic when administered locally (Pipp et al., 2003), however, its overexpression in the skin of transgenic mice leads to inflammation and increased permeability besides marked angiogenesis (Luttun et al., 2002; Odorisio et al., 2002; Oura et al., 2003). The mechanism of PlGF effects is likely mediated by the displacement of VEGF from VEGFR-1, making it available for VEGFR-2, and by the recruitment and stimulation of VEGFR-1 expressing inflammatory cells (Pipp et al., 2003; Fischer et al., 2007). Although *Plgf* gene targeted mice survive, they recover poorly from experimental myocardial infarction or hind limb ischaemia (Carmeliet et al., 2001), pointing towards an important role for PlGF in angiogenesis under pathological conditions.

#### *4.3.1.5 VEGFR-1*

VEGFR-1 is expressed by ECs and monocytes/macrophages (Zachary et al., 2001). VEGFR-1 is a poor mitogen for ECs, however, upon the formation of heterodimers with VEGFR-2, proliferation signalling is potentiated (Fong et al., 1995; Carmeliet et al., 2001; Huang et al., 2001). VEGFR-1 mediated angiogenesis and arteriogenesis are dependent on monocytes (Pipp et al., 2003). VEGFR-1 is indispensable for life, demonstrated by the embryonic lethality triggered by deletion of *Vegfr1* in gene targeted mice (Fong et al., 1995; Fong et al., 1999).

#### *4.3.1.6 VEGFR-2*

VEGFR-2 is the primary receptor transducing VEGF signals, such as permeability in ECs, even though the affinity of VEGF for VEGFR-2 is almost 10 times weaker than for VEGFR-1 (Meyer et al., 1999; Wise et al., 1999; Gille et al., 2001). Also *Vegfr2* gene targeted mice die during gestation (Shalaby et al., 1995; Gille et al., 2001). VEGFR-2 expression is low, but constitutive in the adult blood vasculature (Partanen et al., 1999; Lee et al., 2007).

#### *4.3.1.7 VEGFR-3*

During development, VEGFR-3 is expressed both by vascular and lymphatic ECs, but in the adult it is restricted to the lymphatic ECs and to the special fenestrated vessels of endocrine organs (Partanen et al., 2000). VEGFR-3 can form heterodimers with VEGFR-2 when stimulated by the mature forms of VEGF-C or VEGF-D (Dixelius et al., 2003; Olsson et al., 2006). As described for VEGFR-1 and VEGFR-2, VEGFR-3 is indispensable for mouse development (Dumont et al., 1998; Hamada et al., 2000). However, VEGFR-3 signalling appears not to be necessary for angiogenesis after organogenesis (Mäkinen et al., 2001). Both in humans and in mice, missense mutations in *Vegfr3* have been linked to hereditary lymphoedema (Irrthum et al., 2000; Kärkkäinen et al., 2000; Kärkkäinen et al., 2001).

#### *4.3.1.8 Neuropilins*

NP-1 and NP-2 are transmembrane receptor glycoproteins without enzymatic activity (Takagi et al., 1991; Kolodkin et al., 1997). Their main ligands are class 3 semaphorins that mediate repulsive signals during neuronal axonal guidance (Kolodkin et al., 1997). However, they may also function as receptors for certain VEGFs, hence modulating angiogenesis and lymphangiogenesis (Carmeliet et al., 2005; Klagsbrun et al., 2005). NP-1 forms complexes with VEGFR-2, enhancing VEGF-VEGFR-2 interactions (Soker et al., 2002). Likewise, NP-2 is a co-receptor that enhances VEGF-C/VEGF-D-VEGFR-3 interactions (Kärpänen et al., 2006). *Np1* gene targeted mice are embryonically lethal (Kawasaki et al., 1999), while *Np2* homozygous mutants are viable, though they show lymphatic capillary hypoplasia (Yuan et al., 2002).

### **4.3.2** The Angiopoietins and their Tie Receptors

The angiopoietin (Ang) growth factor family includes Ang1, Ang2, mouse Ang3, and its human orthologue Ang4 (Suri et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The Angs bind to Tie2, a receptor tyrosine kinase, which is expressed predominantly in the ECs and regulates vessel maturation and stability (Thurston, 2003; Brindle et al., 2006; Shim et al., 2007). Ang1 and Ang4 act as Tie2 agonists, however, Ang2 may act as an agonist or an antagonist, in a context dependent manner (Davis et al., 1996; Maisonpierre et al., 1997; Teichert-Kuliszewska et al., 2001). When activated, Tie2 promotes EC survival and migration

(Thurston, 2003; Brindle et al., 2006; Shim et al., 2007). The function of Tie1 is not yet fully understood, although it has a similar expressional pattern to Tie2 and may be activated by Ang1 and Ang4 in a Tie2 dependent manner (Saharinen et al., 2005). Ang1 inhibits endothelial permeability *in vitro* (Wang et al., 2004; Gavard et al., 2008) and *in vivo* (Thurston et al., 2000), whereas Ang2 may lead to destabilised, leaky vessels in some conditions (Fiedler et al., 2004; Fiedler et al., 2006). Loss of *Angpt1, Tie2* or *Tie1* all lead to embryonic lethality (Puri et al., 1995; Sato et al., 1995; Partanen et al., 1996). The *Angpt2* null phenotype is viable, but defective in hyaloid blood vessel regression and lymphatic vessel maturation (Gale et al., 2002).

## 4.4 Distinctive Angiogenic Features of the Central Nervous System

### 4.4.1 Brain Angiogenesis During Development

The blood supply in most organs develops by vasculogenesis, i.e. *de novo* vessel formation during organogenesis. However, the brain obtains its blood supply purely by angiogenesis, i.e. by sprouting of preexisting pial vessels originating from the perineural vascular plexus (Kurz et al., 1996; Kurz, 2000; Hogan et al., 2004; Ruiz de Almodovar et al., 2009; Liebner et al., 2010). Hence the main vessels supplying the brain reside on the surface of the brain, running between the lobes (Figure 12).

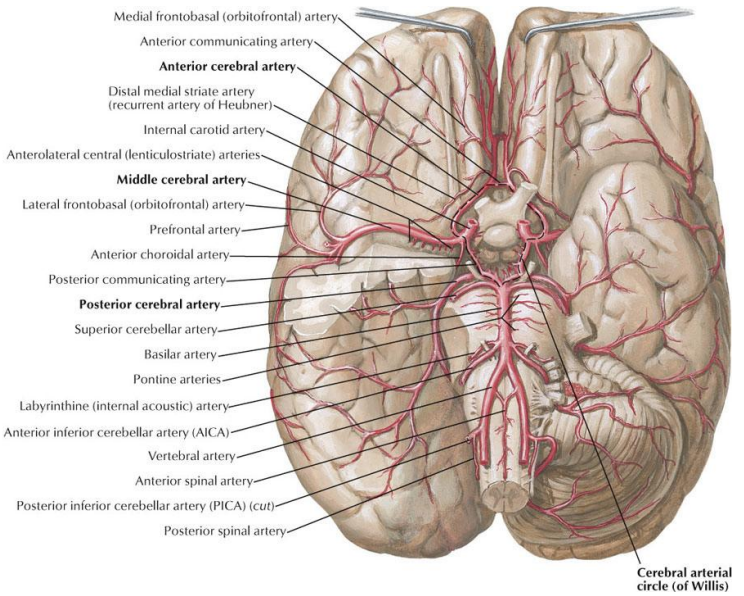
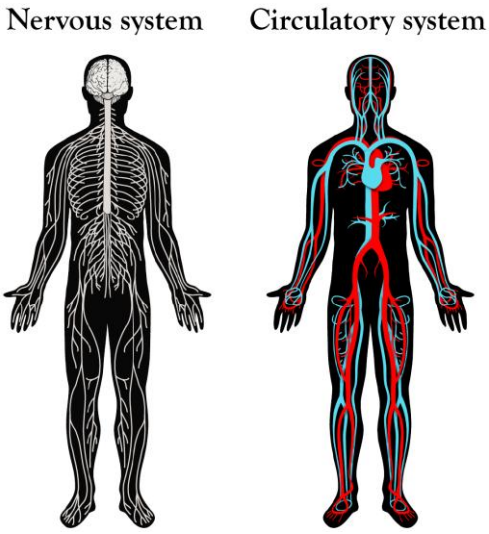



**Figure 12.** Cerebral arteries- inferior view (modified from: www.angiocalc.com).

### 4.4.2 Neuronal Effects of Angiogenic Factors




The vascular and neuronal systems show anatomical and physiological similarities (Figure 13) (Tam et al., 2010). Increasing evidence shows that besides regulating vasculogenesis, angiogenesis and lymphangiogenesis, the VEGF signalling system has a role in neurogenesis and motoneuron survival (Oosthuyse et al., 2001; Carmeliet et al., 2005; Greenberg et al., 2005; Carvalho et al., 2007).

**Figure 13.** Anatomical paralells between the vascular and neuronal systems (modified from (Tam et al., 2010)).

Neuronal cells and blood vessels already cross-talk during development, for example, astrocyte growth provides a template for the growing blood vessels (Ruiz de Almodovar et al., 2009). Furthermore, an increasing amount of evidence shows that VEGF can support neural cells independently of its roles in vessels (Rosenstein et al., 2010). VEGF treatment promotes neuronal survival and neurite outgrowth in explant cultures (Silverman et al., 1999; Rosenstein et al., 2003). Additionally, VEGF is mitogenic for astroglia and Schwann cells *in vitro* and *in vivo* (Silverman et al., 1999; Sondell et al., 1999b, a; Schratzberger et al., 2000; Krum et al., 2002; Mani et al., 2005). Moreover, both glial cell types produce VEGF, to support neuronal growth *in vitro* (Eddleston et al., 1993; Krum et al., 1998), hence it was long suspected that the neuronal effect of VEGF comes indirectly, transmitted by the glial cells. However, the observation that VEGF treatment increases neurite number, length, and size, as well as the size of the soma in primary CNS neuron cultures lacking glia, provided initial evidence that VEGF can directly affect neurons, independently of glia (Rosenstein et al., 2003; Khaibullina et al., 2004). In addition,

abnormal regulation of VEGF is implicated in neurodegenerative disorders (Oosthuyse et al., 2001; Lambrechts et al., 2003; Storkebaum et al., 2004).

## 4.5 Anti-Angiogenic Therapy

### 4.5.1 In Cancer

Studying angiogenesis became a scientific discipline after the landmark paper by Judah Folkman (Folkman, 1971), formulating the hypothesis that beyond a few mm of size, tumours need their own vasculature to grow (Figure 14). Indeed, according to autopsy results, the human body often harbours small, avascular, clinically dormant tumours (Nielsen et al., 1987; Black et al., 1993). Only a rather small proportion (<0.5%) ever become clinically malignant (Feldman et al., 1986; Black et al., 1993; Folkman et al., 2004). A possible explanation for these observations is that once exceeding a certain size, only those tumours that are able to switch to an angiogenic phenotype are able to maintain oxygen and nutrient supplies, thrive and metastasise (Folkman et al., 1991; Naumov et al., 2006). Hence this theory incepts the hope of possibly halting the malignant growth by inhibiting the tumour's ability to recruit blood vessels (Folkman, 2002). Consequently, there was an urge in the scientific community to identify the supposed *tumour angiogenesis factor* (TAF). In 1989 Ferrara and colleagues cloned the TAF and gave it its new name; VEGF (Ferrara et al., 1989).

