Genetic and Observational Subarachnoid Haemorrhage (GOSH) Study - A UK-wide clinical and genetic cohort study of aneurysmal subarachnoid haemorrhage

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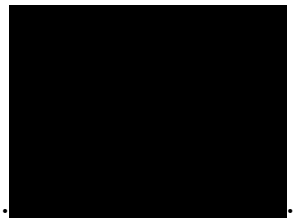
Thesis submitted for the degree of Doctor of Philosophy

2019

Declaration

I, Varinder Singh Alg confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

Signature……  …….

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Abstract

Introduction

Intracranial aneurysms affect 2-5% of the general population.[1] A tiny proportion of these rupture leading to aneurysmal subarachnoid haemorrhage (SAH), which can be fatal instantly or lead to life-changing neurological morbidity in a young group of stroke survivors. Understanding the pathophysiological mechanisms underlying intracranial aneurysms, and being able to predict which patients have a higher risk of rupture has led to intensive research efforts, via epidemiological, molecular and genetic studies. Our aim was to determine the association of candidate genes with intracranial aneurysms in a large UK Caucasian population.

Methods

We performed a case-control genetic association study of single nucleotide polymorphisms (SNPs) in over 1600 patients with intracranial aneurysms from a UK-wide Genetic and Observational Subarachnoid Haemorrhage (GOSH) study and 1500 controls from the Wellcome cohort,[2] utilising a candidate-gene approach. We conducted a literature review and performed a meta-analysis of the existing candidate gene studies to better determine which genes would be suitable for analysis in our cohort. We also performed a new meta-analysis using our data for each SNP examined to determine if our genetic associations with intracranial aneurysms were robust.

Results

We examined 22 SNPs related to vascular endothelial integrity, the extracellular matrix, and inflammation. Two SNPs showed associations with intracranial aneurysms in our UK cohort: the D allele of ACE Insertion/Deletion SNP (associated with vascular endothelial function; OR 1.14 [1.02-1.28], p=0.02) and the MMP-2 C>T rs243865 SNP (associated with extracellular matrix integrity; OR 1.18 [1.04 – 1.33], p=0.012).

Conclusions

We found associations with intracranial aneurysms for the D allele of the ACE I/D SNP, and a potentially functionally significant MMP-2 SNP. Both genes have plausible connections to IA pathophysiology (endothelial function and extracellular matrix integrity, respectively), and could potentially predispose patient to aneurysm rupture as demonstrated by sub-group analysis.

Impact Statement

Our study was a case-control genetic association study examining whether certain gene variants associated with the risk of intracranial aneurysms and subsequent subarachnoid haemorrhage, are prevalent in a UK population. Since the mapping of the human genome, advances have been made in deciphering the genetic causes of various disease. Intracranial aneurysm pathophysiology is complex, in that no single gene is responsible for its occurrence. It is a combination of the small effects of multiple gene variants that can interact with the environment to predispose to intracranial aneurysms. Our study in over 1600 participants with intracranial aneurysms and subarachnoid haemorrhage throughout the UK found two genetic variations with plausible function in the development and rupture of aneurysms. Two genetic variants (ACE I/D polymorphism and the MMP-2 C>T SNP) were associated with IA in a UK Caucasian population, suggesting a role for endothelial dysfunction and extracellular matrix integrity in the pathophysiology of intracranial aneurysms. Our study adds to the current body of literature on intracranial aneurysm genetics; further research should investigate whether these variants are also associated with intracranial aneurysms in other population worldwide. Our results could have relevance for understanding the pathophysiology of intracranial aneurysms, highlighting potentially relevant pathways that could help improve the diagnosis and treatment of devastating subarachnoid haemorrhage.

Acknowledgements

I would firstly and most importantly like to thank Professor David Werring and Professor Henry Houlden for their constant guidance during the course of my PhD studies. I am grateful to them for affording me the opportunity to perform this study. I express my deepest gratitude for their support during the course of my studies, and am conscious of the fact, that without their knowledge, expertise and endeavour, I would not have been able to complete my studies. I am indebted to them both for supporting me through difficult periods in my life and showing utmost compassion and support so I could complete my thesis.

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List of Abbreviations

AAA	Abdominal Aortic Aneurysms
ACA	Anterior Cerebral Artery
ACE	Angiotensin Converting Enzyme
ACOM	Anterior Communicating Artery
ADPKD	Adult Polycystic Kidney Disease
Ap-1	Activator Protein-1
bFGF	basic Fibroblast Growth Factor
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B
CGAS	Candidate-Gene Association Study
COL1A2	Collagen 1A2
COL3A1	Collagen 3A1
COL4A1	Collagen 4A1
COX-2	Cyclooxygenage-2
CRF	Case report form/Clinical research facility
CSPG2	Chondroitin Sulphate Proteoglycan 2
CT	Computerised Tomography
CTA	Computerised Tomography Angiogram
DCI	Delayed Cerebral Ischaemia
DIND	Delayed Ischaemic Neurological Deficits
DNA	Deoxyribonucleic Acid
DSA	Digital Subtraction Angiography
ECM	Extracellular Matrix
EDNRA	Endothelin receptor type A
EDRB	Endothelin-1 B
eNOS	endothelial Nitric Oxide Synthase
ETBR	Endothelin B Receptor
FIA	Familial Intracranial Aneurysm

GAS	Genetic Association Study
GWAS	Genome-wide Association Study
HRT	Hormone replacement therapy
HSPG2	Heparan Sulphate Proteoglycan 2
HWE	Hardy-Weinberg Equilibrium
IA	Intracranial Aneurysms
ICA	Internal Carotid Artery
IEL	Internal Elastic Laminae
IFNγ	Interferon Gamma
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
ISAT	International Subarachnoid Trial
ISUIA	International Study on Unruptured Intracranial Aneurysms
JDP2	Jun 2 Dimerisation Protein
KASP	KBioscience Competitive Allele-Specific
LD	Linkage Disequilibrium
LP	Lumbar puncture
MAF	Minor Allele Frequencies
MCA	Middle cerebral artery
MCP-1	Monocyte chemoattractant Protein - 1
MHC	Myosin Heavy Chain
MMP	Matrix Metalloproteinase
mRs	Modified Rankin Scale
MSA	Multi-System Atrophy
NF-κB	Nuclear factor kappa B
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
OCP	Oral Contraceptive Pill

OR	Odds ratio
PCOM	Posterior Communicating artery
PCR	Polymerase Chain Reaction
PDGF	platelet-derived growth factor
QC	Quality Control
RAF	Risk Allele Frequency
RAS	Renin-Aldosterone System
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SAH	Subarachnoid Haemorrhage
SEPRINA3	Serpin Family A Member 3
SERPINE1	Serpin Family E Member 1
SMC	Smooth Muscle Cells
SNP	Single Nucleotide Polymorphism
SP-1	Specificity factor-1
TGFβ	Transforming Growth actor Beta
TIMP	Tissue Inhibitor of Metalloproteinase
TNFa	Tumour Necrosis factor Alpha
VCAM-1	Vascular Cell Adhesions Molecule-1
VEGF	Vascular Endothelial Growth Factor
VNTR	Variable Number Tandem Repeat
VSMC	Vascular Smooth Muscle Cells
WES	Whole Exome Sequencing
WFNS	World Federation of Neurological Surgeons
WSS	Wall Shear Stress

List of Publications arising from this thesis

1: **Alg VS**, Ke X, Grieve J, Bonner S, Walsh DC, Bulters D, Kitchen N, Houlden H, Werring DJ; Genetics and Observational Subarachnoid Haemorrhage (GOSH) Study Investigators. Association of functional MMP-2 gene variant with intracranial aneurysms: case-control genetic association study and meta-analysis. Br J Neurosurg. 2018 Jun;32(3):255-259. doi: 10.1080/02688697.2018.1427213. Epub 2018 Jan 15. PubMed PMID: 29334797.

2: Hostettler IC, **Alg VS**, Shahi N, Jichi F, Bonner S, Walsh D, Bulters D, Kitchen N, Brown MM, Houlden H, Grieve J, Werring DJ; Genetics and Observational Subarachnoid Haemorrhage (GOSH) Study investigators. Characteristics of Unruptured Compared to Ruptured Intracranial Aneurysms: A Multicenter Case-Control Study. Neurosurgery. 2018 Jul 1;83(1):43-52. doi: 10.1093/neuros/nyx365. PubMed PMID: 28973585.

3: **Alg VS**, Sofat R, Houlden H, Werring DJ. Genetic risk factors for intracranial aneurysms: a meta-analysis in more than 116,000 individuals. Neurology. 2013 Jun 4;80(23):2154-65. doi: 10.1212/WNL.0b013e318295d751. Review. PubMed PMID: 23733552; PubMed Central PMCID: PMC3716358.

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Chapter 1 Introduction

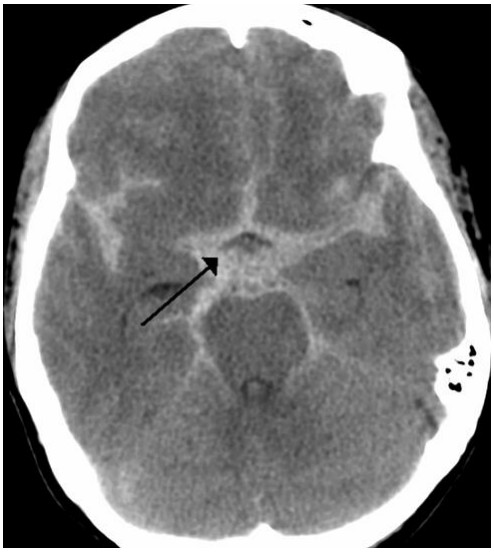
1.1 Background

The first clinical account and autopsy description of aneurysmal subarachnoid haemorrhage (SAH) was by Biumi of Milan in 1765.[3, 4] Aneurysmal SAH is one of the most feared and lethal subtypes of stroke, with approximately 50% (32%-67%) of patients dead or dependent at 30 days post-onset [5, 6]. About 1 in 20 strokes are due to SAH affecting an age range between 17-85 years, with peak prevalence in the 50-65 age group. Despite its relative rarity, aneurysmal SAH has the largest economic burden with an annual estimated cost of £510 million for resources required to care for SAH sufferers in the UK [7, 8], almost double that of ischaemic stroke. Less than one-third of SAH survivors return to previous occupations and lifestyles, as a result of the range of disabilities suffered. These include; cognitive impairment, memory problems to requiring assistance with everyday aspects of care, being unable to walk or stuck in a vegetative state. Some regions, particularly the Netherlands and Japan have higher incidences of SAH than the general worldwide estimate of 8-10 per 100 000 per year.[9]

1.1.1 Clinical presentation, diagnosis and treatment

The diagnosis of SAH remains predominantly clinical with a typical history of sudden onset severe ("thunderclap") headache, which is the most common symptom in up to 97% of patients.[10] There may also be features of meningism and associated symptoms, such as vomiting, blurred vision and temporarily loss of consciousness. Aneurysmal SAH can also cause instant fatality in up to 10-15% patients before reaching medical attention.[11] Aneurysmal SAH can be a difficult diagnosis to make, as 1-3% of all patients presenting to their emergency department with sudden onset headache, are diagnosed with SAH.[12] Confirmation is usually via an unenhanced computed tomography (CT) scan, revealing blood in the subarachnoid space (see figure 1-1) with sensitivity between 93% - 100%.[13] If CT scans are negative than based on the history, a lumbar puncture is usually carried out to detect subarachnoid blood products; in particular, Bilirubin and oxyhaemoglobin which develops between 6 to 12 hours post-ictus. Spectrophotometry is used to detect Xanthochromia, if it is not already visible when initially performing the LP. The CSF must be kept in the dark to avoid bilirubin degradation. Oxyhaemoglobin peaks initially between 4-10 hours and bilirubin will usually appear 10-24 hours post-ictus on spectrophotometry examination, which would clinch the diagnosis.[14] If the LP is performed less than 12 hours or greater than 2 weeks post-ictus, the sensitivity of the LP will be reduced.

Figure 1- 1– Unenhanced CT scan showing widespread subarachnoid Haemorrhage (Black arrow)



Aneurysmal rupture causing SAH accounts for 75-80% of cases[15] and is confirmed via a CT angiogram (CTA) usually at the same time as the diagnostic CT scan or via formal digital subtraction angiography (DSA- see figure 1-2). Treatment for cerebral aneurysms, whether ruptured or unruptured is controversial. In the case of ruptured aneurysms, treatment option includes endovascular coiling where the placement of platinum coils in the dome and neck of an aneurysm can be carried out, thereby, securing the aneurysm from the main circulation (see figure 1-3). The other treatment option of intracranial aneurysms includes neurosurgical clipping via a craniotomy, which prior to the ISAT study was the mainstay of treatment (see figure 1-4).

Figure 1- 2 – Posterior Communicating artery (PCOM) aneurysm on DSA.

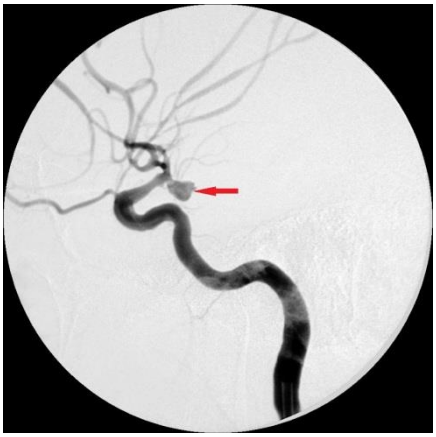


Figure 1- 3 - Endovascular coiling illustration. Adapted from Brisman et al.[16]