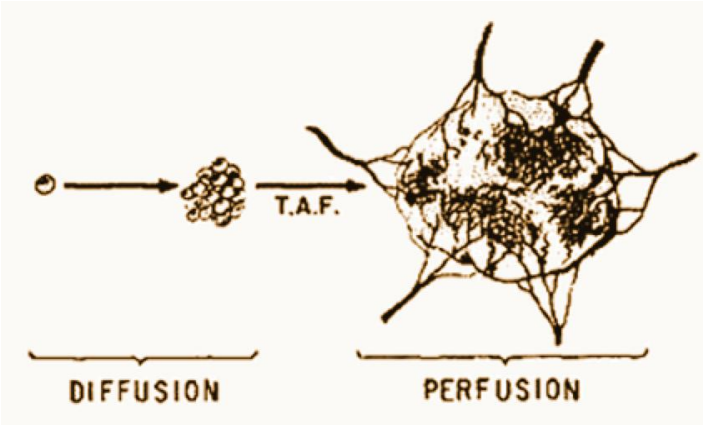



**Figure 14.** Schematic illustration of the theory about the tumour's oxygen supply switching from diffusion to perfusion with the help of the tumour angiogenesis factor (TAF) (modified from (Folkman et al., 1971)).

Blood vessels of most tumours are poorly organised and leaky (Jain, 2005; Weis et al., 2005), as in malignant astrocytic brain tumours (Apuzzo et al., 1981; Anderson et al., 2008). This is likely due to the very high level of VEGF production (Weis et al., 2005, 2011). Anti-angiogenic therapy in model organisms has indeed decreased tumour vascularisation and growth, however, in some cases it simultaneously increased tumour cell invasion (Keunen et al., 2011), and vessel cooption (Holash et al., 1999; Rubenstein et al., 2000).

A significant advancement in anti-angiogenic therapy was achieved in February 2004, when the US Food and Drug Administration (FDA) approved bevacizumab, a humanised anti-VEGF monoclonal antibody, for the treatment of metastatic colorectal cancer in combination with 5-fluorouracil-based chemotherapy regimens (Hurwitz et al., 2004). Unfortunately, the treatment of most malignancies with anti-angiogenic agents is ineffective or just briefly effective, hence limiting their clinical significance. One can theorise that the malignant process could likely be stopped by inhibiting the angiogenic switch. However, it seems that once a malignancy with angiogenic potentials has developed, anti-angiogenic therapy, especially as a monotherapy, is only rarely of clinical benefit (Bergers et al., 2008; Carmeliet et al., 2011). It is possible that starving a tumour of its blood supply is not a feasible approach to suppress cancer progression and alternatively, we may rather turn our focus towards normalisation of the tumour vasculature (Jain, 2005; Weis et al., 2011).

### 4.5.2 In Neovascular Age-Related Macular Degeneration

Anti-angiogenic therapy may be beneficial in diseases other than cancer that are characterised by excess neovascularisation. Such a disorder is neovascular (or wet) AMD. In 2004, the FDA approved pegaptinib, an aptamer that blocks the 165 amino-acid isoform of VEGF, for the treatment of the wet form of AMD (Gragoudas et al., 2004) via intraocular injections. Furthermore, in 2006 the FDA approved ranibizumab, a monoclonal Ab fragment derived from bevacizumab, for wet AMD therapy (Rosenfeld et al., 2006).

## 4.6 Towards Pro-Angiogenic Therapy

There is an important and unmet clinical need for novel treatment strategies of common ischaemic conditions such as ischaemic heart disease (Zachary et al., 2011), critical limb ischaemia (Hastings et al., 2012) and cerebrovascular disorders.

Angiogenic growth factors are promising candidates to correct perfusion by boosting collateral formation in ischaemic tissues. The angiogenic effects of VEGFs appear to be tissue specific, suggesting that the appropriate pro-angiogenic growth factor depends on the tissue type. The biological usefulness of VEGFs in pro-angiogenic therapy have been demonstrated in a variety of tissues in model organisms (Isner, 2002; Vajanto et al., 2002; Anisimov et al., 2009; Bry et al., 2010). However, no pro-angiogenic therapy is approved in humans to date, due to the difficulties in demonstrating clinically significant benefit (Simons, 2005). Suboptimal delivery strategies are an important part of this lack of success (Carmeliet, 2005), and advances in technology are likely to facilitate progress in this aspect as well.

# Aims of the Study

The aim of the studies reported here was to gain further insights into cerebrovascular disorders and to move towards novel therapeutic approaches for these diseases (Figure 15).

The specific aims were to:

1. Elucidate to what extent common genetic variants contribute to the risk of developing IA tested by conducting two multi-national GWASs (I&II).
2. Investigate whether IA and its epidemiological risk factor, high blood pressure, share a common genetic background tested by conducting an association study of IA risk loci with blood pressure in multiple large population based cohorts (n >210 000) (III).
3. Establish which vascular growth factor is the prime candidate for therapeutic vascularisation of the CNS by testing most known vascular growth factors in a rodent model (IV).

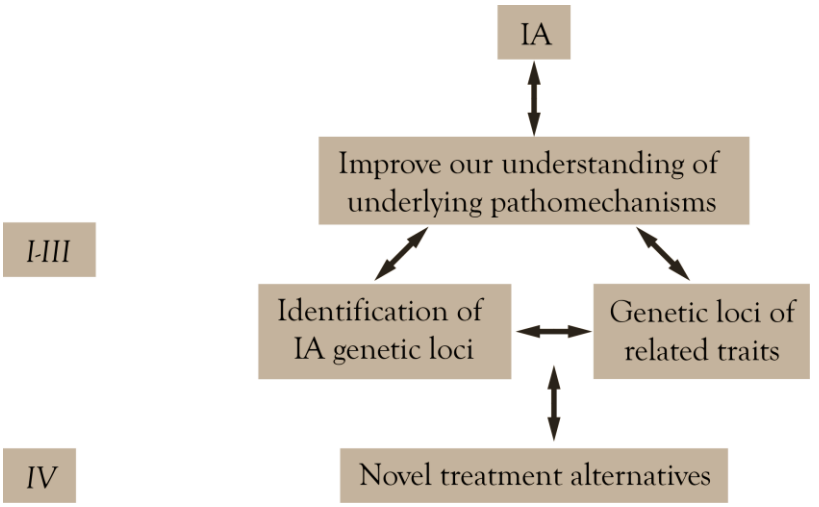



**Figure 15.** Schematic representation of conducted studies.

# Materials and Methods

## *1 Association Studies (I-III)*

A more detailed description of the study samples and methods used can be found in the original publications I-III and the references therein.

### **1.1** Genome-wide Association Studies (I,II)

#### **1.1.1** Population samples in the GWASs

Cohort characterisation and proper diagnosis is crucial for the success of GWA studies. The diagnosis of IA was made with CTA, MRA or DSA. IA rupture was defined by the identification of acute subarachnoid hemorrhage (via CT or MRI) from a proven aneurysm. Cases having at least one first-degree relative with intracranial aneurysm were considered familial, and other cases were considered sporadic. Patients with fusiform aneurysms were excluded.

GWASs are often designed as two-staged, where discovery cohorts are utilised for true genome-wide screening and replication cohorts are used to gain further evidence for the discovery phase results. Hence, the two GWASs presented here (I,II) are both constructed of a discovery- and a replication phase. Furthermore, utilising genetically diverse populations in the study makes it more probable that the identified locus (loci) holds significance for multiple populations (Gudbjartsson et al., 2007).

In the first GWAS (I) three independent cohorts from Finland (920 cases and 985 controls), the Netherlands (781 cases and 6424 controls) and Japan (495 cases and 676 controls) were recruited. Hence, for the number of IA cases, the Finnish cohort dominated. Only Japanese controls were screened for intracranial aneurysm, whilst Finnish and Dutch controls consisted of unscreened population cohorts.

In the second GWAS (II), to increase the power to detect additional loci of similar or smaller effect, two new European case cohorts ($n_{TOTAL}$=2780) and five additional European control cohorts ($n_{TOTAL}$=12 515) were ascertained. Additionally, we increased the size of the Japanese cohort and added a new one (3111 cases and 1666 controls in total). When combining all the old and new cohorts, we had nearly threefold the amount of cases than the original cohort and increased the power to detect variants even with modest effect sizes. When necessary, individuals were removed based on genotyping quality, cryptic relatedness and due to being population outliers.

### 1.1.2 Methods in the GWASs

#### *1.1.2.1 Genotyping and Imputation*

I

Genome-wide genotyping was performed on Illumina platforms, such as the CNV370-Duo, HumanHap300 or HumanHap550 chips. SNPs shared across all platforms (n=314 125) were used in the analysis. Poorly performing genotypes were excluded at a SNP or individual base, by applying pre-specified criteria, which were met by 289 271 SNPs. Over 70 duplicates showed 99.91% genotype identity, confirming the genotyping quality. In the first GWAS (I), only directly genotyped autosomal SNPs were used.

II

Again, whole-genome genotyping for the discovery cohort was performed on Illumina platforms. Replication genotyping was performed using Taqman (Applied Biosystems) or MassARRAY (Sequenom) assays, or multiplex PCR-based Invader assay (Third Wave Technologies Inc.).

Imputation refers to the computed process of estimating non-genotyped genotypes by using known population allele frequencies across a haplotype. In other words, it means educated guessing of untyped SNP genotypes based on their neighbours, with the help of reference data. Imputation always carries uncertainty, and its accuracy varies greatly. We performed imputation analysis with the HapMap phase II

CEU reference panel using the IMPUTE v1 software, calculating posterior probabilities and converting it to the most likely genotype, when it could be estimated with a 90% certainty. The association analysis was based on 831 532 SNPs (both genotyped and imputed) that passed the quality control (QC) filters in all cohorts.

#### *1.1.2.2 SNP Association Analysis*

I.

To test each SNP for association with intracranial aneurysm, we assumed an additive model. We applied the Cochran-Armitage trend test for each cohort and the Mantel extension test for the combined cohorts. We calculated the per-allele and genotype-specific odds ratios and their 95% confidence intervals. Association with IA at a SNP was considered significant if it passed the conservative threshold of $5x10^{-7}$, commonly applied at that time. For each chromosome segment showing significant association with IA, we investigated whether the association signal comes from a lone SNP (making false positive result more likely) or whether more than one SNP had an independent effect. Exactly 15 SNPs from the discovery cohort were carried on to seek their replication in the Japanese cohort. Genotypes were confirmed by two independent methods (Sequenom iPLEX and TaqMan platform (Applied Biosystems)). P values smaller than 0.05 were considered significant in the replication phase. For SNPs with the most significant P values we tested whether confounding variables such as rupture status, family history or gender, had an effect on the association results. Further, we calculated the cumulative effects of risk alleles at the identified susceptibility loci.

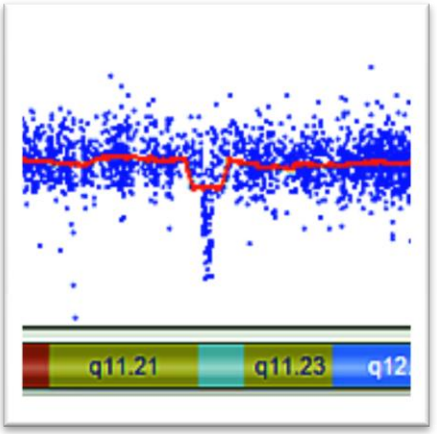
II.

The previous study had limited power to detect loci with genotypic relative risk <1.35, hence we increased cohort size and performed a new GWAS as follows: association with IA was tested for each QC–passed SNP using conditional and unconditional logistic regression for the discovery and replication cohorts, respectively. In the discovery cohort, we used the matched sub-cohort to guide correction for potential confounding due to population stratification and gender, and for the replication cohorts we adjusted for gender. A cohort-wise association analysis of the sub-cohorts (such as the Finnish and other European cohorts) was performed, and results were combined using a fixed-effects model of meta-analysis. We evaluated the

strength of association with intracranial aneurysm by a Bayesian approach, providing by the Bayes factor an alternative that compares the probabilities of the data under the alternative hypothesis of association versus the null hypothesis of no association. Further, we calculated the posterior probability of association (PPA) of the top loci ($P<10^{-5}$). The PPA provides a probabilistic measure of evidence by introducing the prior probability of association, $\pi 1$. We uniformly assumed a $\pi 1$ = 1/10 000, for all SNPs analysed.

### 1.1.3 Copy Number Variation Analysis in the GWAS cohorts

#### *1.1.3.1 Population samples*




A subset of the GWAS cohorts (I,II), with obtainable bead intensity data, was utilised for CNV analysis. Bead intensity data (Picture 5) was available for 2830 IA cases of Finnish (n=974), German (n=1056) and of Dutch (n=800) origins. Population matched controls were available for all cohorts ($n_{TOTAL}$=7460) (Conrad et al., 2010; Casals et al., 2012).

**Picture 5.** Example of bead intensity drop at the site of a deletion at 22q11. SNPs are represented by blue dots plotted for chromosomal location (x-axis) and intensity (y-axis). Red line shows average intensity.

#### *1.1.3.2 CNV Calling and Quality Control*

SNPs present on all three platforms (CNV370-Duo, HumanHap300 and HumanHap550) were utilised in CNV analysis, by pruning down all chips to consensus SNPs. To increase confidence of CNV detection, we used the merged output of three algorithms, namely: PennCNV (Wang et al., 2007), QuantiSNP (Colella et al., 2007) and GNOSIS, the latter being an in-house script at Yale University, School of Medicine. PennCNV applies an integrated hidden Markov model, while

QuantiSNP is based on an objective Bayes hidden Markov model. GNOSIS uses a continuous distribution function to compare the intensity values with the HapMap data to infer copy-number changes. Stringent QC criteria were applied after CNV calling according to algorithms' guidelines. We merged CNV predictions with CNVision, an in-house script at Yale University, School of Medicine. After merging CNV calls, as further QC, samples exceeding the number of average CNV call/sample with over 2 SD were excluded. Altogether 10% of the samples were excluded with QC. Annotation and downstream analysis was performed with the Plink v1.07 software (Purcell et al., 2007). For pathway analysis we used the gene relationships across implicated loci analysis tool (Raychaudhuri et al., 2009). All CNVs of interest were confirmed by q-PCR.

#### *1.1.3.3 Enriching for Dosage Sensitive Genes*

Certain genes are less likely to tolerate copy-number changes, hence CNVs in these genes tend to have phenotypic consequences. These genes are referred to as dosage sensitive genes. Evolutionarily dosage sensitive genes will be protected from copy-number changes, hence CNVs will affect these genes rarely (Schuster-Bockler et al., 2010). In order to increase the chance of finding biologically likely important CNVs in our dataset, we focused our analysis only on deletions that affect possibly dosage sensitive genes (Fujita et al., 2011). We constructed the list of possibly dosage sensitive genes with the help of our population specific CNV cohorts (Conrad et al., 2010; Casals et al., 2012). To define the possibly dosage sensitive genes, the following was done: for computational purposes we combined overlapping isoforms of the approximately 20 000 RefSeq genes to form a single gene. Isoforms that did not overlap were left as duplicates. Genes that were affected by structural variations according to the Database of Genomic Variants (Iafrate et al., 2004) were all excluded from further analysis, excluding about 50% of all genes. Genes affected by 100bp-1kb sized indels in DGV were excluded as well. Furthermore, all genes that were affected by at least one CNV in the control cohorts were excluded, leaving 5771 genes for further analysis. We searched our CNV calls for rare events that are unique to cases and affect one of the 5771 genes not seen with CNV before, hence, possibly being dosage sensitive.

## 1.2 Association Analysis of IA Risk Loci with Blood Pressure (III)

### 1.2.1 Population Samples

The cohorts are characterised in detail in the publication. Briefly, four Finnish population-based cohorts and a mixed-European cohort, not characterised for IA, were used in the study. We included participants with available blood pressure data, and excluded all on blood pressure medication or if blood pressure medication data were unavailable. Similar to GWAS, a multi-stage approach was applied, with a smaller discovery cohort (n=1581), followed by a larger Finnish replication cohort (n=8312) and an exceptionally large mixed-European second replication cohort (n>200 000) of The International Consortium for Blood Pressure Genome-Wide Association Studies (ICBP-GWAS).

### 1.2.2 Methods of IA Risk Loci Association Analysis with BP

#### *1.2.2.1 IA Risk Loci*

The GWASs identified 5 loci with strong association (PPA>0.5) and a further 14 loci with suggestive association (0.1<PPA<0.5) with IA (I,II and (Yasuno et al., 2011)). These 19 loci (Table 6) were tested for association with blood pressure in this study.

#### *1.2.2.2 Genotyping and Imputation*

Finnish cohorts were genotyped using Illumina arrays: Illumina Infinium HD Human610-Quad BeadChip, Illumina HumanCNV370-Duo BeadChip, and Illumina Human670K custom BeadChip. For risk loci SNPs with no directly genotyped data available, we imputed genotypes with MACH using HapMap CEU from Phase II as the reference panel or with IMPUTEv2 using the 1000 Genomes pilot data CEU panel in combination with HapMap Phase 3 haplotypes, extended with Finnish specific HapMap Phase 3 haplotypes.

**Table 6. IA risk loci.**

| *Representative SNPs of loci with PPA>0.5 (II)* | | | | |
|---|---|---|---|---|
| Locus | SNP | Gene | Risk Allele | Finnish P |
| **8q12.1** | rs9298506 | 3'-SOX17 | A | 1.0E-05 |
| **9p21.3** | rs1333040 | CDKN2A/B | T | 5.3E-08 |
| **10q24.32** | rs12413409 | CNNM2 | G | 4.2E-02 |
| **13q13.1** | rs9315204 | STARD13 | T | 1.7E-04 |
| **18q11.2** | rs11661542 | RBBP8 | C | 2.3E-02 |
| *Representative SNPs of loci with 0.1<PPA<0.5 (Yasuno et al., 2011)* | | | | |
| Locus | SNP | Gene | Risk Allele | Finnish P |
| **1p36.31** | rs1876848 | CAMTA1 | G | 1.3E-01 |
| **1p22.2** | rs1725390 | BARHL2-ZNF644 | A | 1.4E-03 |
| **1q21.3** | rs905938 | DCST2 | T | 6.7E-03 |
| **2q33.1** | rs787994 | ANKRD44-SF3B1 | T | 2.9E-04 |
| **4q31.23** | rs6841581 | upstream EDNRA | G | 4.0E-03 |
| **5q23.2** | rs335206 | PRDM6 | C | 5.9E-03 |
| **8p23.2** | rs2045637 | CSMD1 | A | 9.2E-05 |
| **8q24.23** | rs1554349 | FAM135B-COL22A1 | A | 2.3E-02 |
| **11q22.2** | rs2124216 | YAP1-BIRC3 | A | 4.5E-04 |
| **12p13.31** | rs728342 | TMEM16B | G | 4.8E-02 |
| **12q22** | rs6538595 | FGD6 | A | 1.7E-02 |
| **19q13.12** | rs1688005 | FXYD5 | G | 3.8E-02 |
| **20p12.1** | rs1132274 | RRBP1 | A | 1.0E-02 |
| **22q12.1** | rs133885 | MYO18B | G | 2.2E-01 |

Risk alleles are IA risk alleles for the whole GWAS cohort (II and Yasuno et al., 2011) and are aligned to the forward strand of the reference genome. If a SNP is intergenic, Gene represents the nearest gene. Finnish P values are association results of the Finnish sub cohort in the GWASs (II and Yasuno et al., 2011).

*1.2.2.3 Association Analysis with BP and Meta-Analysis of Results*

We tested 41 SNPs from 19 independent loci (Table 6). Quantitative outcome variables were systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and pulse pressure (PP). We tested all 19 loci in a discovery cohort, and those that showed suggestive association with any of the outcome variables were further tested for association in the replication cohorts. We performed association analyses with an additive genetic model with ProbABEL, and with SNPTESTv2, depending on the imputational software used. We adjusted the analyses for age, gender, smoking habits, alcohol consumption, and BMI. We combined association results in a fixed effect meta-analysis with MetABEL, and with METAv1.2. The best result at 5q23.2 in *PRDM6* was further tested for association in the ICBP-GWAS cohort of 200 000 individuals of European descent. In the ICBP-GWAS, association with SBP was tested by linear regression assuming an additive model and correcting for age, age-squared and BMI. To gain the per-allele effect size on blood pressure, we calculated the mean SBP for the three genotypic states for the SNPs tested at 5q23.2, by using Plink v1.07. Fine-mapping of the 5q23.2 region was done by testing all 1000 Genome SNPs (MAF>1%) in the region.

# *2 Growth Factor Induced Angiogenesis in the Murine CNS (IV)*

## 2.1 Methods Used in Model Organism

### 2.1.1 Generation of Adeno-Associated Virus Vectors and Intracranial Injections

Recombinant adeno-associated virus (AAV) vectors were generated from plasmid vectors transfected into packaging cells, purified, quantified by q-PCR and tested in vitro for their transduction efficiencies and protein production.

The AAV vectors were injected intracranially, targeting the septo-diencephalic and septo-striatal subcortex. The injection was carried out as transcranial injections to identical sites, guided by the cranial sutures on anaesthesised 6- to 7-week old female C57BL/6J mice. Four injections were performed per mouse, each delivering 2 µl of the desired vascular growth factor or the control vector (human serum albumin-HSA). The vascular growth factor effect was evaluated two weeks after transduction. All experiments were done in triplicate.

All mouse experiments were approved by the Provincial State Office of Southern Finland and carried out in accordance with the institutional guidelines.

### 2.1.2 Immunohistochemistry

Table 7 summarises the primary antibodies (Abs) used in this study, and the types or molecules that were detected with them. The unconjugated primary Abs were detected with the appropriate secondary Ab conjugates.