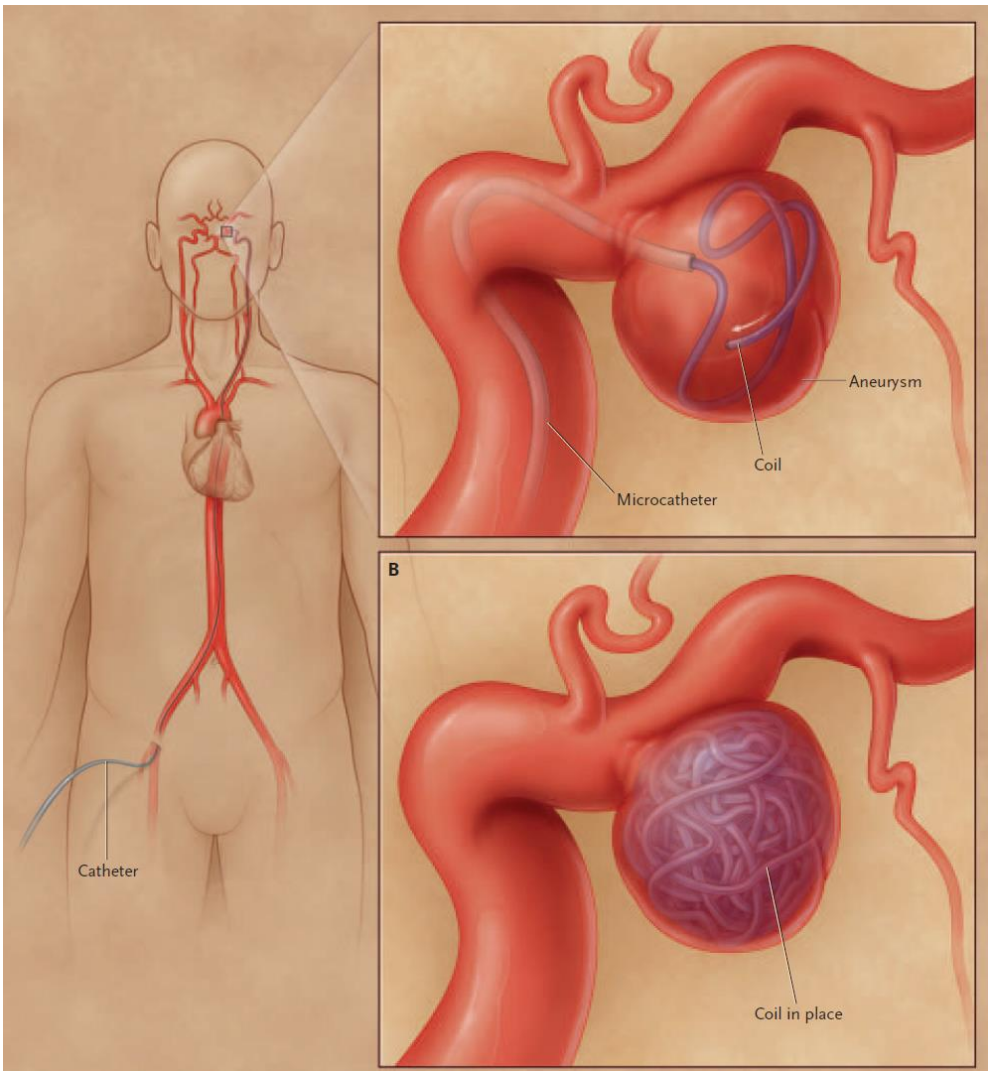
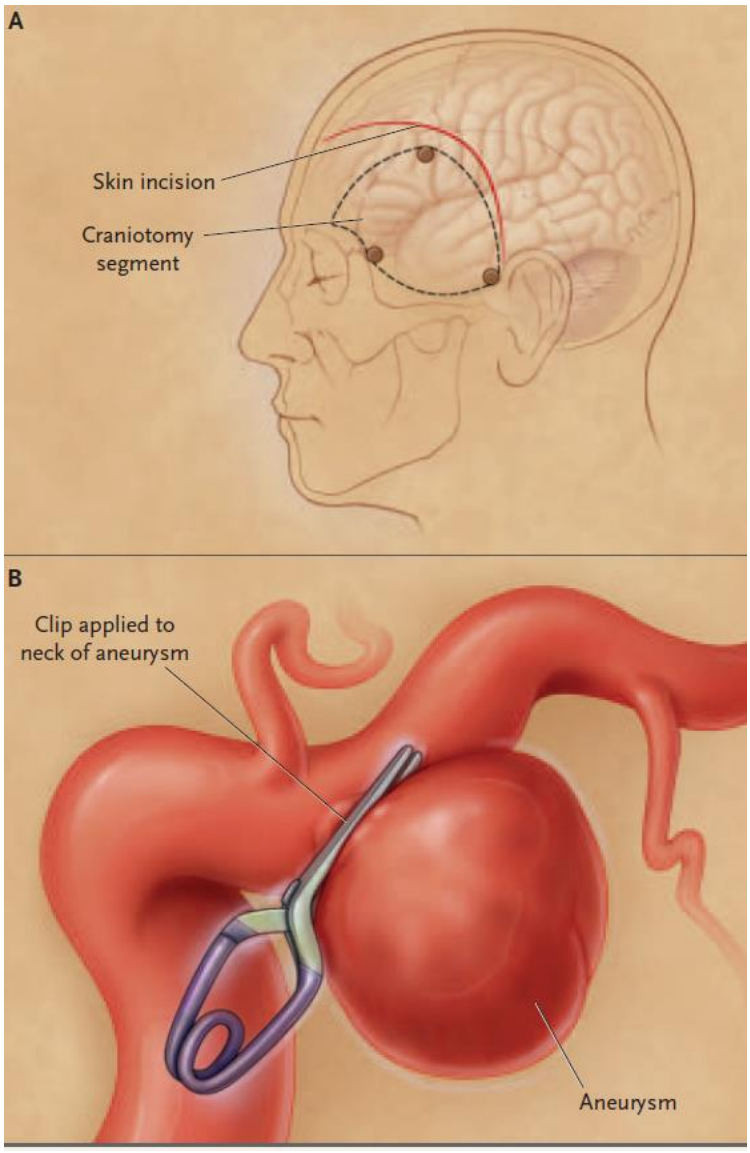


Figure 1- 4 - Neurosurgical clipping illustration. Adapted from Brisman et al.[16]



Neurological sequelae arising from ruptured intracranial aneurysms include; primary parenchymal injury; intracerebral haematoma; acute hydrocephalus; re-bleeding; seizures and cerebral vasospasm – potentially leading to critical ischaemia causing delayed ischaemic neurological deficit (DIND). These complications are primarily managed neurosurgically, and mandate rapid securing of an aneurysm wherever possible.

Since the ISAT trial [17], the majority of aneurysms are treated by endovascular coiling, primarily due to less morbidity associated with a craniotomy and quicker discharge from hospital compared with patients undergoing a craniotomy. Results from the ISAT trial sparked controversy in the neurosurgical field by claiming coiling was superior to clipping. The ISAT study was a randomised controlled trial comparing coiling versus clipping and followed patient outcome 1 year after treatment, via the mRs (modified Rankin scale) score. In total, 2143 patients were recruited into the trial, with 1073 randomised to the coiling arm and 1070 patients randomised to the surgical arm. They demonstrated an absolute risk

reduction of 7.4% [3.6-11.2, p=0.0001] in mortality in favour of endovascular treatment over surgical clipping. However, late re-bleeding was more common in the endovascular group than the coiled group. Concerns regarding these findings, included the higher recurrence rate in coiled aneurysms compared to those treated surgically, and some quarters raised the question, that the comparison was not alike, as inadequately trained surgeons may have performed the clipping, instead of a specialised vascular neurosurgeon compared to 'expert' neuro-interventionalists, hence the poorer outcome in the surgical arm.

Shortcomings of the ISAT were; only including aneurysms less than 10 mm in size and anterior in location. Note was made that aneurysms with wide necks were often better served surgically; due to potential slippage of platinum coils into the parent vessel causing an ischaemic stroke, or aneurysms with difficult configuration, particularly with perforating arteries or major branches arising from the aneurysm. Results post ISAT, demonstrated fewer recurrences of aneurysms in the surgical group compared with those coiled. [5, 18, 19]

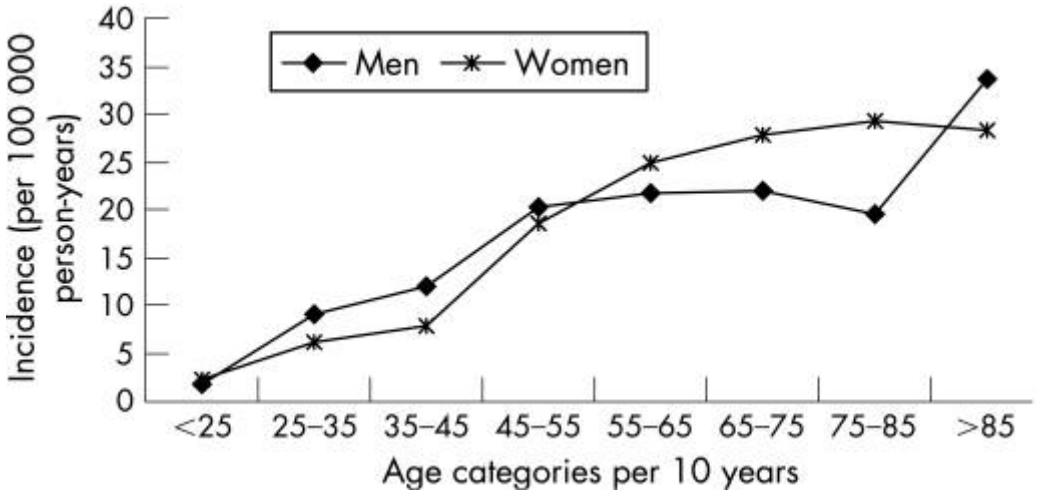
Due to the potential life-years lost for sufferers of the disease, there is an urgent need to better understand the pathophysiology of intracranial aneurysms. Fundamental questions remain, including: (1) how do intracranial aneurysms develop and in whom; (2) what trigger factors cause them to rupture; and (3) which particular patient genotypic and phenotypic traits are associated with vulnerability to rupture?

1.1.2 Epidemiology

Approximately 2-6% of the general population harbour an intracranial aneurysm.[1, 20, 21] A small proportion of these patients will develop a Subarachnoid Haemorrhage (SAH) as a result of aneurysm rupture. SAH comprises approximately 5% of all strokes. 85% of SAH cases are due to rupture of a saccular aneurysm within the cranial cavity. The remaining causes include; trauma, arterio-venous malformations (AVMs), infective (mycotic aneurysms); arterial dissection or coagulopathy[9]. Wide variation exists in SAH incidence, ranging from 2-25 per 100 000 person-years. In general, the age-related incidence of aneurysmal-SAH is approximately 9.1 (CI 8.8-9.5) per 100 000 person-years, excluding Japanese, Finnish and South or Central America [9, 22, 23]. However, in these populations, a higher incidence of 20 per 100 000 person-years has been noted, particularly in Finland and Japan [9, 24]. Rooij[22] *et al* in a recent systematic review estimated overall incidence rates in these populations with 22.7 (CI 21.9-23.5) in Japanese; and 19.7 (CI 18.1-21.3) in Finnish populations. However, Korja and colleagues[25] recently determined the incidence of SAH to be decreasing in the Finnish population, in align to change in smoking habits. From a total of 79,083,579 cumulative person-years, they identified 6,885 people with SAH between 1998-2012. The crude nationwide annual incidence rates had dropped to 6.2-10/100,000 persons, which is lower than that quoted by Rooij.[22]

The incidence increases with age at the time of diagnosis of SAH (see figure 1-5), 50% of patients are under the age of 55, with a peak prevalence age group of 55-60 years. [1, 9, 24, 26] Interestingly, incidence of SAH was shown to be higher in men in the 20-45 years category but higher in women in the 55-85 years age category.[22]

Figure 1- 5 – Incidence of SAH by age and gender, adapted from Rooij 2007.[22]



Aneurysmal SAH has a particularly severe prognosis with approximately 40-44% of patients dead at 30 days post-ictus; [27-30] this statistic includes the 10-15% patients who do not reach medical care.[22] One-third of patients who survive become severely disabled and dependent on rehabilitation services and support for their activities of daily living. Factors influencing outcome include; the severity of the initial bleed, the presence of re-bleeding or hydrocephalus and the occurrence of cerebral vasospasm causing a delayed ischaemic neurological deficits (DINDs).[31, 32]

Definitive treatment of intracranial aneurysm(s) has shifted over the last 10 years from neurosurgical clipping involving a craniotomy to endovascular coiling, via placement of detachable coils into an aneurysm to seal it off and minimise the risk of further rupture causing a re-bleed, which is maximal in the first day, ranging between 4% - 13.6% After the first day, the risk of rebleed is 1.5%/day up to 14 days. 50% of patients will rebleed within 6 months, following which the risk is 3&/year with a mortality of 2%/year.[33]

1.1.3 Associated modifiable risk factors with intracranial aneurysms and rupture

Many risk factors have been linked with the development of intracranial aneurysms and risk of SAH, but the strongest associated modifiable risk factors with intracranial aneurysms include; hypertension, smoking, increased alcohol consumption (see table 1-1); Other risk factors have been tested in association with IA disease and were found not to be associated, these include; oral contraceptive pill

(OCP), hormone replacement therapy (HRT), hypercholesterolemia, lean body mass index (BMI), caffeine intake and diabetes.[34, 35] It is important to appreciate that intracranial aneurysms result from a complex interplay of environmental and genetic factors contributing to the overall phenotype.[36] In the majority of genetic studies to date, the focus has been on the principal association of candidate genes or SNPs, however, contributions of major risk factors have been neglected on overall intracranial aneurysm phenotype.

Table 1- 1 – Risk factors associated with SAH

Modifiable risk factors for SAH	**Non-modifiable risk factors**
Smoking	Female gender
Hypertension	Adult polycystic kidney disease (ADPKD)
Alcohol	Connective tissue disorder e.g. Marfans, Type IV Ehlers Danlos syndrome
Sympathomimetics use/Recreational drugs	Dutch, Japanese populations
	Family history
	Age
	Previous history of SAH

Smoking

Smoking status has been cited as the strongest independent risk factor in the development of aneurysmal SAH.[37] Feigin *et al* [35] performed a meta-analysis on 15 case-control studies and 5 longitudinal studies calculating the influence smoking had on the occurrence of SAH. In the case-control studies an overall odds ratio of 3.1 (CI 2.7-3.5), with an estimate of 2.5 (CI 1.4-4.0) for females and 5.2 (CI 3.0-9.0) for male current smokers against those who had never smoked, was observed.

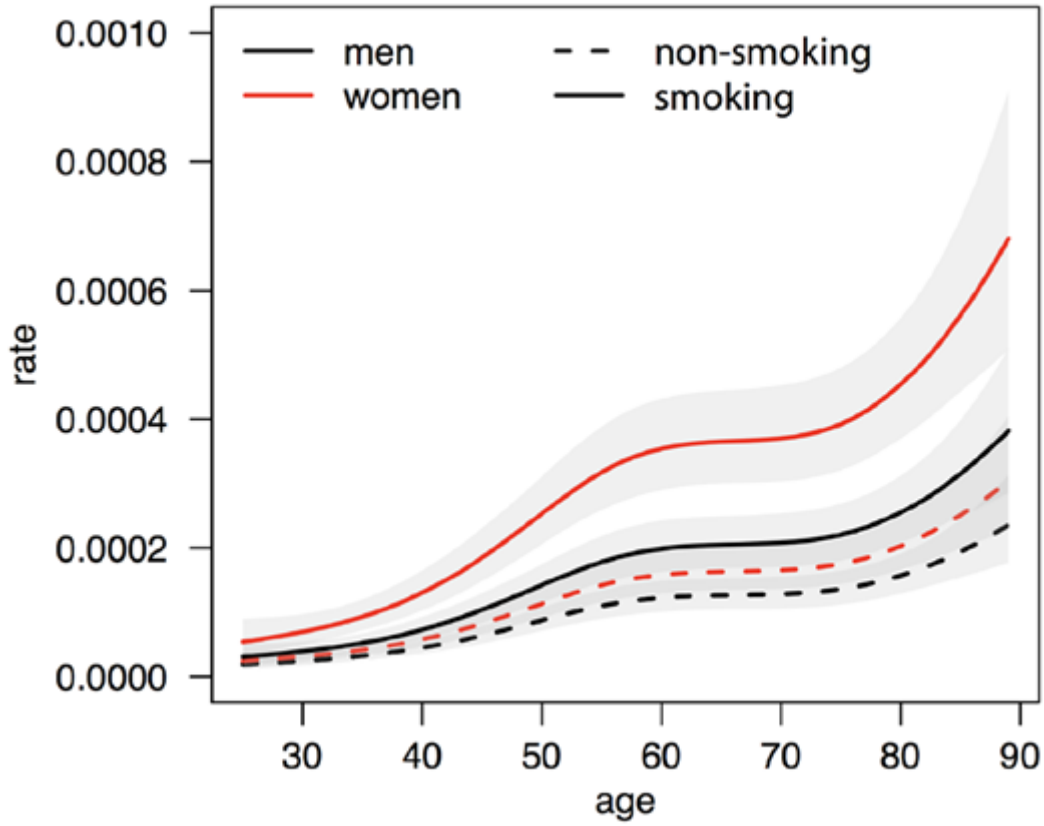
When performing the same analysis in the longitudinal studies, a relative risk of 2.2 (CI 1.3-3.6) was observed overall and individually the relative risks were similar for both sexes; in males the relative risk calculated was 2.2 (CI 1.7-3.0) and 2.2 (CI 1.7-2.8) in females.

The Asia Pacifica Cohort Studies Collaboration demonstrated a hazard ratio of 2.4 (CI 1.8-3.4) existed when comparing current smokers against ex-smokers or non-smokers. In western Caucasian populations, the prevalence of smoking is 45-47% of SAH patients. Forty-percent (40%) of aneurysmal SAH cases have a history of smoking.[27, 37-39]

In a recent study in a Swedish population[40] of 950,000 adults, they demonstrated increased risk of SAH when smoking and gender were combined showing an interaction. Female smokers had a 2.2 fold

increased risk of SAH compared to female non-smokers and male smokers had a 62% higher risk of SAH than no-smoking males (see figure 1-6).