**Table 7. List of Primary Antibodies Used in the Study.**

| Name of Primary Abs | What it Detects |
|---|---|
| anti-podocalyxin | endothelial cells |
| anti-platelet endothelial cell adhesion molecule (anti-PECAM-1) | endothelial cells |
| anti-platelet derived growth factor receptor beta (anti-PDGFR-β) | pericytes |
| anti-α-smooth muscle actin (anti-SMA) | smooth muscle cells |
| anti-CD45 | leukocytes |
| anti-glial fibrillary acidic protein (anti-GFAP) | astrocytes |
| anti-neurofilament 200 | neurons |
| anti-fibrinogen | fibrin |

### 2.1.3 Imaging Modalities and Statistical Analysis

#### *2.1.3.1 Fluorescent Microscopy and Transmission Electron Microscopy*

Compound fluorescent microscopy or confocal microscopy was used in multichannel scanning in frame mode to analyse fluorescently labelled samples with 10x and 40x magnifications. Three-dimensional projections were digitally reconstructed from confocal z-stacks.

For transmission electron microscopy, the brains were post-fixed in osmium tetroxide and embedded in resin. Thin sections were stained with toluidine blue to search for the region of interest, and ultra-thin sections were cut and prepared from the specific region. Magnifications of 20 000- 100 000x were used.

#### *2.1.3.2 Morphological and Quantitative Statistical Analysis of Vessels*

The microvessel area was defined and quantified as the PECAM-1 or podocalyxin positive area from low-magnification confocal micrographs. PDGFR-β positive pericyte-covered microvessel area was measured and compared with the PECAM-1/podocalyxin positive area. Inflammatory cell numbers per injection site were counted from CD45 stained micrographs using low-magnification. The contrast of the images was adjusted using PhotoShop software (Adobe). For statistical analysis,

we used one-way ANOVA from PASW Statistics 19.0. $P<0.05$ was considered statistically significant.

#### *2.1.3.3 Optical Projection Tomography with Lectin Staining*

This experiment was carried out to test for vessel leakiness *in vivo* after vascular growth factor injection. Unilateral growth factor injections were carried out accompanied by contralateral sham injections. Two weeks thereafter, blood vessels were stained with fluorescently labelled tomato lectin injected into the tail vein; the lectin was allowed to circulate for 5 minutes before sacrificing the animal. Lectin stains all exposed surfaces it comes into contact with. Hence, in the case of leaky vessels, the lectin will extravasate and label the surrounding tissue. The brain isolated from the skull was made transparent chemically, embedded in an agarose gel and mounted on a rotatory stage for viewing (Figure 16).

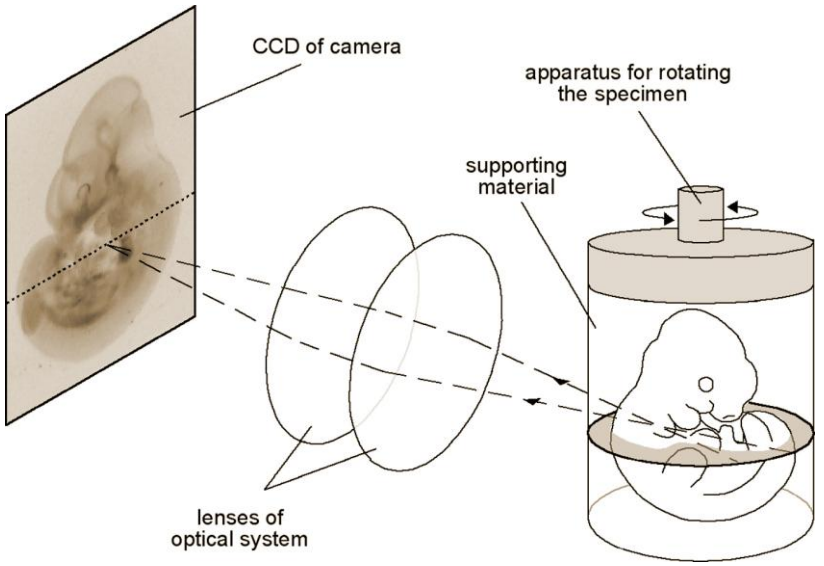



**Figure 16.** Schematic model of OPT imaging (modified from (Sharpe, 2004)).

Image Acquisition. Optical projection tomography (OPT), involving optical microscopy, is a relatively new method, which allows the capture of 360° images of trans-

parent objects of the size-range 1mm- 2cm (Sharpe et al., 2002). The brains were scanned stepwise at a 0.9° resulting in 400 images of projection data over a complete revolution (Figure 16). Appropriate filters were applied to detect autofluorescence and fluorescent signals from the specific dyes. Image stacks and three-dimensional volumetric representations were reconstructed from the raw data.

#### *2.1.3.4 Magnetic Resonance Imaging*

MRI was carried out in order to obtain further insight into the possible *in vivo* side-effects of vascular growth factor treatment. Unilateral growth factor injections were carried out, accompanied by contralateral sham injections and analysed two weeks thereafter. T1-weighted sequences with and without contrast agent, T2-weighted and T2*-weighted sequences were obtained with a 4.7 T MRI scanner from the anaesthetised mice.

# Results and Discussion

## *1 Common Genetic Susceptibility to IA (I,II)*

After candidate gene and linkage studies failed to identify variants strongly increasing the risk of IA, a new approach was needed by scanning the whole genome in a hypothesis generating way, i.e., with GWAS.

### **1.1** The First GWAS Identified Three IA Risk Loci at 2q33.1, 8q11.23-q12.1 and 9p21.3

The discovery phase, consisting of Finnish and Dutch cohorts aimed at identifying intervals harbouring SNPs that surpass the preset threshold of $5x10^{-7}$ for association with IA (Wellcome-Trust-Case-Control-Consortium, 2007). The replication of association was tested in the Japanese cohort, with and expected significance of $P<0.05$ for a positive result (Figure 17). After carefully matching cases and controls, and meticulous QC passed by 289 271 SNPs, the genomic inflation factor was 1.043 for the Finnish and 1.136 for the Dutch cohort. The genomic inflation factor of the combined cohort was 1.114 combined, indicating successful matching (Clayton et al., 2005).

In the discovery stage for the European cohorts, 4 regions harboured at least one SNP surpassing the $5x10^{-7}$ significance threshold, and 16 SNPs showed association of $P<10^{-6}$. The strength of the association came both from the Finnish and the Dutch cohorts. This number is greater than what is expected under the null hypothesis of no association with IA. Fifteen out of the 16 SNPs resided in four intervals, namely: 1q42.3, 2q33.1, 8q11.23–q12.1 and 9p21.3. The strongest association was observed at 9p21.3. This locus has already been shown to associate with IA and AAA (Helgadottir et al., 2008), and the same LD block was shown to be associated with myocardial infarction (Helgadottir et al., 2007; McPherson et al., 2007; Wellcome-Trust-Case-Control-Consortium, 2007). The loci 1q42.3, 2q33.1 and

8q11.23–q12.1 were novel and have not previously been shown to be in association with IA or any other traits.

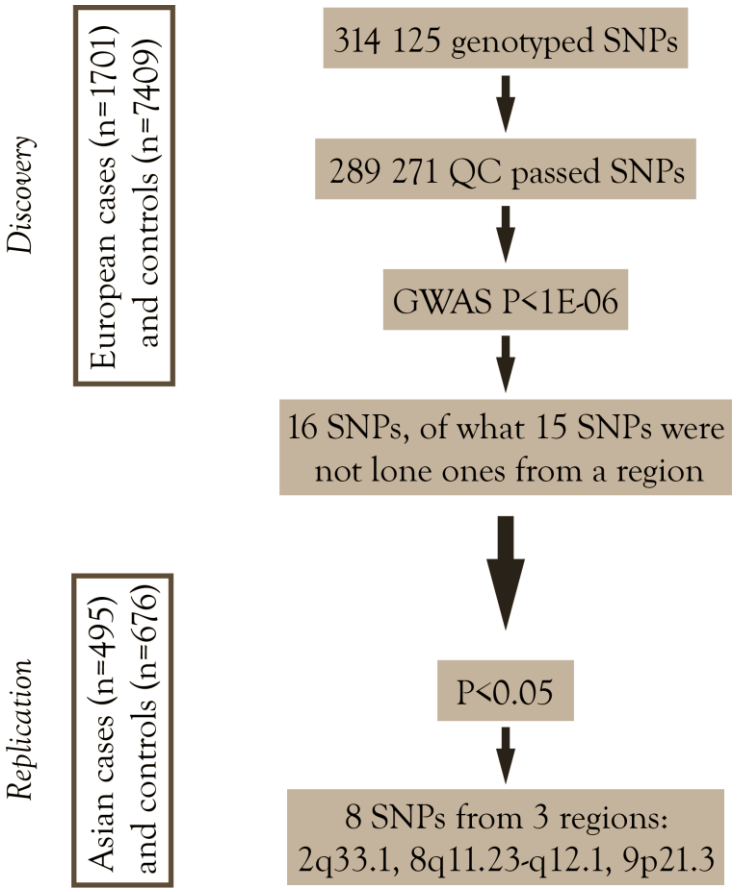



**Figure 17.** Workflow summary of the first GWAS (I).

To test whether these four loci are significant in a non-European population as well, we attempted replication in a Japanese cohort by genotyping the 15 SNPs. Of the 15 SNPs, 8 replicated the significant association with IA, including SNPs from 2q33.1, 8q11.23–q12.1 and 9p21.3. After combining the result from the discovery and replication cohorts, the strongest association with IA was observed at 8q11.23 and at 9p21.3. The loci with representative SNPs are summarised in Table 8.

**Table 8. Representative SNPs of association peaks from the first GWAS (I).**

| Locus | SNP | P |
|---|---|---|
| 2q33.1 | rs700651 | 4.4E-08 |
| 8q11.23 | rs10958409 | 1.4E-10 |
| 9p21.3 | rs1333040 | 1.4E-10 |

P-values are results from the combined discovery and replication cohorts.

### 1.1.1 The Genes at and Nearby 2q33.1, 8q11.23–q12.1 and 9p21.3

The association signal at 2q33.1 lies within a large block of LD in the European cohorts, but in Asian subjects, it is divided into two smaller LD blocks with the association confined to SNPs in the more telomeric block (198.2–198.5 Mb). The two most strongly associated SNPs lie in introns of adjacent genes, *BOLL* and *PLCL1*. *PLCL1* may be of interest due to its significant homology to phospholipase C, involved in the *VEGFR-2* signalling pathways (Shibuya, 2006), which are implicated in central nervous system angiogenesis.

At 8q11.23-q12.1, the two strongest signals (rs10958409 and rs9298506) in the discovery cohort are 110 kb apart and seem to be independent risk alleles, with no LD between them. The Japanese cohort replicated association only at rs10958409. Further studies are necessary to determine whether the association signal at rs9298506 was a true finding. The 8q11.23-q12.1 interval contains a single gene, *SOX17*, between the two European association peaks. Sox17 appears to have an important role in endothelial cell formation and maintenance (Matsui et al., 2006; Kim et al., 2007; Sakamoto et al., 2007).