Figure 1- 6 – Incidence of SAH by smoking and gender status. Adapted from Sundstrom et al.[40]



Smoking status in unruptured aneurysm cases is an independent risk factor for aneurysmal rupture, growth and formation. One study assessed smoking status as a time-dependent covariate in predicting aneurysm rupture risk and found a relative risk of 3.0 [95% CI, 1.2-7.7].[41] Another prospective follow-up study demonstrated smoking to affect aneurysm formation and growth rate, particularly in women, leading to hastened growth and increased prevalence of rupture.[37, 42, 43]

Cigarette smoking has also been shown to affect growth progression of previously unruptured aneurysms, independent of other risk factors. In one study, it was shown that aneurysmal diameter increases by 0.5 mm per year on the background of smoking.[42] However, smoking has also been shown strongly associated with the occurrence of unruptured intracranial aneurysms (UIAs) independently in genetic association studies. In recent genetic studies, 2 candidate SNPs (single nucleotide polymorphisms); rs10757278 on chromosomes 9p and rs10958409 on 8q with previously known associations with IA development were tested in a Caucasian population [44-47] using a logistic regression model on the effect of smoking and IA incidence. The odds ratio of developing an IA in patients with the rs10757278 risk allele in non-smokers was OR 1.40 [1.10-1.78, p=0.007], whereas in patients with a 20 pack-years history, their OR was 5.75 [3.99-8.29, p<0.001]. For the rs10958409 SNP, non-smokers

had an OR 1.48 [1.08-2.07, p=0.023] whereas smokers had on OR 5.04 [3.50-7.61, p<0.001] of developing an IA.
Following treatment of an aneurysm, smoking has also been shown to increase the risk of *de novo* aneurysm development with an odds ratio of 4.[48, 49]

Despite no direct link between smoking and aneurysm formation or growth, many potential mechanisms have been cited for causation. Degradation of elastin due to raised elastase activity has been proposed to affect cerebral vasculature. This could occur in one of two ways; firstly, smoking can cause local inflammation with the migration of granulocytes, which can release elastase or, secondly, inhibition or inactivity of alpha-1 antitrypsin which would lead to a rise in elastase levels.[50, 51]
Haemodynamic effects of smoking include increasing blood viscosity by elevated levels of fibrinogen[52] and raising blood pressure as a result of nicotine stimulation causing catecholamine release, thereby increasing haemodynamic stress. Interestingly, transient hypertension has been linked with aneurysm rupture[53] and has previously been shown to elevate blood pressure 2-3 hours following smoking.[38]

Hypertension
Hypertension is one of the strongest risk factors for aneurysmal SAH with 20-45% of cases having a positive history of hypertension, which is higher than the general population, following adjustment for other well-associated risk factors with SAH.[39, 43] However, interpretation of this association has been complicated by lack of a universal definition. The Asia Pacific Cohort of Studies Collaboration[54] divided their analysis into continuous and dichotomous (Systolic BP<140 mmHg or ≥140 mmHg) categories. The continuous variable analysis showed that with each 10-mmHg increase in SBP there was a 31% [CI 23-38] increased risk of SAH, whilst a hazard ratio of 2.0 (95% CI 1.5-2.7) existed in patients who had a systolic blood pressure >140 mmHg.

In a more recent systematic review of BP association with SAH, Feigin *et al* [35] categorised patients hypertensive or non-hypertensive as declared by their status upon admission to a neurosurgical unit, without a defined cut-off BP being reported. They split their analysis by reporting relative risks for longitudinal and odds ratios for case-control studies respectively. It was noted that SAH patients who were hypertensive had a significantly higher risk of SAH with a relative risk of 2.5 [95% CI 2.0 - 3.1]. They performed a bivariate analysis comparing the relative risk of both sexes. In females, there was a higher relative risk of 3.3 [CI 2.1 - 5.3] compared with males 2.3 [CI 1.8 - 3.0] in the hypertensive group. The same group confirmed their findings when assessing case-control studies. They found odds ratios of 3.3 [CI 2.6 -4 .3] in females compared with 2.1 [CI 1.4 - 3.2] in male hypertensive SAH cases. The increased incidence of SAH in hypertensive patients may reflect an increased risk of aneurysm formation[41] and the possibility that use of anti-hypertensive medication reduces the risk of rupture.[39, 55]
48

Controversy exists whether hypertension affects aneurysm growth or *de novo* aneurysm formation. In one long-term cohort study[42] assessing previous SAH patients with the presence of hypertension or not, there was no difference between the groups for *de novo* aneurysms. However, most of the hypertensive group used anti-hypertensive medication and this may have affected aneurysm growth or formation. Despite this finding, another follow-up study[48] showed, hypertension detected at the time of initial SAH was associated with *de novo* aneurysm formation.

Despite observational evidence supporting a role of hypertension in the development of ruptured or unruptured aneurysms, no clear biochemical or pathological link has yet been established. Certain mechanisms related to hypertension have been proposed as causal with aneurysm formation, these include; disturbance in the synthesis of extracellular matrix proteins by smooth muscle cells, such as collagen and elastin, occlusion of the vasa vasorum or direct endothelial damage from the haemodynamic effects of raised arterial pressure.

Alcohol consumption

Alcohol intake as a risk factor for intracranial aneurysm development has not been as well-quantified compared to smoking or hypertension. There are reports which suggest that increased alcohol consumption increases the risk of rupture rather than affect growth and development of intracranial aneurysms [38, 41, 42]. The Asia Pacific Cohort of Studies Collaboration[54] review split patients into current and previous alcohol consumers. In current alcohol consumers who developed SAH, they calculated a hazard ratio of 1.0 [0.7-1.4]. In a view to quantify alcohol consumption, the Feigin[35] review dichotomised alcohol intake into those drinking less than 150g/week and those consuming >150g/week. The results from this review suggested that there was an increased relative risk of SAH in those patients consuming >150g/week of alcohol, RR 2.1 [1.5-2.8] compared with 1.1 [0.8-1.6] in those drinking less than 150g/week. When analysing difference between gender for incidence of SAH, females had an OR of 5.0 [1.9-14.3] and males 4.5 [2.5-8.0] in comparison to those patients who drank more than 150g/week with non-drinkers. The duration of alcohol consumption has also been cited to be crucial in aneurysmal rupture. In one study, short-term alcohol intake i.e. a few days before SAH was associated with rupture of an intracranial aneurysm compared with those patients who drank moderate-heavy amounts over a longer duration.[38, 56]

1.1.4 Non-modifiable risk factors associated with intracranial aneurysms and rupture

Gender

The incidence of SAH is higher in women than in men, but this sex difference emerges later than 50 years or older.[22] In addition, the prevalence of aneurysms is higher in women and is most pronounced in persons 50 years of age.[7] The difference of incidence between genders, is not explained by increased prevalence of the modifiable risk factors seen in males, including; smoking, excessive use of alcohol, and hypertension.[35]

Family History: potential genetic contribution to intracranial aneurysms

Between 5-10% of aneurysmal SAH cases have a positive family history in their first-degree relatives, providing up to 7 times greater risk of SAH. A prevalence of 9.5% [CI 7.0-12] was reported in a previous study by Rinkel *et al*.[1] The relative risk for patients with a family history compared to a reference population of patients with atherosclerosis or pituitary adenomas were 4.0 [CI 2.7-6.0]. Interestingly, in the same study, 40% of ADPKD patients had a positive family history of SAH or intracranial aneurysms in first or second-degree relatives compared with 14% of patients without ADPKD, suggesting a familial link.[57] In a recent meta-analysis by Vlak[7] *et al*, they found the prevalence ratio of unruptured intracranial aneurysms to be 3.6 [0.4-30] in patients with a positive family history of an intracranial aneurysm or aneurysmal SAH.

Multiple studies have reported an increased incidence of unruptured intracranial aneurysms in first and second-degree relatives of familial and sporadic cases.[58] A crude prevalence of 8.7% of intracranial aneurysm cases among 438 first-degree relatives with ≥ 2 intracranial aneurysms present was found in a study by Ronkainen [59]. In the FIA study,[60] they estimated a prevalence of 19% of unruptured intracranial aneurysms in first degree relatives where at least 2 cases of intracranial aneurysms previously existed. Those patients screened in this study were all over the age of 30; had no previous diagnosis of intracranial aneurysms, and had a history of smoking and hypertension.

Brown demonstrated an important point in terms of the debate about whether to screen individuals with a positive family history for intracranial aneurysms, by effectively examining those individuals at a higher predisposition for aneurysmal development as dictated by the presence of hypertension and smoking.

The prevalence of intracranial aneurysms described in studies based on family history give an inflated prevalence than that of sporadic patients, with a history of a first-degree relative being affected, estimated at 2.3%.[61] However, when examining sporadic cases of SAH, large screening studies demonstrated a prevalence rate of 4% in first-degree relatives of those affected by aneurysmal SAH.[62, 63]

Most familial aneurysms appear to be smaller than 6 mm and occur usually in the regions of the MCA (28%) and ICA bifurcation (44%), which is typical of unruptured intracranial aneurysms distribution.[63, 64] However at time of rupture, familial aneurysms appear larger than sporadic cases.[65] In addition, there appears to be increased frequency of multiplicity of aneurysms in those with familial SAH, 26% in familial cases versus 10% in sporadic cases (RR 2.5 CI 1.2-5.4).[61] In the study by Brown, they reported a frequency of 17.6% in familial cases. Despite the reported raised prevalence in familial intracranial aneurysm disease, these studies are not robust enough; and will need greater recruitment numbers of affected families; and recruitment of relatives of deceased patients who suffered with intracranial aneurysms disease, such as children, siblings and spouses; as well as better risk factor/lifestyle stratification prior to any screening policy being implemented.

In the largest twin study on SAH, the Nordic twin study[66] examined 160,000 twin siblings in Danish, Finnish, and Swedish populations. They recorded an incidence of 8.47 cases per 100,000 follow-up years. Six twin pairs (five monozygotic and 1 opposite sex) were concordant and 492 were discordant for SAH out of 504 pairwise samples. The concordance in monozygotic twins was 3.1% compared with 0.27% in dizygotic twins suffering SAH. The population attributable risk for SAH in monozygotic twins was 5.9% compared with 0.54% in dizygotic pairs. This demonstrated a modest genetic effect on SAH incidence. However, despite this conclusion, the Nordic study did not assess the effect of environmental risk factors on overall disease phenotype, including on the presence of unruptured intracranial aneurysms.

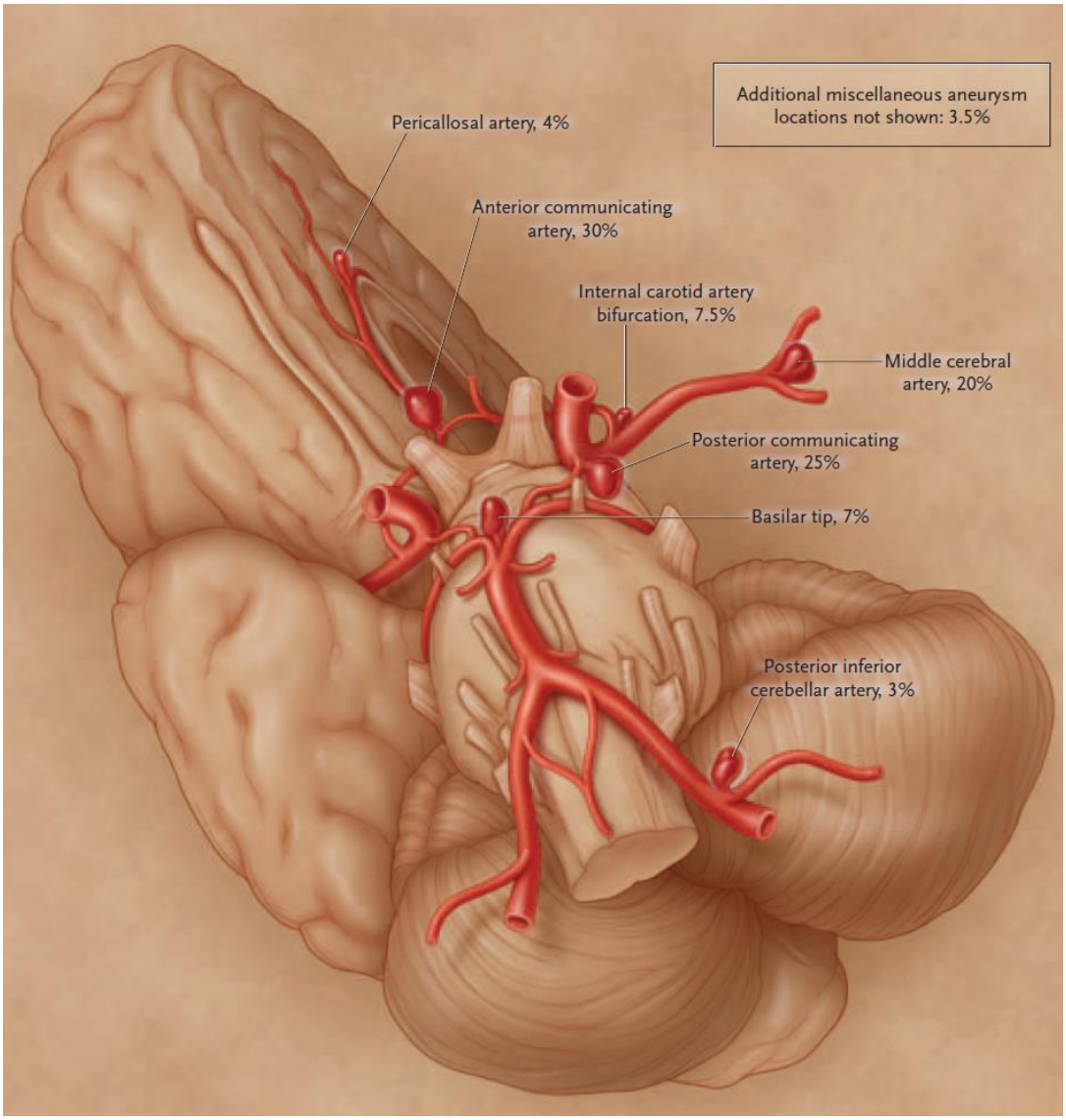
Recently, attention has turned to genetic factors as a possible cause(s) of this cerebral aneurysms. Intracranial aneurysms are complex by nature and it is generally accepted that, like many complex human disease traits the expression of this condition is the result of interaction between the environment and exposure to certain genetic risk factors with low-modest effect.[36] This led initially to hypothesis-driven research looking into plausible candidate genes involved in arterial wall biology that could conceivably cause aneurysm formation or rupture. Advance in genetic research subsequently allowed scanning of the whole genome to detect previously unsuspected genetic variants.

1.1.5 Aneurysm site, size, pathogenesis and prediction of rupture risk

In light of increasing access to neuroimaging modalities like, CT or MRI, increasing numbers of patients are being diagnosed with incidentally found intracranial aneurysms, that otherwise would not have been found. This has forced neurosurgeons and neuro-interventionists alike to weigh up the risks and benefits of offering treatment to those with unruptured aneurysms.

With the high mortality associated with SAH, development and rupture of intracranial aneurysms are very difficult to predict, which has led to a dilemma of whether to treat incidental or additional unruptured aneurysms earlier or place aneurysms under surveillance. In previous years, attempts have been made to predict, if aneurysms at a particular site or given size are more prone to rupture and if so, what is the annual rupture rate? The majority of aneurysms form in the anterior circulation, approximately 80-85% (see figure 1-7). They are typically found at branching sites along the Circle of Willis, where it is thought that higher levels of haemodynamic stress are present, either predisposing to or initiating intracranial aneurysm formation. Understanding the pathogenesis of aneurysmal development and their interaction with environmental and genetic factors is key in being able to better predict which phenotype of patients are at increased risk of intracranial aneurysm development or rupture.

Figure 1- 7- Distributions of Intracranial aneurysms. Adapted from Brisman et al.[16]



Studies prior to ISUIA

Rinkel[1] and Morita[54] performed a systematic review on all unruptured intracranial aneurysms based upon site and size in an attempt to calculate the relative and annual risk of aneurysm rupture. Using anterior communicating artery aneurysms as the reference point, one group found an annual rupture rate of 1.1% [0.4-2.5] for ACOM aneurysms, for MCA aneurysms the rupture rate was 1.1% [0.7-1.8] again with a relative risk (RR) of 1.0 [0.4-2.8]. For internal carotid aneurysms the annual rupture rate was 1.2% [0.8-1.7] providing a relative risk of 1.1 [0.4-2.9]. Finally, for the posterior circulation aneurysms, the annual rupture rate was highest at 4.4% [2.7-6.8] with a relative risk of rupture of 4.1 [1.5-11].