The most strongly associated SNP at 9p21.3 was rs1333040, from the region that previously showed association with IA and multiple other vascular diseases (McPherson et al., 2007; Wellcome-Trust-Case-Control-Consortium, 2007; Helgadottir et al., 2008) (Figure 10- page 53). Adjacent SNPs that are associated with type 2 diabetes mellitus (Saxena et al., 2007; Scott et al., 2007; Zeggini et al., 2007) showed no significant association with IA. The strongest association signal is

located between genes *CDKN2A* and *CDKN2B*, encoding cyclin-dependent kinase inhibitors. Later studies in model animals suggested the mechanism of this locus being excessive proliferation and diminished senescence in vascular SMCs via modified expressional levels of *CDKN2A* and *CDKN2B* (Visel et al., 2010).

## 1.2 The Second GWAS Confirmed 8q12.1 and 9p21.3 and Identified Three New Loci

To increase statistical power to detect susceptibility loci with smaller effects, the discovery cohort was enlarged with further European cases and controls (Figure 18).

The strength of the association was measured by the PPA, calculated through the Bayes factor that provides a probabilistic measure of the evidence of association (Wakefield, 2007; Stephens et al., 2009). To further increase power, we imputed not genotyped SNPs using HapMap phase II CEU (Frazer et al., 2007) as reference panel, after which 831 532 SNPs passed QC. After performing association analysis, three regions showed very high PPAs (>0.995) and two additional regions showed high PPAs (>0.5). These results confirmed strong association with IA at 8q11.23–q12.1 and 9p21.3, however, the 2q33.1 locus remained suggestive in this second study. The strongest signal at 2q33.1 in the first GWAS came from the Finnish cohort. The relative contribution of the Finns decreased in the second GWAS, whilst the locus simultaneously lost significance. This suggests that the 2q33.1 is predominantly a Finnish specific risk locus, although further studies are needed to confirm this. The three newly identified loci were at 10q24.32, 13q13.1 and 18q11.2 (Table 9). Furthermore, these new results confirmed the presence of two independent loci at 8q11.23–q12.1, as suspected in the previous study. Thus, the five chromosomal regions were comprised of six independent association signals.

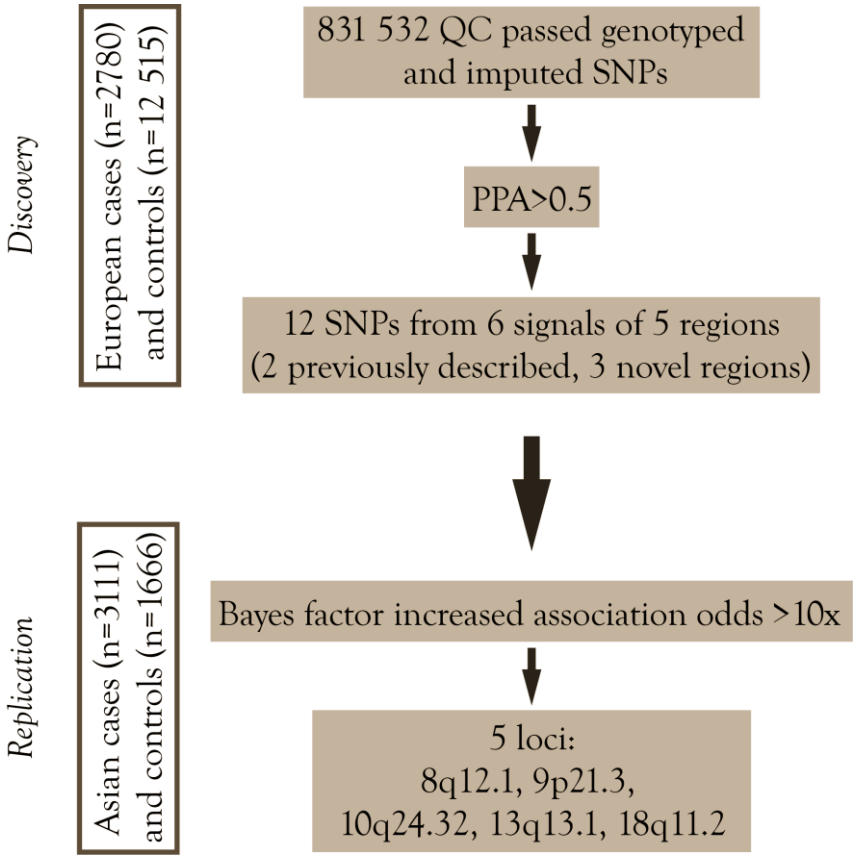



**Figure 18.** Workflow summary of the second GWAS (II).

We sought replication in two Japanese cohorts by genotyping 1-4 top SNPs from each independent signal. The replication was considered successful if the Bayes factor increased the odds of association more than tenfold with the replication data. Five out of the six candidate loci replicated, with only 8q11.23 failing replication. After combining the discovery and replication results with a fixed-effects model, all five loci surpassed the conventional threshold for genome-wide significance ($P<5\times10^{-8}$) (Table 9). The 9p21.3 locus showed a $10^{10}$ times stronger association with IA compared with the other loci.

**Table 9.** **Top SNPs of association peaks from the second GWAS (II).**

| Locus | SNP | P |
|---|---|---|
| 8q12.1 | rs9298506 | 1.3E-12 |
| 9p21.3 | rs1333040 | 1.5E-22 |
| 10q24.32 | rs12413409 | 1.2E-09 |
| 13q13.1 | rs9315204 | 2.5E-09 |
| 18q11.2 | rs11661542 | 1.1E-12 |

P-values are results from the combined discovery and replication cohorts.

### 1.2.1 The Genes at and Nearby 10q24.32, 13q13.1 and 18q11.2

Among the newly identified loci, the strongest association was at 18q11.2, within an extended LD interval harbouring a single gene, *RBBP8*. The protein encoded by *RBBP8* is involved in cell cycle progression via *BRCA1* (Yun et al., 2009). The second strongest new signal came from the associations at 10q24.32, residing in the first intron of *CNNM2*, with a role in magnesium homeostasis (Stuiver et al., 2011). The third new locus was at 13q13.1 in the intron 7 of *STARD13*. The overexpression of the STARD13 protein leads to the suppression of cell proliferation (Leung et al., 2005).

## 1.3 The Five Loci Together Explain Only Little of the Familial Risk

When assuming a fourfold increase of IA risk among siblings (Schievink, 1997; Cannon Albright et al., 2003) and furthermore that the SNPs increase disease risk in an additive fashion, we estimate that together the five IA risk loci account for 3.5-5.2% of the familial risk of IA.

## 1.4 No Top Loci Associated with Gender, Family History or Age

When trying to dissect the mechanism of the risk loci, we examined whether any of them show independent association to known risk factors, such as gender, family

history of IA and age. The results showed no significant independent contribution to risk by any of these factors. Unfortunately BP measurements and data on smoking habits were not available from most cohorts.

## 1.5 No Loci Associated with SAH

Although biologically it is a fascinating question why aneurysms form, the most clinically relevant question is why they rupture. We tested whether any of the identified risk loci independently increase the risk of rupture. However, we found no significant contribution. Currently there is no known common risk locus of SAH.

## 1.6 No Association within Previously Identified Linkage Intervals

A tempting hypothesis is that rare, strongly penetrant mutations of a gene cause Mendelian forms of the disease, and less penetrant mutations of the same gene may contribute to population risk. Identifying mutations in a gene by two fundamentally different approaches, such as linkage and association, would strongly increase the likelihood of the genes' true involvement in the diseases. Hence, we examined if any of the identified common IA risk loci reside in previously described linkage intervals (Table 4- page 48-50). Consistent with GWASs in other traits, no association was seen with common variants at regions identified in familial linkage studies.

## 1.7 CNVs in IA

It is hypothesised that part of the missing heritability might be explained by rare structural and sequence variants. It might be due to a few variants with major effects or a vast number of rare variants all with individually modest effect size. The vast number of CNVs in apparently healthy individuals seems to confer that most CNVs are not disease causing *per se*. However, large (>500kb) CNVs affecting multiple genes are likely to have phenotypic consequences (Pietiläinen et al., 2011).

Since common susceptibility loci account for only a minority of the genetic risk to IA, as is commonly seen with most complex disorders (Visscher et al., 2012), we examined whether rare variants, such as CNVs could explain some proportion of

the putative hidden heritability. We found multiple rare, case-specific variants that delete or severely disrupt genes. Altogether 111 likely dosage sensitive genes were affected by these events. Most of these events were seen once, i.e. unique events. A few recurrent events were observed, affecting the genes *SMARCA2* and *TRAM1L1*. Unfortunately, deep resequencing of these genes in over 90 cases did not reveal further mutations in the genes. At the time of the analysis, there were no further IA cohorts available to replicate their involvement in IA.

### 1.7.1 Rare and Common Variants Affect the Same Pathways

Although no CNVs were seen in the association regions, interestingly, some of the unique deletions affected the same pathways as did genes at GWAS loci. The two pathway axes that were hit by both common (GWAS) and rare (unique CNV) events are the *CDKN2B-CDKN2A-E2F1-PMAIP1* axis effecting vascular SMC proliferation and survival (Visel et al., 2010) and the *STARD13-ITSN1-SRGAP2* axis pointing towards the possible involvement of the Rho GTPase cycle (Leung et al., 2005). Confirmation of their role warrants further studies.

## 1.8 Possible Mechanism of IA Formation Based on GWAS Results

The strongest common risk locus of IA is 9p21.3, a general vascular risk locus. Based on model animal studies it is suspected to effect via excessive proliferation and decreased survival of vascular SMC (Visel et al., 2010). The genes within the other association regions appear to be involved in progenitor cell–mediated vascular development and/or repair (Matsui et al., 2006; Kim et al., 2007; Sakamoto et al., 2007). These findings point towards IA being part of a generalised vasculopathy prone to intracranial manifestation, rather than a disease of its own.

# *2 Suggestive IA Risk Locus is Associated with High Blood Pressure (III)*

The two GWASs identified 5 loci strongly associated with IA and further loci with suggestive association (Table 6- page 73). However, they could not provide evidence of supposed mechanisms of how these loci contribute to disease risk. The purpose of this study was to test in multiple independent, population based cohorts whether any of the IA risk loci show association with BP, an epidemiological risk factor of IA. The study design was three staged: the positive results from the discovery cohort (n=1581) were tested first in three further Finnish population-based cohorts (n=8312) and then tried for a second replication in a mixed European cohort (n>200 000).

## **2.1** IA Risk Loci Association Analysis with BP

Exactly 41 SNPs from 19 independent IA loci were tested in the discovery cohort for association with blood pressure, adjusted for age and gender. We observed tendency of association at 2q33.1 with DBP and with MAP, at 4q31.23 and 19q13.12 with DBP, and at 5q23.2 with SBP, DBP, and MAP. We did not detect association with PP.

To analyse the independence of the association signals observed, we tested all SNPs from the 19 loci adjusting for further BP risk factors, such as smoking habits, alcohol consumption, and BMI. After adjusting for these factors, the strength of the association with DBP decreased at 4q31.23 and 19q13.12. At the 2q33.1 locus, after adjusting for the above mentioned factors, the strength of the association increased marginally both with DBP and MAP. After adjusting the analysis for smoking habits, alcohol consumption and BMI, the strength of association increased with SBP, DBP, and MAP substantially at 5q23.2, with all three SNPs tested (rs570682, rs2287696, rs335206). At all three 5q23.2 SNPs, the risk allele of high blood pressure was the same as for IA.

In the three Finnish follow-up cohorts, the significant association with DBP and MAP, at both 2q33.1 and at 5q23.2, failed to replicate. However, all tested SNPs at 5q23.2 showed strong association with SBP in all Finnish replication cohorts ($P_{FIN}$= $3.01x10^{-5}$). Furthermore, the 5q23.2 SNPs showed strong association with SBP in the mixed European cohort of ICBP-GWAS (n>200 000) (Ehret et al., 2011). When combining the results from all cohorts in a fixed-effect meta-analysis, the strongest association was observable at rs2287696 ($P=8.13x10^{-7}$). The effect size of the locus on SBP is small, which is typical for all common risk loci of BP (Ehret et al., 2011), and of most complex diseases. In this study, participants homozygous for the risk allele, "A" in the case of rs2287696, had on average 1.3 Hgmm higher SBP compared to those who were homozygous for the protective allele, and 0.9 Hgmm higher than those with the heterozygous genotype.

## 2.2 Fine-Mapping the 5q23.2 Region

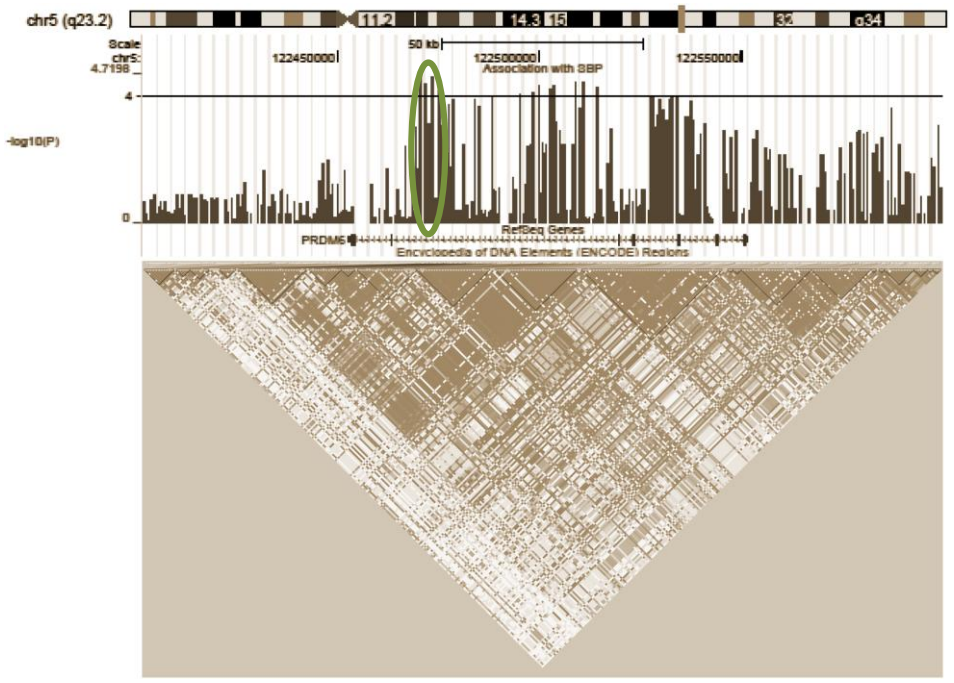



**Figure 19.** LD structure of the 5q23.2 locus with –ln(P) projected above them. The SNPs showing the strongest association with BP reside in the second intron of *PRDM6* (encircled with green).

IA risk loci SNPs tested at 5q23.2 reside in the intronic regions of *PRDM6*. To further explore the associated region we examined all 1000 Genomes variants (The-1000-Genomes-Project-Consortium, 2010) in and around *PRDM6* in the four Finnish cohorts. The strongest association was observed in the second intron with rs163189, near rs570682 and rs2287696. The most significantly associated SNPs clustered within a 4.7kb region (Figure 19), surrounding a transcription factor binding site.

## 2.3 Possible Mechanism of IA and High SBP Risk

*PRDM6* encodes an epigenetic modulator of transcription with roles in ECs (Wu et al., 2008) and vascular SMCs (Davis et al., 2006), where it is highly expressed. When active, PRDM6 inhibits differentiation and promotes proliferation of vascular SMCs (Davis et al., 2006), hence, intriguingly, having a very similar effect as is supposed for the strongest common IA risk locus at 9p21.3 (Visel et al., 2010).

## 2.4 Causality or Pleiotropy?

Based on these results, it is not possible to tell whether the identified risk variant at 5q23.2 increases the risk of developing IA as a consequence of elevated SBP or if the variant modifies the vessel wall in a way that elevates SBP and as a pleiotropic effect concurrently increases IA risk (Figure 20). Likely, the mechanical effect of elevated SBP on the vessel wall exacerbates IA formation. To further dissect the question of causality vs. pleiotropy, one would need to study a cohort characterised both for IA and blood pressure. To the best of our knowledge, such a large-scale cohort currently does not exist.

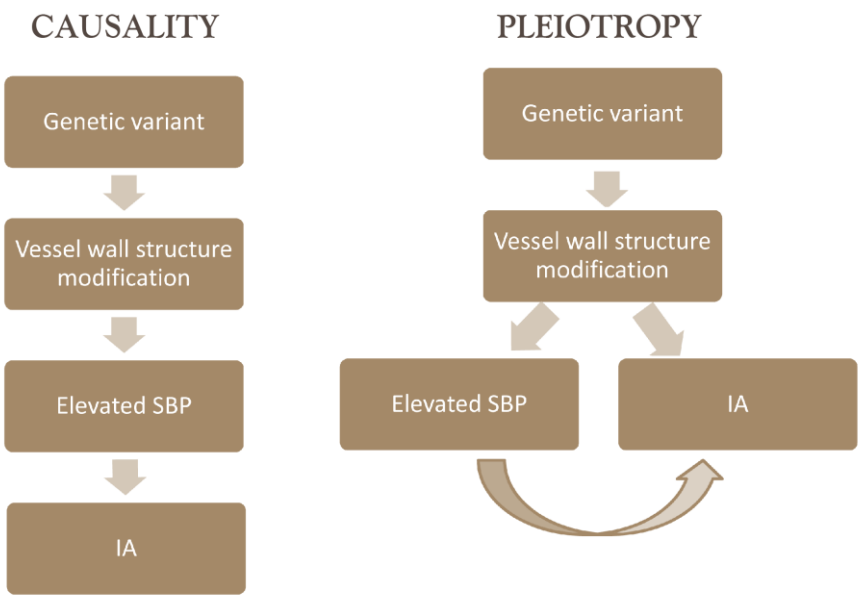



**Figure 20.** Causality vs. pleiotropy as possible explanations for the overlapping association with IA and SBP at 5q23.2.

# *3 CNS Angiogenesis in a Model Organism (IV)*

## 3.1 Therapeutic Brain Revascularisation

Different types of pathologies, such as CCH or complex IA, may necessitate revascularisation in the CNS. Current CNS revascularisation treatment strategies are predominantly surgical and focus on re-opening or bypassing the occluded or ill vessel segment. The surgical risks and the anaesthesiological risks of such procedures may be prohibitively high. Furthermore, in the case of chronically compromised cerebral blood flow, abrupt correction carries risks. There is a clinical demand for a low risk, gradual revascularisation procedure.

In order to address this, we systematically screened, for the first time, the angiogenic potentials of most known vascular growth factors, in the murine CNS. The aim was to identify the vascular growth factor most suitable for therapeutic brain revascularisation.

## 3.2 PlGF Promotes the Formation of Arterialised Microvessels

### 3.2.1 Significant Increase of Microvessel Density

AAV9s encoding the human vascular growth factors were delivered to the septo-diencephalic and septo-striatal subcortex of the murine brain by four separate injections. Two weeks following the injection, we observed that PlGF, VEGF and the short, activated form of VEGF-C (VEGF-CΔNΔC) had stimulated a comparable increase in the vascular area, as quantified by both podocalyxin and PECAM-1 immunofluorescence (Figure 21A and B). However, VEGF and VEGF-CΔNΔC promoted the formation of hemangioma-like glomeruloid structures (Figure 21C and F), comprised of likely leaky, dysfunctional vessels. Hemangiomas were not observed in brains expressing the other factors. In contrast, PlGF promoted the formation of organised microvessels (Figure 21D). VEGF-B did not promote angiogenesis (Figure 21A,B and G). As seen from the control (HSA) injected brain, the injection itself did not provoke a significant angiogenic response (Figure 21A,B,E and H).

In summary- PlGF was the only vascular growth factor that strongly promoted angiogenesis without hemangioma formation.

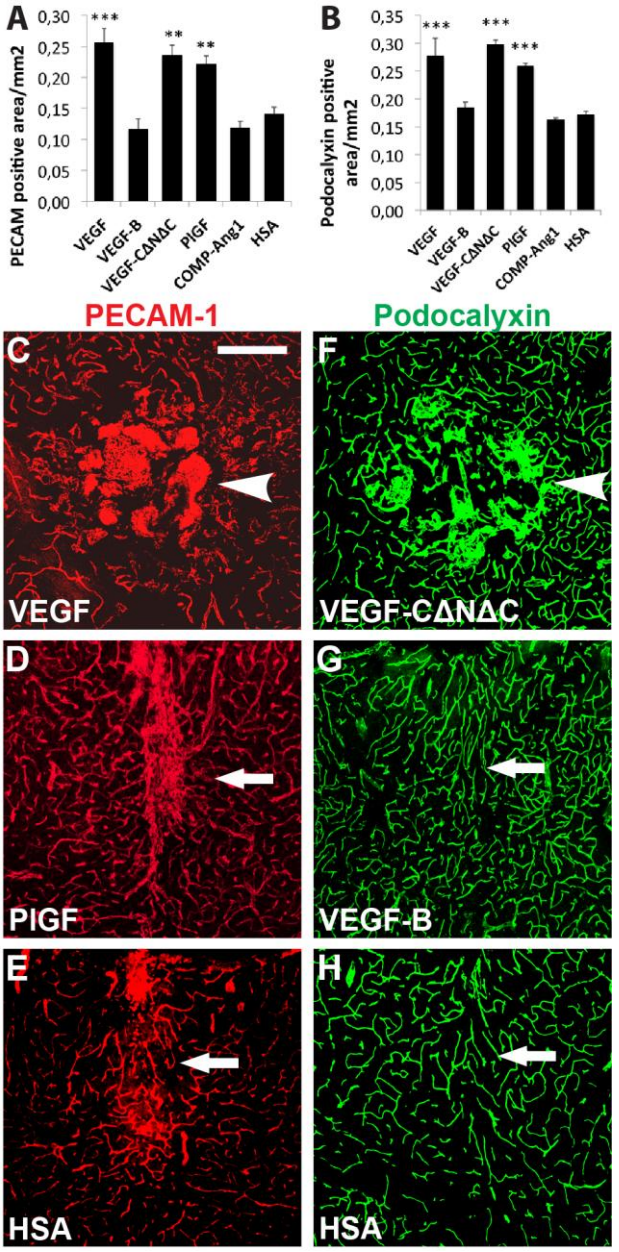



**Figure 21.** Effects of vascular growth factor gene transduction on the murine brain vasculature. Immunostaining of the targeted brain regions two weeks after transduction. Quantifications of PECAM-1 (A) and podocalyxin (B) positive areas. **P<0.01 and ***P<0.001, when compared to HSA control. Arrowheads point to hemangiomas (C,F). Arrows point out needle path (D,E,G,H). Scale bar: 200 μm.