Morita et al[67] reviewed Japanese patients with unruptured intracranial aneurysms and categorised patients into having anterior and poster circulation aneurysms. They had similar findings to the Rinkel group, with anterior circulation aneurysms having an annual risk of rupture of 1.8% [1.3-2.3] compared

with 3.2% [2.0-5.0] in the posterior circulation. The relative risk of rupture for posterior compared to anterior circulation aneurysms was 2.3% [1.4-3.7]. However, posterior (vertebrobasilar) circulation aneurysms account for 10% of all aneurysms which would inflate the estimated rupture risk. In both the Rinkel[1] paper and ISUIA study, Posterior Communicating artery Aneurysms (PCOM) were placed in the posterior circulation group and not in the anterior circulation as should be the case, which again will lead to inflation of rupture risk.

Prediction of rupture risk based upon the size of unruptured intracranial aneurysms was also reviewed by the Morita and Rinkel. They dichotomized patients into those with < 10 mm and ≥ 10 mm aneurysms. Both studies demonstrated larger aneurysms (≥ 10 mm) to have a higher annual rupture risk with 4% [2.7-5.8] versus 0.7% [0.5-1.0] and a relative risk of rupture of 5.5% [3.3-9.5]. The Morita cohort revealed an annual rupture risk in larger aneurysms of 9.3% [6.4-13.1] versus 1.5% [1.0-2.2] in aneurysms less than 10 mm with a relative risk > 10 mm aneurysm rupture of 6.4 [4.0-10.4]. In these studies, the contribution of significantly associated risk factors for aneurysm development was not tested to check their contribution to overall aneurysm rupture risk.

The ISUIA (International Study on Unruptured Intracranial Aneurysms): prognosis for rupture risk of unruptured aneurysms[64]

ISUIA was a large observational study attempting to determine the natural history of unruptured aneurysms and outcome following definitive aneurysm treatment; either by neurosurgical clipping or coiling.[64, 68] This reopened the debate on how best to manage unruptured intracranial aneurysms. Two publications were released by the group; one in 1998;[68] and the other in 2003.[64] The first publication concluded that aneurysms less than 1cm had a low rupture rate of 0.05% per year, those greater than 1cm had a higher likelihood of rupture between 0.5%-2.5%. Despite the predictably higher rupture rate in larger aneurysms, it was felt this group represented a disproportionate number of high-risk phenotypes, as they were selected on basis of having suffered a previous SAH. The outcomes, principally clipping were worse with the suggestion that risks of intervention in aneurysms; less than 1cm or in patients aged greater than 50 years, outweighed the benefits. Following this initial report, publications appeared citing better surgical outcomes.[69-71]

The results from the second publication by ISUIA were published in 2003 (see table 1-2). The size bands of aneurysms were changed from the previous study. The results from the 2nd paper was in broad agreement with those in the 1st except the conclusion from the 1st paper that aneurysms less than 10 mm were safe did not match with the second paper, as SAH was noted in the 7-9 mm range. Significantly, aneurysms of the PCOM had a higher risk of rupture which was in agreement with earlier findings of Rinkel[1] and Morita[67]. This should be interpreted with caution as the ISUIA placed PCOM artery aneurysms in the posterior (vertebrobasilar) instead of the anterior circulation aneurysm group, which

would have been correct and provided a rupture rate of 0.1% per year, but by being in the posterior circulation group the risk of rupture rises to 0.5%, which would justify treatment.

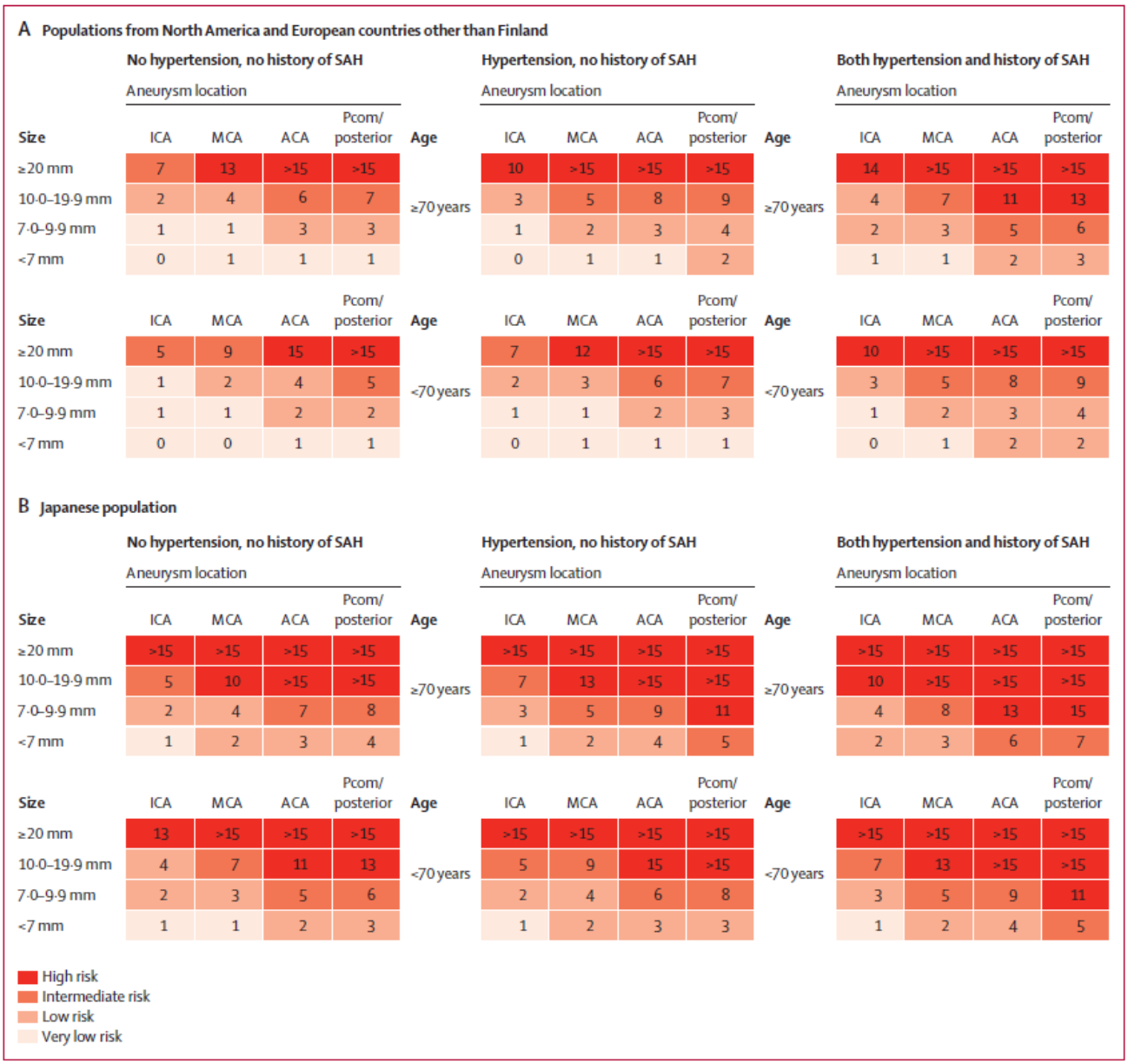
Table 1- 2 - Summary of results from the ISUIA study 2003[64]

Annual rupture risk of unruptured intracranial aneurysms		
Aneurysm size range (mm)	**Incidental (%)**	**Additional aneurysms (%)**
<7	0.1	0.4
7-12	1.5	0.8
13-24	2.7	1.2
>24	5.3	-

The PHASES STUDY[72]

Recently an international collaboration pooled their unruptured aneurysm follow-up data in an attempt to better predict aneurysm rupture. They used a set of six routinely collected risk factors to calculate rupture risk. These predictors included; age, hypertension status, previous history of subarachnoid haemorrhage, aneurysm size, location and geographical region. Other risk factor data including, sex, smoking status and the presence of multiple aneurysms did not impact significantly on the risk of aneurysm rupture. Pooled data to develop this tool came from 6 previous studies[64, 73-76] that followed up the natural history of unruptured aneurysm cases (see figures 1-8 and 1-9).

Figure 1- 8 – Adapted from Greving et al.[72]



A Populations from North America and European countries other than Finland

Size	No hypertension, no history of SAH — Aneurysm location: ICA	MCA	ACA	Pcom/ posterior	Age	Hypertension, no history of SAH — Aneurysm location: ICA	MCA	ACA	Pcom/ posterior	Age	Both hypertension and history of SAH — Aneurysm location: ICA	MCA	ACA	Pcom/ posterior
≥20 mm	7	13	>15	>15	≥70 years	10	>15	>15	>15	≥70 years	14	>15	>15	>15
10·0–19·9 mm	2	4	6	7		3	5	8	9		4	7	11	13
7·0–9·9 mm	1	1	3	3		1	2	3	4		2	3	5	6
<7 mm	0	1	1	1		0	1	1	2		1	1	2	3
Size	ICA	MCA	ACA	Pcom/ posterior	**Age**	ICA	MCA	ACA	Pcom/ posterior	**Age**	ICA	MCA	ACA	Pcom/ posterior
≥20 mm	5	9	15	>15	<70 years	7	12	>15	>15	<70 years	10	>15	>15	>15
10·0–19·9 mm	1	2	4	5		2	3	6	7		3	5	8	9
7·0–9·9 mm	1	1	2	2		1	1	2	3		1	2	3	4
<7 mm	0	0	1	1		0	1	1	1		0	1	2	2

B Japanese population

Size	No hypertension, no history of SAH — Aneurysm location: ICA	MCA	ACA	Pcom/ posterior	Age	Hypertension, no history of SAH — Aneurysm location: ICA	MCA	ACA	Pcom/ posterior	Age	Both hypertension and history of SAH — Aneurysm location: ICA	MCA	ACA	Pcom/ posterior
≥20 mm	>15	>15	>15	>15	≥70 years	>15	>15	>15	>15	≥70 years	>15	>15	>15	>15
10·0–19·9 mm	5	10	>15	>15		7	13	>15	>15		10	>15	>15	>15
7·0–9·9 mm	2	4	7	8		3	5	9	11		4	8	13	15
<7 mm	1	2	3	4		1	2	4	5		2	3	6	7
Size	ICA	MCA	ACA	Pcom/ posterior	**Age**	ICA	MCA	ACA	Pcom/ posterior	**Age**	ICA	MCA	ACA	Pcom/ posterior
≥20 mm	13	>15	>15	>15	<70 years	>15	>15	>15	>15	<70 years	>15	>15	>15	>15
10·0–19·9 mm	4	7	11	13		5	9	15	>15		7	13	>15	>15
7·0–9·9 mm	2	3	5	6		2	4	6	8		3	5	9	11
<7 mm	1	1	2	3		1	2	3	3		1	2	4	5

High risk
Intermediate risk
Low risk
Very low risk

Figure 1- 9 -Adapted from Greving et al.[72]

PHASES aneurysm risk score	Points
(P) Population	
North American, European (other than Finnish)	0
Japanese	3
Finnish	5
(H) Hypertension	
No	0
Yes	1
(A) Age	
<70 years	0
≥70 years	1
(S) Size of aneurysm	
<7·0 mm	0
7·0–9·9 mm	3
10·0–19·9 mm	6
≥20 mm	10
(E) Earlier SAH from another aneurysm	
No	0
Yes	1
(S) Site of aneurysm	
ICA	0
MCA	2
ACA/Pcom/posterior	4

To calculate the PHASES risk score for an individual, the number of points associated with each indicator can be added up to obtain the total risk score. For example, a 55-year-old North American man with no hypertension, no previous SAH, and a medium-sized (8 mm) posterior circulation aneurysm will have a risk score of 0+0+0+3+0+4=7 points. According to figure 3, this score corresponds to a 5-year risk of rupture of 2·4%. SAH=subarachnoid haemorrhage. ICA=internal carotid artery. MCA=middle cerebral artery. ACA=anterior cerebral arteries (including the anterior cerebral artery, anterior communicating artery, and pericallosal artery). Pcom=posterior communicating artery. posterior=posterior circulation (including the vertebral artery, basilar artery, cerebellar arteries, and posterior cerebral artery).

1.1.6 Pathophysiology and biology of intracranial aneurysms

Current knowledge on IA formation is very limited; the specific pathophysiological lesions that cause IA initiation, growth and rupture are not fully understood. Many theories describe an imbalance of haemodynamic forces, inflammatory processes and vascular remodelling as possible reasons for the development of IA.

Intracranial aneurysms are arterial lesions defined by thinned and dilated regions of the vessel wall, which exhibit loss of the internal elastic lamina, tunica media thinning and subsequent degradation and remodelling of the extracellular matrix (ECM) proteins throughout the affected part of the vessel wall.[77] Different morphology of aneurysms exist, these include; saccular, fusiform. infective and dissecting. Fusiform aneurysms tend to bulge on all sides of a vessel wall, secondary to atherosclerosis. Infective aneurysms usually occur in cases of sepsis, particularly infective endocarditis and can resemble saccular aneurysms but are usually located more distally within the arterial tree. Dissecting aneurysms result

from a tear in the intimal layer, usually due to trauma and blood enters, causing progressive dilatation at the site of injury. For the purpose of this study, we are concentrating on saccular 'berry' aneurysms of the cerebral circulation. These usually occur at branch points and are berry like in their dilatation on one side of the vessel. Due to the unique configuration of the Circle of Willis aneurysms consistently occur at arterial bifurcations; such as the Basilar and ACOM arteries.[77-79] These areas are particularly prone to changes in haemodynamic patterns that could lead to intracranial aneurysm development.

Wall shear stress (WSS) and hydrostatic transmural pressure are two major theories that have been regularly tested in aneurysmal development. These will be reviewed in turn.

1.1.6.1 *Wall Shear Stress*

Due to the high flow and small radii of cerebral vessels, these vessels are exposed to higher WSS than other vascular beds. Localised dilatation by either physical damage or ECM degradation can occur pathologically due to long-term raised WSS. It is postulated that ECM degradation and smooth muscle apoptosis occur in response to vascular endothelium triggering a biochemical cascade. Insults such as; chronic hypertension, smoking or cocaine abuse leads to oxidative stress in surrounding cerebral tissue, which may impair endothelial cell function, thereby not being able to reduce WSS to maintain homoeostasis. It has not been possible to quantify the direct effects of WSS on human models and the majority of studies investigating this effect have been conducted on non-biological models. Mantha[80] *et al* used patient angiographic data to examine WSS in virtual vessels containing sidewall aneurysms with virtual reconstructions of the same vessels using techniques of computational fluid dynamics.[80] They found WSS to be low prior to aneurysm initiation, representing a stagnation zone in the parent vessel which potentially nullifies random force vectors in each direction. These stagnation zones would promote leukocyte migration and adhesion, thereby potentially initiating immune and inflammatory cascades that breakdown the ECM and vascular smooth muscle cells (VSMCs).[81] It was also observed, that WSS was reversed during different phases in the cardiac cycle. From this experimentation, it was hypothesised that this reversal could generate pathological triggers initiating the first steps in sidewall aneurysm formation.

At arterial bifurcations within the Circle of Willis, high WSS has been noted which could lead to aneurysm development. The parent vessel radius and bifurcation angle in these areas dictate the severity of WSS. Blood flow imbalance at these bifurcations could trigger a cascade of cellular reactions which could lead to vascular remodelling, even under normal physiological conditions.[81-86] Fukuda *et al* demonstrated that Nitric Oxide inhibition via nitric oxide synthase during conditions of high WSS could suppress aneurysm formation,[87] because high WSS inhibits VSMC (Vascular smooth muscle cell) proliferation through raised NO (Nitric Oxide) and transforming growth factor-beta (TGFβ) levels, which can give rise to aneurysm formation by allowing physical forces to locally dilate an artery.

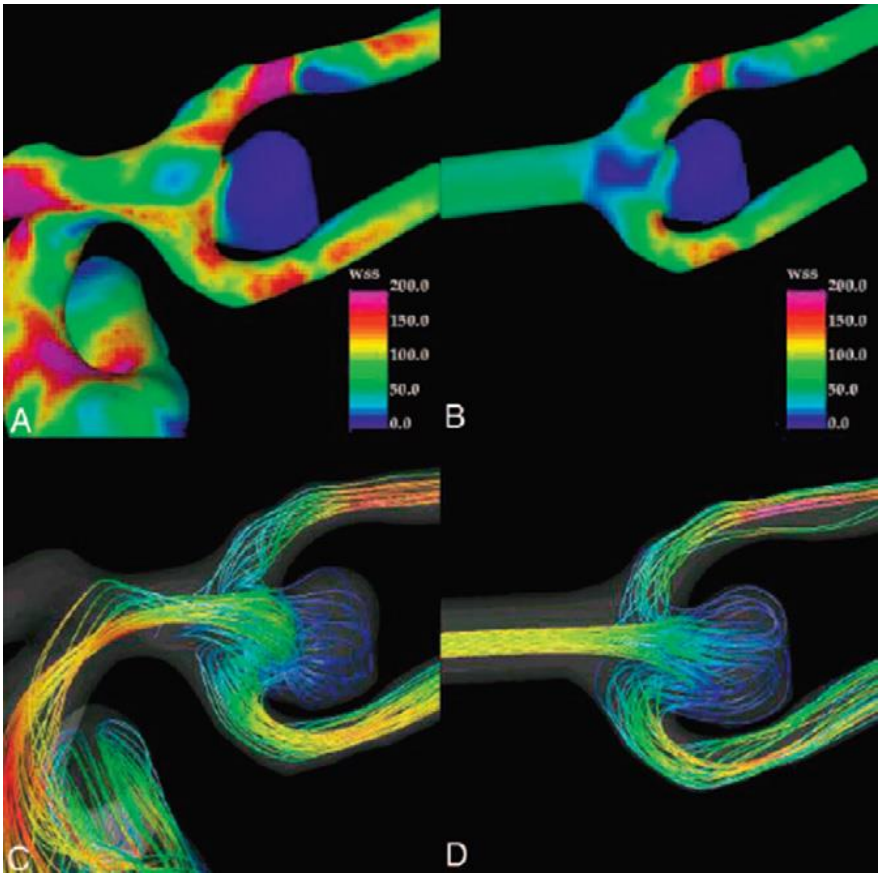
1.1.6.2 *Hydrostatic and transmural pressure*

Endothelin-1 is a well-known potent vasoconstrictor, its release is stimulated in response to mechanical stretch as a mechanism to normalise blood flow. Also, activation of the endothelin-1 B (EDRB) receptor promotes apoptosis of VSMCs. Cataruzza[88] and colleagues demonstrated in rats, that antagonism of the ETBR inhibits the development of intracranial aneurysms under conditions of increased pressure.[89] This could yet provide a pharmacological target in the future to treat the disease. Interestingly, in the most recent GWAS,[47] the SNP rs6841581 on chromosome 4q31.23 coding for the Endothelin receptor type A (EDNRA) was significantly associated with IA patients in Dutch, Finnish and Japanese populations. This variant may represent one aspect of aneurysm formation by triggering VSMC apoptosis and causing local vasoconstriction.

Once aneurysm formation has been initiated various haemodynamic patterns within the aneurysm neck and dome have been demonstrated giving rise to areas of high and low WSS. One study demonstrated a one-to-one linear relationship existed between systemic mean arterial pressure, mean arterial pressure in the parent vessel and intra-aneurysmal pressure. Therefore, with increasing mean arterial pressure, via the Law of LaPlace, this could potentially increase growth of the aneurysm.[90]

Shojima[91] *et al* showed high WSS and flow velocities present at the neck of the aneurysm and lowest at the tip of the aneurysmal dome (see figure 1-10).[83, 91]

Figure 1- 10 - Haemodynamic flow patterns observed in apical aneurysms occurring at the bifurcation of the middle cerebral artery

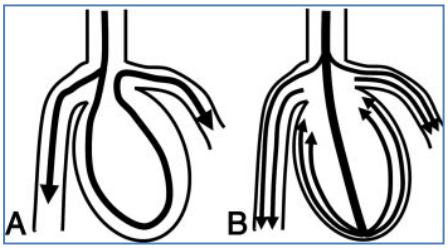


(A, B) areas of high wall sheer stress (WSS) around the neck of the aneurysm and in the distal vessels depicted by light spots, and the areas of low WSS in the dome of the aneurysm depicted by dark areas; and (C, D) the turbulent flow patterns in the dome. Adapted from Penn et al.[77]

The fact there is low WSS at the dome tilts the balance towards endothelial cascading reactions rather than direct physical forces as cause of aneurysm rupture. Earlier studies demonstrated that if a certain level of WSS is not present, then endothelial function can deteriorate which in turn will affect arterial integrity.[91]

In a canine model of intracranial aneurysms,[92] two distinct flow patterns were observed; these included the recirculation flow pattern, and jet flow pattern (see figure 1-11). The re-circulation pattern was associated with higher WSS but lowers intra-aneurysmal pressure as blood entered through one channel and left through a separate channel at the neck of the aneurysm. The jet flow pattern caused blood to enter directly into the aneurysm without hitting the neck and scattering back of the dome and out through many channels in the neck. The pressure gradients were higher in the jet flow pattern compared with the recirculation pattern. This may explain why some aneurysms tend to rupture and others less so.

Figure 1- 11– (A) Recirculation pattern and (B) Jet flow pattern in bifurcation aneurysms. Adapted from Moftakhar[92]



However, despite these theories on haemodynamic stress leading to aneurysm development, questions still remain, as why some aneurysms remain stagnant and do not grow or rupture over one's lifetime, particularly smaller aneurysms. Also, this does not explain why some smaller aneurysms rupture compared to some giant aneurysms. Histopathologically, some aneurysms demonstrate focal areas of wall thinning and propensity to rupture, whilst the rest of the aneurysm is thickened and fibrotic.

1.1.7 Outcome following aneurysmal subarachnoid haemorrhage

In the past three decades, the chances of a patient surviving aneurysmal SAH has increased by 17% to approximately 65%, despite the disease incidence being consistent.[93] This possibly reflects better awareness, prompt diagnosis and better/more treatment options available. However, what has remained stable are the two-thirds of patients who regain functional independence following SAH. Despite this, half of all SAH survivors have cognitive impairments with only a one third of survivors resuming the same level of work pre-SAH.[32, 94, 95] Various outcome scores have been applied to patients, in particular the Glasgow Outcome Scale (GOS) and modified Rankin scale (MRS), in proving an index of outcome following SAH. A few studies have shown that a mRs score of 0-3 (good outcome) varies between 36%-55% at 1-12 months after SAH.[8, 32] Other such predictors of outcome include the use of clinical – WFNS[96] and radiological – Fisher[97] scales. Clinical condition at admission is an important predictor of outcome, with 1 in 5 patients comatose on admission going on to have a full neurological recovery, and in those patients with no verbal or motor responses on admission; they have a 5% chance of regaining independent functions.[98] If patients are discharged to a nursing home due to poor recovery, one in three patients will recover independence within 2 years of being admitted there.

Cerebral vasospasm (CVS) is a well reported complication of SAH, leading to possible delayed neurological deficits (DINDs)/delayed cerebral ischaemia (DCI) in aneurysmal SAH patients.[99] CVS is thought to occur anywhere between 3-21 days post-ictus in approximately 11-33% of SAH patients, but usually peaks between days 4-14, with maximal onset at day 7. The incidence rates of vasospasm vary between 40%-70%.[100] CVS can be present without DINDs, even with severe arterial narrowing. Severe permanent neurological deficits from DCI can occur in up to 60% of SAH patients,[101] with fatality in approximately 7%.[102] Delayed cerebral ischaemia with radiographic vasospasm occurs in approximately 30%.[102] It appears that DINDs have reduced in incidence over the last 2 decades,

particularly with the increased use of calcium-channel antagonists such as, Nimodipine. Also, better fluid management to maintain adequate cerebral perfusion pressure and the use of radiological and clinical grades of SAH to accurately predict patients most likely to suffer from CVS.

Therefore, better prediction at admission of aneurysmal SAH patients likely to develop DCI could help clinicians plan treatment more intensively. Such predictors include genetic factors possibly causing some patients to be more susceptible to CVS/DIND. To date there is paucity of research in this field with anecdotal evidence existing in at least 2 candidate genes suggesting a possible link with CVS.[103, 104] However, this evidence pool is small and is hypothesis driven based upon initial testing of candidate genes on aneurysmal disease presence, further work would be required to test candidate SNPs in other populations or implement a non-hypothesis driven approach in using genome-wide association studies (GWAS) to detect novel genetic variants that could predispose aneurysmal SAH sufferers to a poor outcome.

The exact time period of when survivors can anticipate maximal recovery is contentious but in general with time post-discharge, quality of life improves. In one study, quality of life continued to improve between 5 and 12.5 years after the initial SAH, despite functional impairment.[105]

Chapter 2 – Overview of Intracranial aneurysm genetics

2.1 Literature Review on Intracranial Aneurysm Genetics

2.1.1 Genetic Studies on Intracranial Aneurysms

In the last decade since the mapping of the Human Genome,[106-108] there has been an increasing emphasis on identifying genetic factors involved with various diseases. Like most common complex vascular traits, intracranial aneurysm pathophysiology is complex and it is thought that no one particular gene is responsible for intracranial aneurysms, except for polycystin 1 in ADPKD. This has led to various approaches in trying to elucidate genetic factors that could be responsible or contribute to intracranial aneurysmal development. Intracranial aneurysms are a complex interplay of genetic and environmental factors affecting overall disease phenotype and it is important to appreciate this when planning any subsequent genetic association study.[109] Genetic association studies thus far, have failed to identify a single gene causing mutation that directly predisposes to intracranial aneurysms. However, the hypothesis remains, that multiple low risk SNP variants interplay with known environmental risk factors that may predispose a patient to the intracranial aneurysm phenotype.[45, 46]

As mentioned in chapter 1, modifiable risk factors for intracranial aneurysms include, smoking, hypertension and excessive alcohol intake, along with non-modifiable risk factors such as female gender and family history.

Between 5-10% of aneurysmal SAH cases have a positive family history in their first-degree relatives, providing up to 7 times greater risk of SAH. A prevalence of 9.5% (CI 7.0-12) was reported in a previous study by Rinkel *et al*.[1] The relative risk for patients with a family history compared to a reference population of patients with atherosclerosis or pituitary adenomas was 4.0 (CI 2.7-6.0). Interestingly, in the same study, 40% of ADPKD patients had a positive family history of SAH or intracranial aneurysms in first or second-degree relatives compared with 14% of patients without ADPKD, suggesting a familial link.[57]

Most familial aneurysms appear to be smaller than 6 mm and occur usually in the regions of the MCA (28%) and ICA bifurcation (44%), which is typical of unruptured intracranial aneurysms distribution.[63, 64] In addition, there appears to be increased frequency of multiplicity of aneurysms in those with familial SAH, 26% in familial cases versus 10% in sporadic cases (RR 2.5 CI 1.2-5.4) [61]. In the study by Brown, a frequency of 17.6% in was reported in familial cases.[60] Despite the raised prevalence in familial intracranial aneurysm cases, these studies are not robust enough; and will need greater recruitment of affected families; and recruitment of relatives of deceased patients who suffered with ruptured intracranial aneurysms. In a recent meta-analysis by Vlak[7] *et al*, they found the prevalence ratio of

unruptured intracranial aneurysms to be 3.6 [0.4-30] in patients with a positive family history of intracranial aneurysm or SAH.

Contrary evidence depicting a modest genetic effect on aneurysm development in familial cases was shown in the largest twin study on SAH, the Nordic twin study.[66] This study examined 160,000 twin siblings in Danish, Finnish and Swedish populations. They recorded an incidence of 8.47 cases per 100 000 follow-up years. Six twin pairs (five monozygotic and 1 opposite sex) were concordant and 492 were discordant for SAH out of 504 pairwise samples. The concordance in monozygotic twins was 3.1% compared with 0.27% in dizygotic twins suffering SAH. The population attributable risk for SAH in monozygotic twins was 5.9% compared with 0.54% in dizygotic pairs. This demonstrated a modest genetic effect and a larger effect of environmental factors on SAH incidence. However, despite this conclusion the Nordic study did not assess the effect of environmental risk factors on overall disease phenotype

However, despite this observational data suggesting a genetic component to intracranial aneurysms, researchers have tried to explain this link through genotyping familial and sporadic aneurysm cases, via genetic association studies.

Three major approaches have been employed including; candidate gene association studies, linkage-association and genome-wide association studies (GWAS).

2.1.2 Linkage Association studies

This type of genetic association study examines whole-genome material against DNA markers of known location, in affected families. Traditionally, the search for a disease-causing gene begins with linkage association studies. Linkage analysis determines approximate location of a disease gene relative to another DNA sequence called a genetic marker, whose position within the genome is already known. Linkage of intracranial aneurysm causing gene(s) with a specific DNA marker means the marker and the gene are located in a nearby chromosomal loci. This type of analysis is usually conducted in large family pedigrees, where intracranial aneurysms are known to have afflicted individuals. This type of study has its limitations in so far that; large families with SAH are rare; high case fatality rates; late onset; and low prevalence make finding suitable cases difficult. This can be further complicated by locus heterogeneity, which can reduce the analysis power. It is important to appreciate that once certain loci are found to be associated with intracranial aneurysms, knowledge about them and genes they herald are required to determine what the disease-causing variant is.

Several linkage-based analyses have been conducted mostly in Japanese and Caucasian populations[36, 61, 110-136] which have found certain chromosomal loci containing plausible candidate genes such as Elastin, Collagen type 1A2 and Versican (see figure 2-1). Usually if loci are significantly associated, a calculated LOD (logarithm of odds) score > 2 signifies this. The genes uncovered from these linkage

studies point to extracellular matrix genes involved in vascular repair and maintenance, which are crucial in maintaining vascular integrity, particularly at sites of vascular injury.

Figure 2- 1- Taken from Ruigrok, Stroke 2008.[61]

	Study Population (reference)	Genetic Loci	LOD or NPL Score	Potential Candidate Genes Within These Loci
Suggestive loci*				
1p	Single Northern-American family (20)	1p34.3–p36.13	LOD 4.2	Perlecan
	Single Dutch family (24)	1p36.11–p36.13	NPL 3.2	
5p	Single French- Canadian family (22)	5p15.2–14.3	LOD 3.6	
7q	Japanese sib-pairs (15)	7q11	NPL 3.2	Elastin, Collagen type 1 A2
	Northern-American families (17)	7q11	LOD 2.3	
11q	Single Northern-American family (21)	11q24–25	LOD 4.3	
14q	Single Northern-American family (21)	14q23–31	LOD 3.0	
	Japanese sib-pairs (15)	14q23–31	NPL 2.3	
19q	Finnish relative pairs (16)	19q13.3	LOD 2.6	
	Finnish sib pairs and relative pairs (19)	19q13.3	LOD 4.0	
	Japanese families (18)	19q13	NPL 2.2	
	Japanese families (23)	19q13.3	LOD 4.1	
Xp	Single Dutch family (24)	Xp22.2–p22.32	NPL 4.5	
	Japanese families (18)	Xp22	NPL 2.2	
	Finnish relative pairs (16)	Xp22	LOD 2.1	
Possible loci#				
5q	Japanese sib-pairs (15)	5q22–31	NPL 2.2	Versican
17 cen	Japanese families (18)	17 cen	NPL 3.0	TNFRSF13B

*loci with LOD scores >2.2 and nonparametric LOD (NPL) score >3.2.
#loci with LOD and NPL scores above 2 but under the levels for "suggestive linkage."

2.1.3 Candidate gene studies

This approach is hypothesis-driven involving the characterisation of genes based on their function(s), which are suspected to be involved in the pathophysiology of aneurysmal development. These studies are usually conducting following on from linkage studies to determine plausible gene associations from the discovered loci. Typically, association studies are carried out where an allele frequency (risk variant) is analysed between cases and matched-controls. If a particular allele is statistically more common in the patient group, it is said to be associated with that disease[36]. A drawback of this type of approach is that other genes possibly involved in the pathogenesis of aneurysms are overlooked, because focus was only on one *candidate* gene. Another hindrance, is that if a particular allele is associated with one ethnicity it may not be associated with another, this is a feature of all genetic studies, hence the requirement for examination of large numbers of cases, in different populations in order to achieve significant power. Certain genes examined in this approach include extracellular matrix (ECM) protein genes such as; Elastin, [36, 61, 110, 123, 126, 129, 137-141] Collagen,[36, 61, 137, 142-153] inflammatory mediator genes

such as, IL-6 and TNF-alpha[109, 141, 154-156] and vascular endothelial factors such as the ACE gene (please see chapter 3 on systematic review).