### 3.2.2 PlGF Induced Microvessels Arterialise

Blood vessels formed in response to PlGF displayed hallmarks of mature vessels. Newly formed microvessels in PlGF transduced brains were covered by PDGFR-β positive pericytes. Furthermore, the number of arterialised microvessels increased significantly after PlGF gene transductions. The PlGF treatment-induced small arteries were surrounded by a continuous layer of SMA positive smooth muscle cells.

## 3.3 PlGF Induced Angiogenesis Does Not Incite Significant Side Effects

### 3.3.1 No Strong Inflammation

VEGF family growth factors may promote inflammation, an unwanted and potentially life-threatening side-effect in the CNS. Furthermore, inflammation can indirectly contribute to angiogenesis, confounding the direct pro-angiogenic effect of the vascular growth factor. When evaluating the extent of inflammatory cell recruitment from the blood circulation upon vascular growth factor expression, we observed that human VEGF and VEGF-CΔNΔC potently recruited CD45 positive leukocytes, whereas neither human nor mouse PlGF significantly increased inflammatory cell numbers in the injection area, when compared to the control (Figure 22). However, as expected, human PlGF recruited somewhat more inflammatory cells in the murine CNS than the murine PlGF. The difference, though, was insignificant. Further experiments indicated that the angiogenic effect of PlGF was not due to the inflammation it evoked.

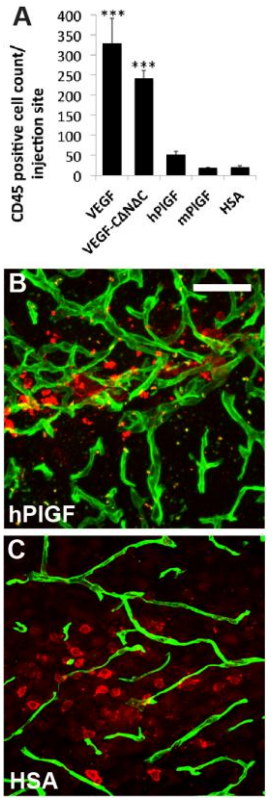



**Figure 22.** Inflammatory effects of vascular growth factor expression. Quantitative analysis of inflammation by CD45 positive cell count per injection site (A). ***P<0.001, when compared to HSA control. B,C: Immunostaining for CD45 positive cells (red). Vascular counterstaining for podocalyxin (green). Scale bar: 50 μm.

### 3.3.2 No Marked Gliosis or Neuronal Rearrangement

Tissue damage induced proliferation of astrocytes is referred to as gliosis, resulting in scar formation in the CNS. Gliotic scars have the potential to provoke epileptic activity, an undesired side-effect of vascular growth factor treatment. To test whether the vascular growth factors promote gliosis, we examined the astrocyte- and neuronal architecture by immunohistochemical staining, two weeks after gene transfer. We did not observe marked gliosis after PlGF treatment and the neuronal architecture was not visibly affected when compared to the HSA control. In contrast,

the VEGF or VEGF-CΔNΔC gene transductions were associated with substantial gliosis, and distortion of neuronal structures. Thus, in summary, VEGF and VEGF-CΔNΔC induced hemangioma formation, glial scars and a mispatterned neuronal architecture, which are all undesired side effects of pro-angiogenic therapy.

### 3.3.3 BBB is Intact and Functional

Vascular growth factor treatment may interfere with the BBB without further obvious side-effects. Thus, we examined BBB anatomy by electron microscopy and its functionality by in vivo extravasation assays. Electron micrograms showed that capillaries formed upon PlGF treatment displayed mature basal laminae, associations with mural cells and contacts with astrocyte foot processes, i.e. anatomically the BBB appeared to be intact.

To further, functionally investigate the BBB of newly formed microvessels, we tested for plasma leakage by staining for extravasated fibrinogen. Fibrinogen, once extravasated, gets deposited as fibrin in the perivascular tissues. We observed fibrin outside of the vessels in the VEGF and VEGF-CΔNΔC treated brains, indicating substantial vessel leakage. In contrast, no significant leakage was observed after PlGF treatment.

To further test BBB functional integrity *in vivo*, we stained for plasma extravasation by fluorescently labeled tomato lectin and visualised the results using OPT. After VEGF gene transfer we observed strong extravasation at the site of the VEGF-induced angiomas. Importantly, the dense capillary network formed upon PlGF treatment did not show signs of plasma extravasation.

### 3.3.4 Intact In Vivo Anatomy

We compared the *in vivo* effects of VEGF, VEGF-B (as negative control) and PlGF treatments by MRI, two weeks after right-sided vascular growth factor and left-sided sham injections. After VEGF treatment, a major distortion of anatomical structures including a brain midline shift was observed in the T1 sequence. Gadolinium-based contrast agent revealed the breakdown of the BBB in a strikingly wide region around the VEGF transduction site and the T2 series demonstrated severe oedema

over the majority of the corresponding hemisphere. The T2* series visualised a large area of microhaemorrhages after VEGF treatment. On the contrary, after PlGF and VEGF-B gene transfers, the *en gross* anatomy appeared to be normal, without significant oedema or enhancement, indicative of an intact BBB.

## 3.4 PlGF Is the Prime Candidate Vascular Growth Factor for CNS Revascularisation

Taken together, these results indicate that both mouse and human PlGF induce the formation of mature and arterialised vessels comprising an intact BBB, with minimal inflammation, gliosis and vessel leakage.

# Present State and Future Perspective

## *1 Overhauling the Status Quo of IA*

### **1.1** Is IA a Disease of its Own? Is IA Genetic?

A few decades ago we assumed IAs to be congenital, however, further studies showed that they form *de novo* during one's lifetime (Rinkel et al., 1998). A few years ago we assumed IA being (1) a distinct disease with a (2) significant genetic component to its risk. In this thesis, I summarise the latest results on the subject, showing that common genetic risk factors do not appear to explain a significant proportion to the risk of IA. Furthermore, the strongest common risk locus of IA is 9p21.3, a common cardiovascular risk locus. Based on these results, I suggest that we start to reshape our thinking about sporadic IA and consider it as (1) an intracranial manifestation of a generalised vasculopathy, as supported by epidemiological evidence (Rinkel et al., 1998; Huttunen et al., 2011; Vlak et al., 2011; Pyysalo et al., 2012). Additionally, sporadic IA, as most complex diseases, appears to lack a genetic contribution that would guide us in day-to-day clinical decision-making (2).

## *2 Quo Vadis IA Research?*

Some potential future projects that I outline here represent a logical continuation of the research results presented in this thesis, others are signalling the old-new era of genetics: the Mendelian boom No 2.

## 2.1 Search for Finnish Specific IA Loci

Although the two GWASs succeeded in describing most of the common IA risk loci that hold significance in multiple nations, there are likely population specific loci in isolates, such as the Finns. As mentioned earlier, the 2q33.1 locus appeared to be a predominantly Finnish-specific IA locus. Furthermore, the Finnish population is broken into multiple subpopulations, like the Kuusamo isolate. It is possible that the common genetic risk in these isolates is carried only by a few, higher impact variants. These variants may have the traditional value of highlighting disease-associated pathways. Studies aiming to answer these questions are under way by our collaborators at Kuopio Neurosurgery. Furthermore, Finnish specific studies on SAH genetics may bring us closer to understanding the background of the higher-than-average rupture rate of IA in Finland.

## 2.2 Mendelian Boom No 2 and Beyond

The costs of whole-exome and whole-genome sequencing have come down exponentially. This facilitates the sequencing of families exhibiting the rare, Mendelian forms of IA and it is very possible that causative variants will be identified from the linkage regions. These studies are potentially pending, yet since most humans are expected to carry approximately 20 completely inactivated genes in their genome (MacArthur et al., 2012), further proof of causality will be required from, for example, functional studies. It is likely that these mutations are family-specific, and their global value will lie in highlighting disease related pathways. It should be noted, that in a Mendelian form of thoracic AAA complicated with IA, whole-exome sequencing has already yielded results (Regalado et al., 2011). This, revisiting family research, is what I refer to as Mendelian boom No 2.

Furthermore, sequencing studies are accompanied by expressional assays with a growing frequency and will be likely followed-up by works investigating the role of methylation and imprinting, for example.

## 2.3 Genetic Diagnostics in Sporadic IA is Unlikely

Currently, only a few biologically plausible IA genes are known, such as *ENDRA* (Yasuno et al., 2011), *SMAD3* (Regalado et al., 2011) or *PRDM6* (III), and a handful of associated loci. Even if these gain functional confirmation and the list of genes grows considerably, a clinically significant genetic test evolving for sporadic IA is unlikely, particularly in the near future.

## 2.4 Shaping Future Therapies

As mentioned previously, since the contributions of the individual risk loci are small, likely no therapy can be based directly on them. However, the improved understanding of the diseases and their genetic background, may aid public health decisions.

## 2.5 Further Epidemiological Studies are Needed

The number of IA is the same in Finland, Japan and Northern Sweden as the rest of the world, however, IAs appear to be more rupture-prone in these regions (Ohkuma et al., 2002; Sivenius et al., 2004; Stegmayr et al., 2004). Since the genetic risk does not have a major contribution to SAH (Ruigrok et al., 2001; van Gijn et al., 2007; Korja et al., 2010), one can speculate that the higher-than-average rupture rate is due to environmental- and life-style related factors. Such a risk factor in Finland may be the population's infamous alcohol consumption with a tendency towards binge drinking (Popova et al., 2007) or perhaps the worldwide highest per capita coffee consumption is to blame (Argano et al., 2012; Wikipedia, 2012). Likewise, the incidence of smoking in Japan is notably high (Avila-Tang et al., 2009). In model organisms by environmental manipulation these questions may be teased apart. However, the approach to decipher this puzzle in human populations is more perplexing. Nevertheless, life has created circumstances that highly mimic the ideal, laboratory settings (Table 10). In experimental genetics the real-life equivalents of environmental manipulation are migrant- and adaptation studies.

Studying the SAH rates of Finnish and Japanese emigrants around the world, or of foreign immigrants to Finland and Japan, could provide further evidence about the

aetiology of the higher-than-average IA rupture rates (Marmot et al., 1984). Indeed, first generational Finns in Sweden were shown to have a higher risk of SAH than the general population (Khan et al., 2004). However, these results are limited by the facts that first generational migrants tend to keep the habits of their original country (Kagan et al., 1980). Furthermore, Japanese Americans are more likely to suffer from SAH than the general population, although SAH showed correlation with smoking (Klatsky et al., 2005).

**Table 10. Naturally occurring equivalents of experimental situations** (modified from (Crawford, 2006)).

| Experimental Genetics | Observational Genetics |
|---|---|
| Saturation mutagenesis | Mutation screening near nuclear disasters |
| Gene knockouts | Autosomal recessive diseases |
| Inbred organisms | Populations with low numbers |
| Clonal populations | Twin Studies |
| Environmental manipulation | Migrant studies/Adoption studies |
| Random mating experiments | Family studies |
| Enormous pedigrees by design | Searching for populations with large family size |
| Selection for extreme traits | Populations with extreme bottlenecks due to historical events |
| Designed breeding experiments | Selective ascertainment on phenotypes |
| Inter-strain variation studies | Population/anthropological genetic studies |
| Highly inbred species | Cultures with inbreeding by design |

## 2.6 The Haves and the Have-Nots

The significance of the genetic contribution to a trait differs for everyone, depending on the unique genetic architecture and environmental factors affecting an individual. Many may smoke, enjoy alcohol excessively and have untreated high blood

pressure; still, never develop IA. Their genetic risk is likely very low. Sadly, on the other end of the life-style spectrum, some develop IA and even succumb to SAH without ever smoking, disliking alcohol and with normotension. They likely have a high genetic risk of IA. When aiming to understand the genetic risk of IA, and not the genetic background of risk factors such as nicotine addiction, it would be more efficient if one would focus on patients that develop IA without having life-style related risk factors. Additionally, when trying to improve the grim outcome of IA and of SAH, one should give more emphasis to studiing the genetic components to the risk of post-SAH cerebral vasospasm (Ducruet et al., 2010).

# *3 The $1000 Genome*

The $1000 genome is a meme that scientists in the field of genetics have long aimed for. Sequencing prices have gone down significantly, however, they are still above $1000. Currently, whole-exome and whole-genome sequencing prices are around $2000-8000, which is low enough to facilitate large-scale sequencing studies and projects such as individual tumour sequencing. Furthermore, whole-genome SNP genotyping is available from the price-range of $300-500, sold direct-to-consumer.

## **3.1** Recreational Genetics

Recreational genetics is based on the concept of direct-to-consumer genotyping. This is currently dominated by genome-wide SNP platforms. Results are evaluated primarily based on GWAS findings and they calculate an increased or decreased risk of common diseases, such as hypertension, compared to the general population, or predict phenotypes, such as eye colour. As mentioned before, risk loci identified via GWAS predict disease at the individual level weakly, making most of the disease predictions by these platforms clinically irrelevant, serving rather as a genetic horoscope. Hence the name; recreational genetics.

# *4 Preventive Indirect Bypass Surgery in Local Anaesthesia: Go-go Bypass*

## 4.1 Challenges of Current Cerebral Revascularisation Procedures

Chronic cerebral hypoperfusion due to atherosclerosis or MMD (Scott et al., 2009), or the treatment of certain complex aneurysms (Hopkins et al., 1979; Spetzler et al., 1980; Sanai et al., 2009) may necessitate cerebral revascularisation surgery, to ensure vessel perfusion distal to the pathology. Currently, direct bypass surgeries form a significant part of cerebral revascularisation procedures. Cerebral bypass surgeries are technically challenging, hence confined to a limited number of centres nationally and internationally (Langer et al., 2008; Mesiwala et al., 2008). Factors that limit the clinical usefulness of current bypass procedures are their apparent lack of efficacy in athero-occlusive diseases (The-EC/IC-Bypass-Study-Group, 1985; Vilela et al., 2008; Powers et al., 2011) and the risk of the potentially life-threatening hyperperfusion syndrome (Kim et al., 2008). Additionally, general anaesthesia, necessary for current bypass surgeries, carries a risk of stroke due to the readily preoperatively compromised cerebral blood flow (Kaisti et al., 2003). Therefore, there is an unmet clinical need for a technically robust, gradual revascularisation of the brain. Our study identified PlGF as a potentially safe and effective angiogenic factor in the murine CNS, hence establishing it as a prime candidate for therapeutic cerebral angiogenesis.

## 4.2 PlGF Enhanced Multiple Bur Hole EC-IC Bypass

I envision a treatment, where indirect multiple bur hole bypass will be enhanced with PlGF, thus making indirect revascularisation sufficient in adults. The procedure would consist of the following steps: (1) drilling multiple bur holes over the region where revascularisation is desired, (2) careful opening of the dura and arachnoid membrane followed by the (3) delivery of PlGF expressing AAVs in slow-releasing biodegradable nanoparticle polymers (Leary et al., 2006), placed into the bur holes (Figure 23).

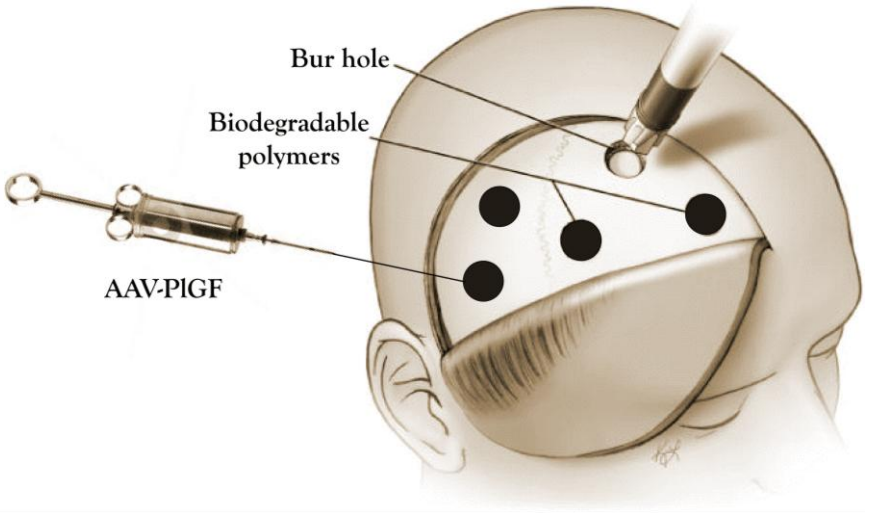
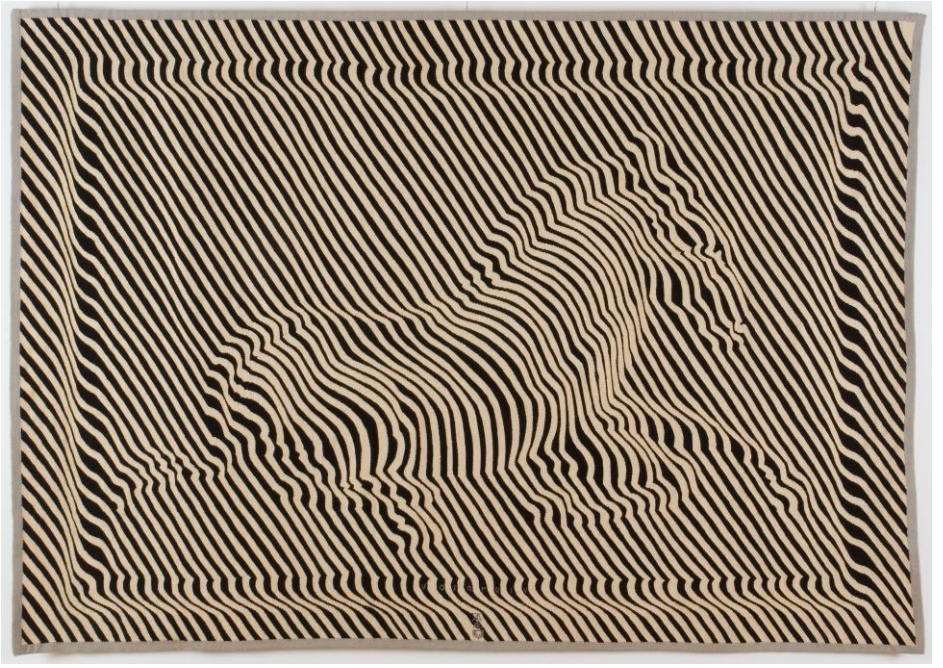



**Figure 23.** Nanoneurosurgery and neurosurgery combined. Schematic image of vascular growth factor enhanced EC-IC indirect bypass surgery (modified from (Baaj et al., 2009)).

According to our results, newly formed vessels in mice are already detectable two weeks after growth factor treatment. However, AAV mediated gene transfer ensures long term expression in the transduced cells and their progeny (Koeberl et al., 1997). Likely, the haemodynamic needs of the cerebrum would dictate which vessels stabilise and which get pruned over time. Both intracerebral injections (Kaplitt et al., 2007) and systemic administration of AAV were shown to be free from significant side-effects in humans (Nathwani et al., 2011). However, in the case of aiming to revascularise a well-defined region, local administration is likely superior to the systemic one. Hence we advocate the delivery of AAV locally, adjacent to the brain parenchyma.

PlGF enhanced multiple bur hole bypass could be offered as a prophylactic treatment due to its simplicity and consequently low risk rate. Furthermore, it could be performed under local anaesthesia and would likely necessitate only a few days of perioperative hospitalisation. In the future, PlGF enhanced multiple bur hole indirect EC-IC bypass could possibly be utilised in diseases such as VD, MMD, post-

SAH vasospasm, or during rehabilitation after stroke, since revascularisation of the ischaemic brain tissue would likely improve functional outcome (Taguchi et al., 2004). Looking further into the future, PlGF enhanced indirect EC-IC bypass may be combined with techniques such as progenitor cell or stem cell transplantation, in order to gain biological and functional restoration of ischaemic brain regions (Moe et al., 2005; Farin et al., 2009; Olstorn et al., 2011). The road of translating research results into improved patient care is always long, however, these results represent important steps in this process.



*Zebra* (1944) by **Victor Vasarely**. Reproduced with the kind permission of the Victor Vasarely Museum, Pécs, Hungary.

# Conclusions

The main findings from the studies presented in this thesis may be summarised as follows: based on its genetic background, sporadic IA emerges as an intracranial manifestation of a generalised vasculopathy. Furthermore, IA and its traditional risk factor, high SBP, share an overlapping genetic background.

The strongest common risk locus to IA is the 9p21 locus ($P=1.5x10^{-22}$), a well-studied general cardiovascular risk locus. This, and the lack of a comparably strong IA-specific risk locus suggests, that in contrary to prior views, IA does not appear to be a disease of its own but rather a part of a generalised vasculopathy prone to intracranial manifestation. Importantly, GWASs were not designed to identify risk alleles that are significant at the individual level. The identified loci explain 5.2% of the familial risk of developing IA in the Finnish population, hence likely no clinically meaningful genetic screening or cure will be based on them. However, the contribution of the common IA risk loci is significant at a population level. By identifying common IA risk loci and by dissecting the pathomechanisms behind them, we gain better understanding of the pathways increasing disease risk, hence possibly shaping future public health policies.

Nevertheless, to the best of our current knowledge, the most effective way to lower IA and SAH risk at the individual level is treatment of hypertension, smoking cessation and lowering alcohol consumption to a moderate level. Healthy life-style is the best way to get the most out of our genes.

Let it be emphasised once again, that IA formation is an intermediate phenotype when aiming to tackle the deadly SAH. Since it is a small, and likely specific subgroup of IA that ruptures, future studies should focus more on SAH.

Decades of vascular research taught us about angiogenesis and vascular growth factors and the key elements of therapeutic revascularisation. Translating research results into improving the quality of life of patients suffering from cerebral hypoperfusion is now ever closer. The results presented here represent the next significant step towards therapeutic angiogenesis in the CNS, by identifying PlGF, via systematic screening, as the safest, most efficient vascular growth factor in the CNS of a model organism.

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