Due to the potential of missing unknown genes involved in IA development, this has driven genetic association studies which utilise a non-hypothesis approach; as is the case with linkage and GWAS studies.

2.1.4 Genome-Wide Association Studies (GWAS)

In recent years (2008 -) focus has shifted on another non-hypothesis approach to look for genetic associations with intracranial aneurysms. GWAS studies utilise similar principles as seen with linkage analysis but this allows for scanning the entire genome in unrelated individuals or sporadic cases. This has the advantage of finding novel variants or SNPs whose involvement was previously unknown in intracranial aneurysm disease. This approach allows greater precision of where risk mutations may be present and whether one or more variants contribute to overall disease phenotype. However, to perform such studies, a large number of cases and controls are required to achieve statistical power or GWAS level of significance ($<5x10^{8}$), hence power calculations need to be accurate prior to planning any new project. Also, GWAS chips are very expensive and different chips have different resolutions in that some may scan for 500,000 variants and others over 1 million. Significance level is set very high as many variants may be associated with a particular disease phenotype at 95% confidence interval, to ensure only those with the strongest association are chosen and to allow for multiple testing. Genome-wide association studies have 2 phases; the 1st phase examines the discovery cohort and any positive SNP associations seen in this batch are then checked in phase 2, in a replication cohort, to check if positively associated variants are indeed associated with the overall disease phenotype.

Six such GWA studies have been carried out examining association with intracranial aneurysms. These studies comprised of Japanese, Finnish and Dutch populations in a large international collaboration.[45-47] In total >20 new variant SNPs were found to be associated with intracranial aneurysm phenotype across all GWAS (see chapter 3 on systematic review and meta-analysis). The most significant association was seen on chromosomal loci 9p21.3 which harboured the rs1333040 SNP encoding the CDKNA2B gene involved in cell signalling. The exact mechanism of this variant remains to be fully understood, but interestingly this same loci has been repeatedly associated in patients with abdominal aortic aneurysms (AAA)[157] and patients with myocardial infarction and diabetes.[116] This gene could possibly be a vascular risk factor and predispose patients with cardiovascular or cerebrovascular disease.

2.2 Genetic Principles and considerations when designing a genetic association study

Genetic association studies test the correlation between genetic variants within genes against disease status. For reliable CGAS, certain conditions have to be met. Understanding the population and ancestry to be studied is paramount to limit population stratification. It is advisable to perform a genetic association study with similar ethnicities to the indigenous population to be studied, and additional ethnicities which have less numbers can be used as replication cohorts. However, by analysing them together in the same cohort, can lead to population stratification and make results less reliable. Therefore, it is important to match cases and controls by ethnicity, otherwise confounding can be introduced.[158] Significant sample size for the study in hand, should be determined early on, to reduce the the risk of false positive results. This is usually done by using a genetics power calculator, in this study we used to calculator created by Shaun Purcell at Harvard University.[159] When multiple testing is implemented, correction methods would need to be employed but this would mean greater numbers of cases and controls would be required to determine a modest effect.

Hardy-Weinberg Equilibrium (HWE)
Alleles must segregate randomly within a population, allowing expected genotype frequencies to be measured from the allele frequencies. This is known as HWE. Departure from HWE, may reflect;

1. Non-random mating, HWE requires random mating when testing any SNP for association. This can be violated for example, in consanguineous breeding.

2. Genotyping error, if there is a high rate of missingness from genotyping samples, this can distort HWE.

3. Multiple testing, if multiple SNPs within a gene are tested, correction for this must be applied to prevent the risk of false positives.

4. Admixturing within the main population can produce genetically distinct populations which do not share the same risk allele frequency in the general indigenous population.

Violations of any of these factors can cause departure from HWE, making genetic association studies unreliable.
The Hardy–Weinberg equilibrium, states that allele and genotype frequencies in a population will remain constant from generation to generation in the absence of other evolutionary influences. These influences include; mate choice, mutation, selection, genetic drift, gene flow and meiotic drive. Because

one or more of these influences are typically present in real populations, the Hardy–Weinberg principle describes an ideal condition against which the effects of these influences can be analysed.[158]

Linkage Disequilibrium

LD is where alleles at 2 or more different loci are non-randomly associated with each other. The measure of correlation between SNPs in the same genomic region is also known as linkage disequilibrium. It produces correlation over short distances of the genome, with other variants/SNPs that may be associated with the disease in question.[160]

The level of linkage disequilibrium is influenced by a number of factors, including genetic linkage, selection, recombination rate, the rate of mutation, genetic drift, non-random mating, and population structure.

2.3 Objectives

The primary question which our study will address, is whether previously studied candidate gene polymorphisms in other populations; are significantly associated in our UK-based cohort. The Genetic and Observational study of Subarachnoid Haemorrhage (GOSH), will investigate a large UK-wide clinical and genetic cohort of patients with sporadic intracranial aneurysm disease. We expect our data will not only confirm or refute previous genetic associations with the disease, in a UK population, but also investigate how genetic variants are linked to detailed phenotype information including aneurysm location, environmental risk factors, and clinical outcome. This will increase our understanding of aneurysm pathogenesis, and ultimately help predict rupture risk, in an attempt to reduce the burden of aneurysmal SAH.

The study will consist of:

1. A meta-analysis of all genetic association studies; whether CGAS or GWAS on intracranial aneurysms (see chapter 3).
2. A Candidate Gene Association Study (CGAS) including participants with intracranial aneurysms in a multicentre UK-wide cohort and Wellcome control DNA.
   Multi-variable analysis to assess interactions with known risk factors for intracranial aneurysms (smoking and hypertension)

Chapter 3 - Genetic risk factors for intracranial aneurysms: A meta-analysis in over 116,000 individuals

3.1 Abstract

Background: There is an urgent need to identify risk factors for sporadic intracranial aneurysm (IA) development and rupture. A genetic component has long been recognised, but firm conclusions have been elusive given the generally small sample sizes and lack of replication. Genome-wide association studies (GWAS) have overcome some limitations, but the number of robust genetic risk factors for intracranial aneurysms remains uncertain.

Methods: Comprehensive systematic review and meta-analysis of all genetic association studies (including GWAS) of sporadic IA, conducted according to STREGA and HuGENET guidelines. We tested the robustness of associations using random-effects and sensitivity analyses.

Results: Sixty-one studies including 32,887 IA cases and 83,683 controls were included. We identified 19 SNPs associated with IA. The strongest associations, robust to sensitivity analyses for statistical heterogeneity and ethnicity, were found for the following single nucleotide polymorphisms: on chromosome 9 within the cyclin-dependent kinase inhibitor gene CDKNA2BAS (rs10757278; OR 1.29 [1.21-1.38] and rs1333040; OR 1.24 [1.20- 1.29]), on chromosome 8 near the SOX-17 transcription regulator gene (rs9298506; OR 1.21 [1.15-1.27] and rs10958409; OR 1.19 [1.13-1.26]), and on chromosome 4 near the Endothelin receptor A gene (rs6841581; OR 1.22 [1.14-1.31]).

Conclusions: Our comprehensive meta-analysis confirms a substantial genetic contribution to sporadic IA, implicating multiple pathophysiological pathways, mainly relating to vascular endothelial maintenance. However, the limited data for IA compared to other complex diseases necessitates large-scale replication studies in a full spectrum of populations, with investigation of how genetic variants relate to phenotype (e.g. IA size, location and rupture status).

3.2 Introduction

Intracranial aneurysms (IA) are present in 2-5% of the general population; approximately 0.7-1.9% of cases rupture, causing subarachnoid haemorrhage (SAH).[1] The annual incidence of SAH is 8-9/100,000, yet because of the high risk of death or severe disability[32] and younger age of onset compared with other stroke types (40-65 years),[161] SAH has a disproportionately large socio-economic impact.

SAH has been associated with common modifiable risk factors including hypertension, smoking and alcohol intake.[34, 35, 39] However, a genetic component has long been recognised; for example, first-degree relatives of patients suffering with aneurysmal SAH have a four-to seven-fold increased risk of being affected compared with the general population.[58, 118] Candidate gene association studies (CGAS) of sporadic intracranial aneurysms have identified numerous potential risk loci, but these studies have been limited by small sample sizes and lack of replication. More recently, Genome-wide association studies (GWAS) have identified novel genetic loci strongly associated with intracranial aneurysms,[45-47, 162-164] but these still explain only a fraction of the genetic risk.

Thus, the number of true genetic risk factors, and the strength of their associations with intracranial aneurysms remains uncertain. The pooling of data from all genetic association studies allows risk estimates to be determined with more precision than is possible from any single study, and assessment of robustness to statistical heterogeneity and population ethnicity differences across studies. We therefore undertook a comprehensive systematic review and meta-analysis of all published CGAS and GWAS of sporadic intracranial aneurysms, including sensitivity analyses for statistical heterogeneity and ethnicity.

3.3 Methods

Our meta-analysis was conducted in a accordance with the HuGENet (Human Genome Epidemiology Network) guidelines,[165] and followed published recommendations to improve the quality of meta-analyses of genetic association studies.[166] We assessed the quality of reporting of genotyping based on the STREGA (Strengthening the Reporting of Genetic Association Studies) statement.[165]

3.3.1 *Search strategy*

Electronic databases; PubMed, EMBASE and Google Scholar were used to retrieve potentially relevant articles on human genetic studies of IAs and SAH that had been published up to December 2012. Search terms used under the Medical Subject Headings (MESH) were:
Aneurysm(s), intracranial aneurysm(s), subarachnoid haemorrhage, genetics, SNPs, Polymorphism, GWAS, Linkage and candidate gene(s).

Articles in all languages were searched (see Figure 3-1 for flow diagram of search strategy) and

translated as necessary. After retrieving relevant articles, references were also checked for other potentially relevant articles not found in the initial search.

3.3.2 *Selection Criteria*

We included cohort or case-control studies evaluating associations of genetic polymorphisms with proven intracranial aneurysms (using CT/MR angiograms or digital subtraction angiography) including unrelated individuals from candidate gene (CGAS) and genome-wide association studies (GWAS), in all ethnicities. Where duplicate datasets or cohorts existed, only the largest study was included. All SNPs evaluated in at least 2 published studies (CGAS or GWAS) were included in meta-analysis. Exclusion criteria were: history of inherited conditions associated with intracranial aneurysms, e.g. adult polycystic kidney disease (ADPKD) or Ehlers-Danlos syndrome.

3.3.3 *Data extraction*

Data was extracted by two of the authors (VSA, DJW) and differences resolved by consensus. Where available, genotype frequencies for each SNP were recorded. Where unavailable, we estimated the number of cases per genotype category using published information on risk allele frequencies and odds ratios for intracranial aneurysms.

Statistical Analysis

For each genetic polymorphism with more than one publication, meta-analysis was carried out to determine a pooled odds ratio (OR) and 95% confidence intervals (CI) using a fixed-effect model (Mantel-Haenszel method). Inter-study heterogeneity p-values (p_{Het}) were measured from Cochran's Q test and quantified using Higgins I^2 statistic. SNP associations showing significant inter-study heterogeneity ($p_{Het} < 0.05$ and $I^2 > 50\%$) were examined under a random-effects (Der Simonian and Laird) model, and, where appropriate, subjected to sensitivity analysis to determine the effect of individual studies on the pooled odds ratio. Ethnic subgroup analysis was also performed were appropriate.

All genetic models (dominant, recessive and additive) were assessed in studies recording genotype frequencies. Bonferroni ($p_{corrected}$) correction was applied for multiple testing ($p<0.01$). In studies not recording genotype frequencies, per-allele (additive) odds ratios were pooled with calculation of confidence intervals. Controls were checked to be in Hardy-Weinberg equilibrium for each study prior to meta-analysis.

Publication bias was assessed using the Egger regression asymmetry test and visualisation of funnel plots. Review Manager (RevMan)[167] version 5.2 from the Cochrane Collaboration, 2011 and Comprehensive Meta-Analysis (2.2) from Biostat™ were used to create the forest plots, perform sensitivity and publications bias analysis.

3.4 Results

Our search identified 642 articles of which 134 articles met initial inclusion criteria. Two studies published in Chinese were translated. After screening for duplication and eligibility, data from 66 case-control studies (60 candidate-gene and 6 GWAS) were included. Genotype and allele frequencies were reported in 48 publications; the remaining 18 reported additive odds ratios. In total, 32,887 cases and 83,683 control subjects were included, evaluating 41 polymorphisms in 29 genes (Figure 3-1). The studies encompassed mainly Caucasian European, Chinese and Japanese populations. Risk allele frequencies and control Hardy-Weinberg equilibrium status for each study of all SNPs meta-analysed are shown in Appendix I Table 1.

We identified 19 SNPs (from CGAS and GWAS) that were significantly associated with intracranial aneurysms in at least one genetic model (Table 3-1). The most robust associations were seen in 11 of 12 SNPs discovered from GWAS (Table 3-1), with the strongest associations for loci on chromosome 9 (rs1333040 and rs10757278), chromosome 8 (rs9298506 and rs10958409) and chromosome 4 (rs6841581) (Figure 3-2). Other GWAS loci variants associated with intracranial aneurysms included 9p21.3 (rs2891168), 2q33 (rs1429412 and rs700651), 7q13 (rs4628172), 12q22 (rs6538595) and 20p20.1(rs1132274) (Appendix I Table 1, Figures 1 and 2).

Eight SNPs from CGAS were significantly associated with intracranial aneurysms; SERPINA-3 (rs4934) and two variants relating to collagen genes (COL 1A2 [rs42524 G>C] and COL 3A1 [rs1800255 G>A]) showed the most robust associations. The remaining significantly associated CGAS SNPs included; HSPG2 (rs3767137), Versican (rs251124 and rs173686), ACE I/D and IL-6 G572C (See Table 3-1, Figure 3-3 and Appendix I Figure 2).

3.4.1 SNPs demonstrating statistical heterogeneity

Nine of the 19 significantly associated SNPs (SERPINA-3, ACE I/D, IL-6 G572C, CSPG2 - rs173686, rs1333040, rs700651, rs9298506, rs10958409, and rs4628172) demonstrated significant statistical heterogeneity; random effects and sensitivity analyses was only possible for the 7 variants reported in more than 2 publications (see Table 3-2). Following sensitivity analysis, the SERPINA-3, ACE I/D and IL-6 G572C SNPs (see Figure 3-3F) derived from CGAS, no longer showed an association with intracranial aneurysms.

3.4.2 Ethnicity Analysis

Nine SNPs (2 from CGAS, 7 from GWAS) were subjected to subgroup analysis to determine the effect of ethnicity (see Table 3-3 and Appendix I Figures 3-6). Associations with intracranial aneurysms in Japanese, Chinese and Caucasian populations were compared for each SNP, following which 5 of these

9 SNPs (all from GWAS) remained robustly associated with intracranial aneurysms in all groups (Table 3-3).

3.4.3 Publication Bias

Inspection of funnel plots and calculation of Egger p-values confirmed that none of the SNPs (CGAS or GWAS) associated with intracranial aneurysms (Table 3-1) demonstrated publication bias. Please see supplementary figures 7-11 for funnel plots of all SNPs (CGAS and GWAS) associated with IA.

Candidate-gene association studies not confirming associations with intracranial aneurysms

Twenty-two candidate SNPs reported in more than one study were not associated with IAs after meta-analysis (see Appendix I Table 2) including the following: eNOS VNTR 4a/4b, eNOS T786C, eNOS G894T, Endoglin intron 7 Wt/Ins, MMP-3 5A/6A, Elastin intron 20 T>C; Elastin intron 23 C>T, IL-1β C5111T, IL-6 G174C, PAI-1 4G/5G,GP IIIa A1/A2, Factor XIII valine/leucine; APOE ε2, ε4, COL4 1A C>T (rs3783107), Fibrillin-2 C>G (rs331079), MMP-2 C>T (rs243865), MMP-9 C1562T (rs3918242), COL 3A1 (rs2138533 C>T and rs11887092 A>G), rs10757272 C>T and rs11661542 A>C.

3.5 Discussion

This comprehensive systematic review and meta-analysis has identified 19 SNPs associated with intracranial aneurysms among 32,887 cases and 83,683 controls. The variety of genes implicated by these SNPs suggests that multiple pathophysiological pathways, mainly involved in vascular endothelial maintenance and extracellular matrix integrity, are likely to contribute to intracranial aneurysm development and rupture. The most robust SNPs identified arose from variants on chromosomes 4,8 and 9, derived from GWAS and confirmed by subsequent replication case-control studies.

3.5.1 Genetic variants associated with intracranial aneurysms identified by GWAS

3.5.1.1 SNPs with potential roles in vascular endothelial maintenance

The SNP rs1333040 located on chromosome 9p21.3 in the CDKNA2BAS (cyclin-dependent kinase inhibitor 2B antisense) gene was strongly associated with intracranial aneurysms. Two major linkage disequilibrium (LD) blocks exist within the 9p21.3 locus; one associated with vascular diseases, and the other associated with diabetes. This locus is associated with a wide spectrum of arterial disorders, including coronary artery disease and abdominal aortic aneurysms,[116] raising the possibility that there are as yet unidentified common processes involved in all these common cardiovascular pathologies. The CDKNA2BAS gene, on intron 12, is associated with cell cycle signalling, although its exact

biological roles are not fully understood.[45, 46] Association of the rs1333040 SNP with intracranial aneurysms was found to be independent of smoking and hypertension in one study.[168] In multivariable analysis, this SNP was preferentially associated with posterior communicating artery aneurysms, providing preliminary evidence that aneurysm site could be related to genetic factors.[168] However, larger cohorts will need to be studied in addition to scanning the entire region to check which SNPs are in linkage disequilibrium (LD) with the rs1333040-T allele and whether they have any cumulative (epistatic) effect on intracranial aneurysm phenotype including location.

The SNP rs10757278 on chromosome 9p21.3, located in a non-coding RNA region called ANRIL (Antisense noncoding RNA in the INK4 locus), showed robust association with intracranial aneurysms, but its function remains unclear. Moreover, the observed association of rs10757278 with intracranial aneurysm may arise from moderate linkage disequilibrium with the known risk variant rs1333040 SNP (r=0.59), from which it is approximately 41kbp upstream.[169] However, these two SNPs were not in strong LD in a Japanese population, raising the possibility of a smaller LD block associated with intracranial aneurysm. In a Japanese study,[168] haplotype analysis of both risk alleles of these SNPs, demonstrated significant association with IAs (OR 1.56 (1.32-1.85, $p=2.9x10^{-07}$). Functional studies show altered ANRIL expression as a result of variations within the 9p21.3 region,[164] with reduced expression of RNA encoded by CDKN2A and CDKN2B in mice;[164] these two genes, adjacent to the 9p21 region, encode tumour suppressor proteins (p14-16), which are involved in cell proliferation.[170] Moreover, p14 levels correlate with ANRIL activity and modulate matrix metalloproteinase-3 levels, which may influence extracellular matrix repair.[170]
In the most recent GWAS[164] in a Caucasian population of familial and sporadic IA, 6 SNPs, also located in the 9p21.3 region, were associated with intracranial aneurysms; one of these (rs6475606) achieved GWAS level statistical significance (OR 1.34, p-value=$4.29x10^{-07}$) for association with sporadic as well as familial intracranial aneurysms. Association with the rs1333040 SNP was also confirmed in this study,[14] further strengthening association of this locus with intracranial aneurysms.

Two SNPs on chromosome 8q (rs10958409 and rs9298506), surrounding the SOX-17 gene, were studied in European and Japanese populations; rs10958409 was associated in both populations, but the rs9298506 SNP was associated with intracranial aneurysm in the European but not Japanese population. SOX-17 plays an important role in generation and maintenance of stem cells of endothelial and haematopoietic lineages, crucial in the formation and maintenance of vascular endothelium.[171] Furthermore, SOX-17 knockout mice show multiple vascular abnormalities reflecting defective endothelial remodelling.[171] Defective stem cells involved in vascular maintenance are thus plausible targets in intracranial aneurysm pathogenesis.
The heterogeneity observed in the meta-analysis of these two SNPs may partly be explained by the difference in risk allele frequencies (RAF) in the Caucasian (see Appendix I table 1)[44] compared to the

other populations studied (rs10958409 – RAF 0.88 vs. ~0.2; rs9298506 – RAF 0.17 vs. ~0.80). Further support implicating chromosome 8 loci was shown in a recent GWAS,[164] in which a novel SNP, also in the region of the SOX-17 gene, was significantly associated with intracranial aneurysms (rs1072737 - OR 1.22 [1.07-1.39]), independent of smoking. For both SOX-17 loci, in the most recent GWAS,[164] smoking was an independent risk factor, acting in a multiplicative manner (chromosome 8 – rs1072737, OR 2.12 [1.76-2.56]) and chromosome 9 – rs6475606, OR 2.11[1.77-2.52]).

A further recent GWAS[47] found the SNP rs6841581 A>G on chromosome 4q31.23, coding for the Endothelin receptor type A (EDNRA) gene, was significantly associated with intracranial aneurysms in Dutch, Finnish and Japanese populations (OR 1.22 [1.14-1.31], $p=1.95x10^{-8}$). Another GWAS[163] in a Japanese population showed that the rs6842241 SNP, located at 4q31.22 near the EDNRA gene, was significantly associated with intracranial aneurysm (OR 1.25 [1.16-1.34], $p=9.58x10^{-09}$). EDNRA is a G-protein-coupled receptor for endothelins, which modulate vasoconstriction and vasodilatation following haemodynamic insult. Endothelin-1 (EDN-1) - the predominant isoform in vascular smooth-muscle cells - activates EDNRA. Endothelin signalling is activated at the site of vascular injury,[47, 172] causing cell proliferation; indeed, following subarachnoid haemorrhage, EDNRA and EDN1 protein levels are both raised in CSF.[173-175] Alternatively, down-regulation of EDNRA signalling could lead to defective vascular repair allowing aneurysms to develop following injury, a hypothesis supported by functional analysis of EDNRA variants showing lower transcriptional activity for the rs6841581 risk allele (G).[163]

SNPs with unknown function or not examined in more than two studies.

Five SNPs from GWAS do not correspond to genes with any known functions relevant to intracranial aneurysm development. Two of these SNPs (rs1429412 and rs700651), at locus 2q33, flank the BOLL gene (involved in germ cell development) and the PLCL1 gene (involved in intracellular cascade reactions with abolished phospholipase C activity). PLCL1 lies close to the VEGFR2 (Vascular endothelial growth factor receptor-2), which is involved in central nervous system angiogenesis and is a marker for endothelial progenitor cells. Three further SNPs on chromosome 2 were associated with familial intracranial aneurysms: rs11693075 (close to the histone deacetylase complex SAP130 gene), rs1897472 and rs3769801 (both located within the PDEA1 [phosphodiesterase 1] gene).[164]

One associated SNP located on chromosome 7 (rs4628172 G>T) is within the TMEM 195 gene, which encodes trans-membrane proteins, possibly involved in fatty acid biosynthesis and iron binding.[162, 163] Further associated SNPs from the latest GWAS include variants on chromosome 12q22 (rs6538595, rs11112585 and rs2374513) within C12orf75 gene.[164] The rs1132274 G>T SNP on chromosome 20p12.1 was also associated with intracranial aneurysms.[46] However, none of these SNPs yet have known clear functional relevance to intracranial aneurysms.

Three further loci not included in this meta-analysis (due to being reported in only one study) showed

significant association with intracranial aneurysms.[46] These loci included 18q11.2 (rs11661542, 10q24.32 (rs12413409) and 13q13.2 (rs9315204), but their mechanisms of association remain unclear.

3.5.1.2 Genetic variants associated with intracranial aneurysms identified by candidate gene association studies

Eight SNPs from CGAS were associated with intracranial aneurysms in our meta-analysis, albeit with significant statistical heterogeneity. These SNPs relate to genes with plausible relevance to intracranial aneurysm biology, including those important for extracellular matrix (ECM) integrity, inflammatory mediators, and vascular endothelium maintenance. Extracellular matrix gene variants may predispose to intracranial aneurysms development by weakening arterial walls. In our meta-analysis, six such SNPs showed significant associations with IA: COL1A2 (rs42524), COL 3A1 (rs1800255), SERPINA-3, Versican (rs251124 and rs173686), and HSPG2 (heparan sulfate proteoglycan2; rs3767137). The COL1A2 rs42524 SNP is located on chromosome 7q22.1 close to the elastin gene – a protein component of arterial walls, previously associated with intracranial aneurysms in a linkage-association study.[126] Collagen type 1 is usually found in the adventitial layer of cerebral arteries and provides tensile strength, making it a plausible factor in intracranial aneurysm development. Nevertheless, caution is needed in generalizing this result, as all three studies identified were in Far-East populations; for robust association to be confirmed this SNP has to be examined in other populations, including Europe, as risk allele frequencies may differ. Collagen type 3A1 (COL 3A1) was investigated in three Chinese populations; rs1800255 (G>A) was the only SNP significantly associated intracranial aneurysms, but replication in other ethnicities and larger cohorts is required to confirm these findings.

We also found associations between two Versican genetic variants (rs251124 and rs173686) and intracranial aneurysms in Dutch, Japanese and Chinese populations.[151, 176, 177] In a previous linkage study on a Japanese cohort, chromosome 5q14.3, very close to the Versican gene, was implicated in intracranial aneurysms.[176] *In vivo* studies on AAA samples found reduced Versican mRNA expression,[178, 179] which could reflect a decline in smooth muscle cells associated with weakness in the vascular wall. Moreover, a GWAS[180] on thoracic and aortic dissections, showed significant association with the 5q13-14 locus, corresponding to the Versican gene.

The pro-inflammatory cytokine IL-6 G572C SNP had an overall protective association with intracranial aneurysms in a recessive model. However, there was significant statistical heterogeneity, and sensitivity analysis revealed no association, once one Chinese study[155] was excluded, which may partly reflect the greater RAF (0.79 vs. 0.05-0.09) and inclusion of only unruptured intracranial aneurysms in this study.[155] Another source of heterogeneity may be gender, as one other study[154] included only male controls which may not be appropriate since SAH has a female predominance. Therefore, the overall

association of this SNP with intracranial aneurysms is not robust. Sustained abnormal vascular remodelling in association with inflammation is hypothesized to be a key mechanism in intracranial aneurysm development.[171] IL-6 can injure vessel walls by inhibiting collagen synthesis, thus increasing wall fragility and increasing the risk of aneurysmal dilatation. IL-6 levels are increased in the CSF and plasma following SAH.[181, 182] but whether this is directly related to the G572C SNP remains uncertain as in these studies patients were not genotyped for the mutant allele; moreover, IL-6 may be released as part of a systemic inflammatory response in the acute phase following SAH.

3.5.2 Strengths of our study

Our systematic review and meta-analysis are the largest and most comprehensive to date on genetic variants associated with intracranial aneurysms. We assessed strength of association using multiple genetic models and carefully assessed the robustness of all associations by performing random effects, sensitivity and ethnic subgroup analyses. We conducted our analysis and reporting according to quality guidelines for genetic association studies including HuGENET[165]and STREGA.[165]

3.5.3 Limitations of our study

Our assessment of inter-study heterogeneity by visual inspection of the Forest plots and sensitivity analyses only partly addresses potential sources of heterogeneity between studies. Further individual patient data is required on other factors that could also contribute to heterogeneity, such as gender proportions, age, genotyping methods or errors, disease phenotype (aneurysm size, location and rupture status) and environmental risk factor status. Our meta-analysis was only able to pool reported positively associated SNPs from the six GWAS studies, but data on many SNPs sequenced not reaching the threshold for GWAS statistical significance was not available, limiting our meta-analysis to 41 SNPs in total. Indeed, for many of the candidate-gene studies, pooling all available data from GWAS might allow more robust risk estimates to be calculated; despite eight candidate gene SNPs being associated with intracranial aneurysms, on average only 2.7 publications were available for each variant.

We have identified 19 candidate SNPs associated with intracranial aneurysms, of which 16 were robust to random effects or sensitivity analyses. The odds ratios suggest that the genetic contribution to aneurysm development is substantial, but the variants identified implicate multiple pathophysiological mechanisms, particularly involving vascular endothelium maintenance. Nevertheless, the amount of data on genetic associations with intracranial aneurysms remains much smaller than for other complex diseases, so further large replication studies in a full spectrum of populations are required ideally using GWAS or Exome sequencing to identify rarer functional variants. A limitation of most studies included is that both ruptured and unruptured intracranial aneurysms were analysed together; since only a minority of IAs are destined to rupture, future studies should address genetic risk factors specifically related to rupture status. Another important area for future research is to explore how genetic factors are related to aneurysm location, size or morphology, since there may be specific genetic associations

for particular aneurysm phenotypes (which may also relate to rupture risk). Finally, validation studies of risk models in prospective cohorts (e.g. of unruptured intracranial aneurysms followed up for rupture risk) would be helpful in determining the relevance of genetic risk loci in addition to well-established risk factors for overall disease phenotype, to ultimately reduce the devastating burden of aneurysmal SAH.

FIGURE LEGENDS

Figure 3-1 – **Flow chart illustrating the number of studies evaluated in this meta-analysis.**

Table 3-1 – **SNPs associated with intracranial aneurysms.** p_{Het} - Inter-study heterogeneity p-value. I^2 – Higgins statistic demonstrating degree of inter-study heterogeneity. FE- Fixed effects model.

Figure 3-2 – **Forest plots (additive model) for GWAS in all populations**
2A – rs1333040 SNP; **2B** – rs10757278 SNP; **2C** – rs9298506; **2D** – rs10958409 and **2E** – rs6841591.

Figure 3-3 - Forest plots for candidate gene SNPs associated with intracranial aneurysms
3A– Dominant model forest plots for SERPINA-3 SNP respectively. **3B** – Collagen 3A1 (rs1800255) Dominant model forest plot. **3C** – Collagen 1A2 (rs42524) Dominant model forest plot. **3D** – ACE I/D recessive model forest plot. **3E** – IL-6G572C SNP recessive model forest plot prior to sensitivity analysis. **3F** – IL-6 G572C SNP recessive model forest plot following sensitivity analysis.

Table 3-2 – R**andom effects and sensitivity analyses of associated SNPs which showed significant statistical heterogeneity under fixed effects modelling**
[a] 4 studies including one Caucasian and three Japanese populations, were excluded.
The odds ratios in **bold** show significant association with IA.

Table 3-3 - Subgroup ethnicity analysis.
The odds ratios in **bold** show significant association with IA.

Figures and Tables

Figure 3- 1 – PRISMA Flow diagram of search strategy

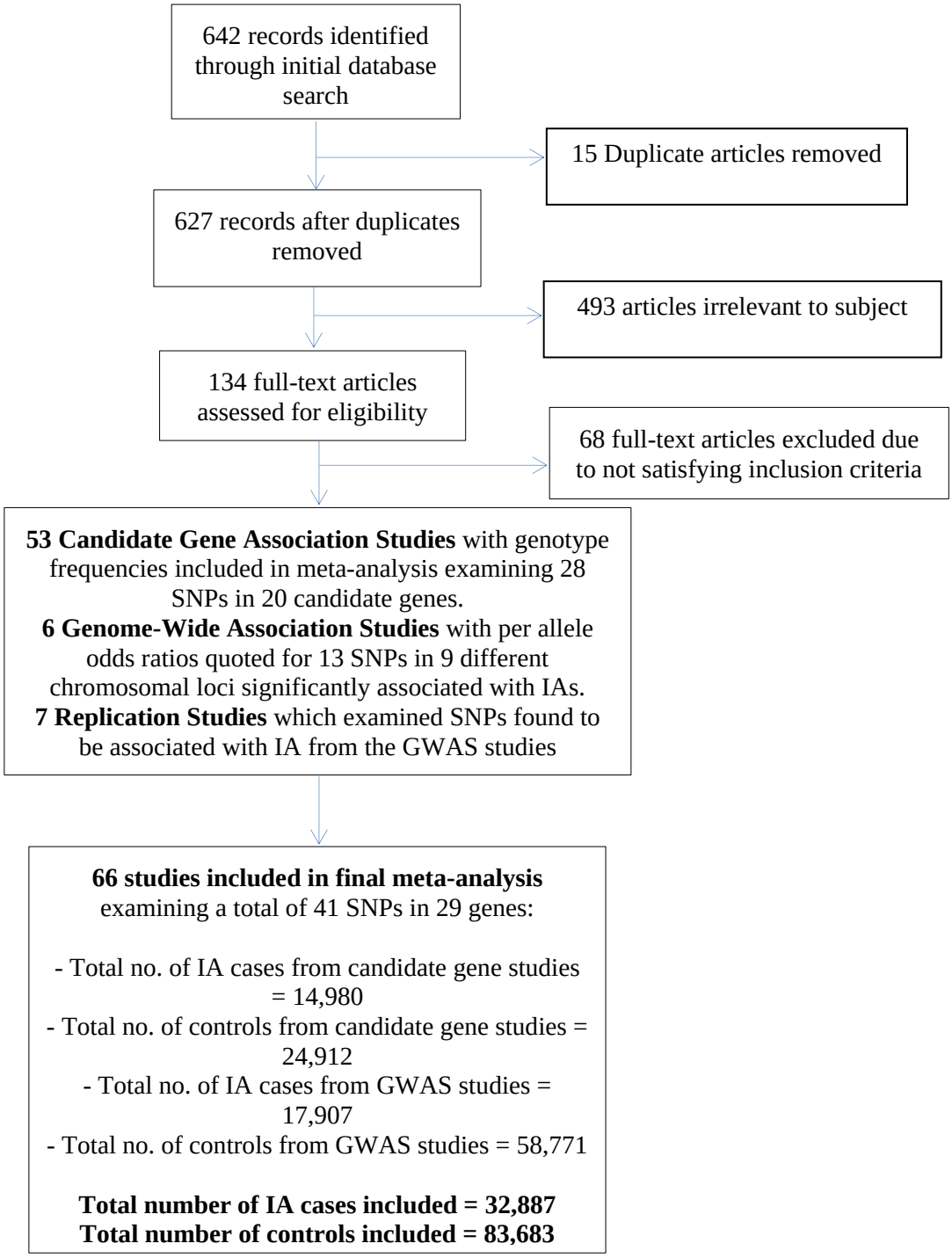


Figure 3- 2 - Forest plots (additive model) for GWAS in all populations

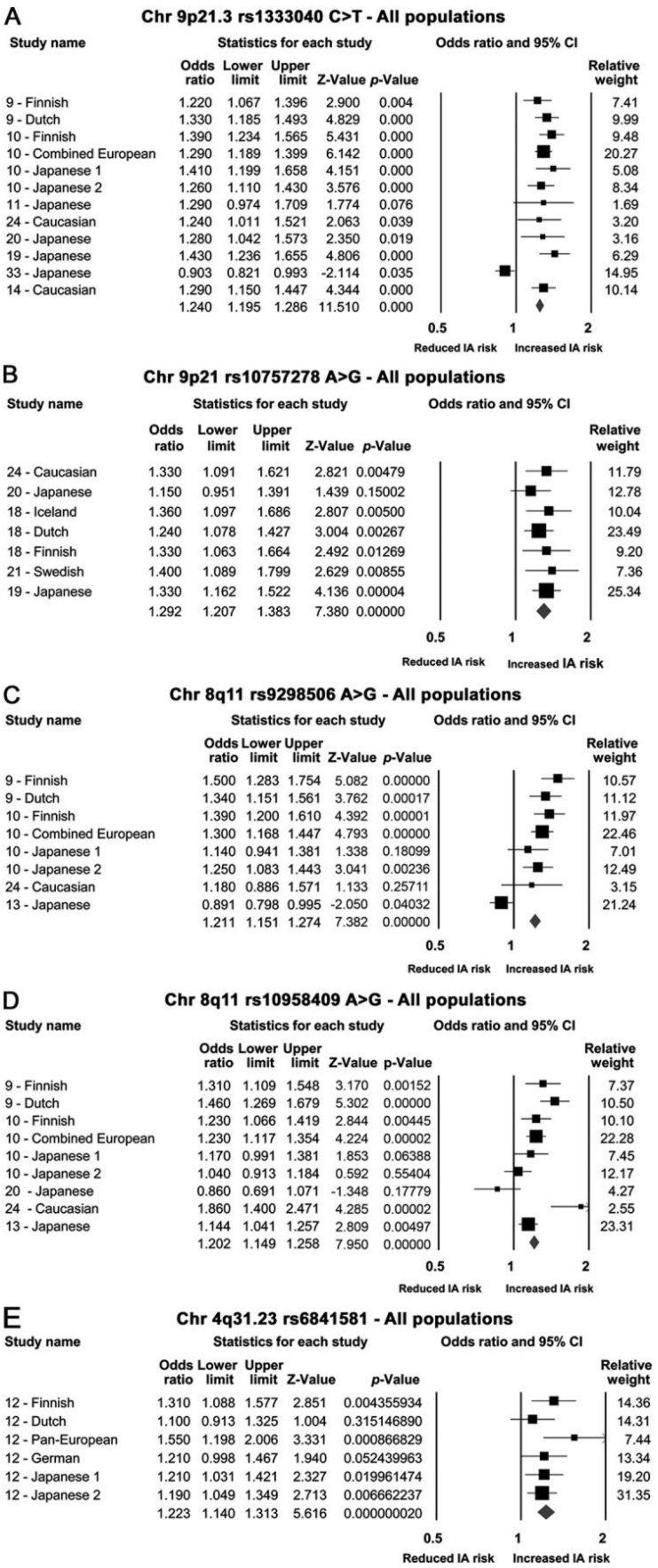


Figure 3- 3 - Forest plots for candidate gene SNPs associated with intracranial aneurysms

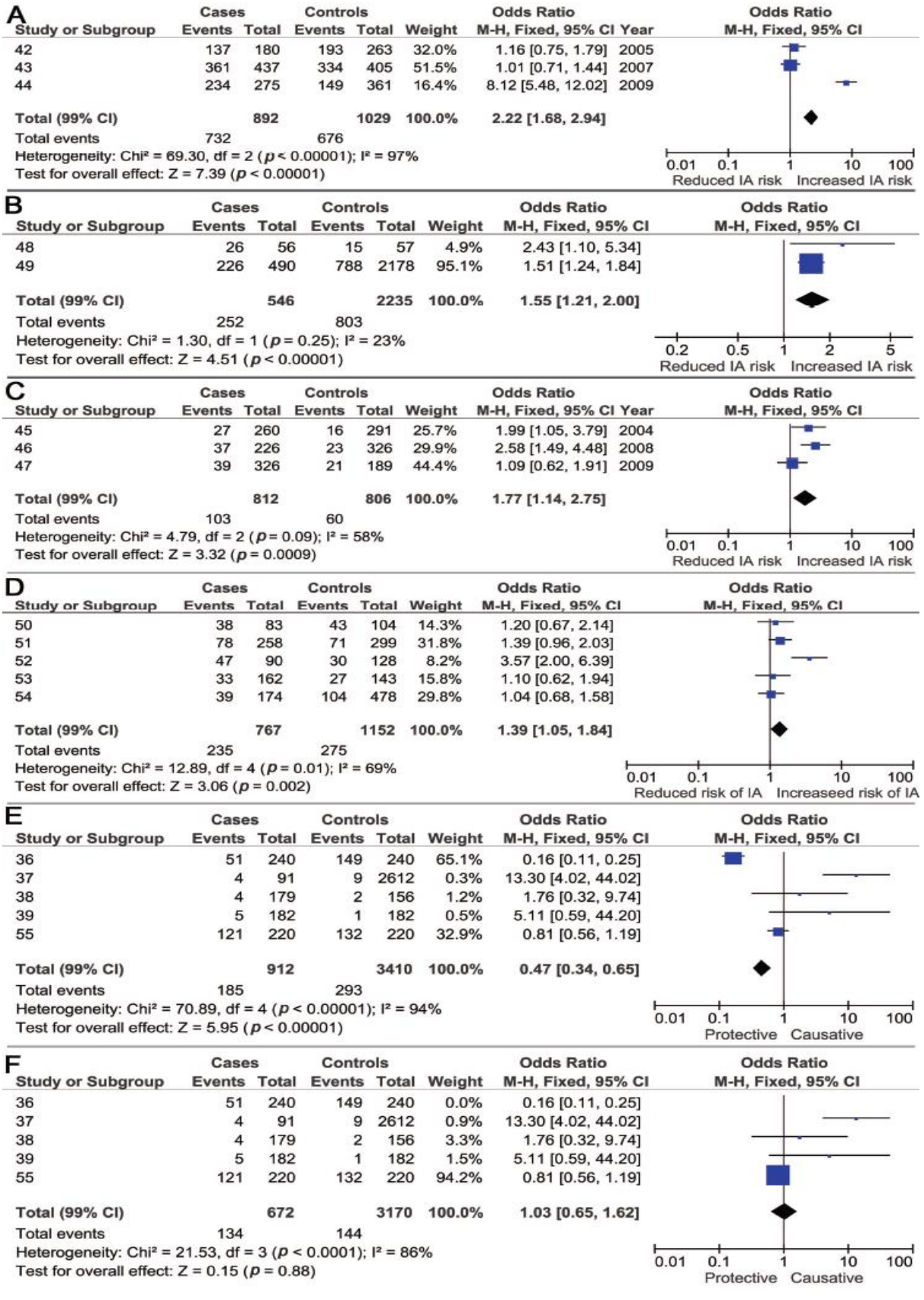


Table 3 - 1 – SNPs associated with intracranial aneurysms. pHet - Inter-study heterogeneity p-value.

Gene/ Locus	SNP	Genetic Model	Number of Studies	Cases	Controls	FE-OR (95%)	$p_{corrected}$ (Bonferroni)	p_{Het} (I^2)	Robust association after random effects/ sensitivity analyses	Potential biological mechanism of association with IA
Genome-wide association study (GWAS) SNP Associations										
9p21.3	C>T (rs1333040)	Additive	12[44-46, 162-164, 168]	11949	29014	1.24 [1.2-1.29]	$9.83x10^{-08}$	$5.40x10^{-08}$ (80%)	√	Vascular endothelium
9p21	A>G (rs10757278)	Additive	7[44, 116, 168-170]	3394	17075	1.29 [1.21-1.38]	$1.59x10^{-13}$	0.84 (0%)	N/A	Vascular endothelium
8q11	A>G (rs9298506)	Additive	8[44-46, 163]	9246	26331	1.21 [1.15-1.27]	$1.55x10^{-13}$	$1.90x10^{-07}$ (84%)	√	Vascular endothelium
8q11	A>G (rs10958409)	Additive	8[44-46, 162, 163, 169]	9873	27029	1.20 [1.15-1.26]	$1.78x10^{-15}$	$6.87x10^{-05}$ (76%)	√	Vascular endothelium
4q31.23	A>G (rs6841581)	Additive	6[47]	4370	14181	1.22 [1.14-1.31]	$1.95x10^{-8}$	0.39 (4%)	N/A	Vascular endothelium
9p21.3	C>G (rs2891168)	Additive	2[164, 168]	2076	1985	1.32 [1.21-1.44]	9.84x10-10	0.98 (0%)	N/A	Vascular endothelium
2q33	G>A (rs1429412)	Additive	4[44, 45, 162]	2675	7632	1.20 [1.12-1.30]	$1.07x10^{-06}$	0.30 (18%)	N/A	Unknown
2q33	G>A (rs700651)	Additive	6[44, 45, 163, 169]	4283	13236	1.11 [1.06-1.18]	$7.13x10^{-05}$	$1.17x10^{-02}$ (66%)	√	Unknown
7q13	G>T (rs4628172)	Additive	2[162, 163]	2443	6376	1.11 [1.03-1.19]	$4.04x10^{-03}$	$4.71x10^{-03}$ (87%)	N/A	Unknown
12q22	G>A (rs6538595)	Additive	6[47]	4370	14181	1.16 [1.10-1.23]	$1.12x10^{-7}$	0.98 (0%)	N/A	Unknown
20p12.1	G>A (rs1132274)	Additive	5[47]	4370	14181	1.19 [1.11-1.28]	$8.29x10^{-7}$	0.52 (0%)	N/A	Unknown
Candidate gene SNP Associations										
SERPINA 3	A>G (rs4934)	Dominant Recessive Additive	3[183-185]	892	1029	2.22 [1.68-2.94] 0.80 [0.49-1.31] 1.27 [1.07-1.50]	<0.00001 0.25 0.0002	<0.00001 (97%) 0.04 (69%) <0.00001 (94%)	×	Extracellular Matrix
COL 1A2	G>C (rs42524)	Dominant Recessive Additive	3[186-188]	812	806	1.77 [1.14-2.75] 1.14 [0.16-7.94] 1.69 [1.11-2.57]	0.0009 0.86 0.001	0.09 (58%) 0.31 (4%) 0.06 (64%)	N/A	Extracellular Matrix

COL 3A1	G>A (rs1800255)	Dominant Recessive Additive	2[189, 190]	546	2235	1.55 2.00] 1.31 2.38] 1.40 1.72]	[1.21- [0.72- [1.14-	<0.00001 0.25 <0.0001	0.25 (23%) 0.70 (0%) 0.26 (21%)	N/A	Extracellular Matrix
HSPG2	A>G (rs3767137)	Additive	2[151, 177]	1316	1742	1.22 1.39]	[1.08-	0.002	0.122 (58%)	N/A	Extracellular Matrix
Versican (CSPG2)	C>T (rs251124)	Additive	3[151, 176, 177]	1489	1687	1.25 [1.1-1.41]		0.0007	0.201 (38%)	N/A	Extracellular Matrix
Versican (CSPG2)	A>G (rs173686)	Additive	2[176, 177]	857	879	1.23 1.43]	[1.05-	0.008	0.013 (84%)	N/A	Extracellular Matrix
ACE	I/D	Dominant Recessive Additive	5[191-195]	767	1172	0.82 1.12] 1.39 1.84] 1.23 1.40]	[0.61- [1.05- [1.07-	0.66 0.002 0.003 (0.003)	0.002 (76%) 0.01 (69%) 0.47 (0%)	×	Vascular endothelium
IL-6	G572C	Dominant Recessive Additive	5[154-156, 196, 197]	912	3410	0.68 0.96] 0.47 0.65] 0.61 0.75]	[0.48- [0.34- [0.49-	0.004 <0.00001 <0.00001	<0.00001 (92%) <0.00001 (94%) <0.00001 (95%)	×	Inflammatory mediator

I^2 – Higgins statistic demonstrating degree of inter-study heterogeneity. FE- Fixed effects model.

Table 3 - 2 – Random effects and sensitivity analyses of associated SNPs which showed significant statistical heterogeneity under fixed effects modelling

SNP	No. of Studies	Studies excluded	FE-OR (95%) prior to adjustment	RE-OR (95%)	FE-OR (95%) Post sensitivity analysis	Genetic Model
SERPINA-3	3	Liu[185]	**2.22 [1.68-2.94]**	2.12 [0.36-12.31]	1.07 [0.74-1.53]	Dominant
			1.27 [1.07-1.50]	1.25 [0.61-2.59]	0.97 [0.79-1.19]	Additive
ACE I/D	5	Slowik[193]	**1.39 [1.05-1.84]**	1.44 [0.86-2.42]	1.20 [0.88-1.62]	Recessive
IL-6 G572C	5	Sun[155]	**0.47 [0.34-0.65]**	1.49 [0.24-9.27]	1.03 [0.65-1.62]	Recessive
			0.61 [0.49-0.75]	0.95 [0.31-2.85]	1.04 [0.79-1.39]	Additive
rs1333040	11	Low[163]	**1.23 [1.19-1.28]**	**1.26 [1.15-1.39]**	**1.31 [1.26-1.37]**	Additive
rs700651	6	Low[163]	**1.11 [1.06-1.18]**	**1.14 [1.03-1.25]**	**1.19 [1.11-1.28]**	Additive
rs9298506	8	Low[163]	**1.21 [1.15-1.27]**	**1.24 [1.08-1.41]**	**1.32 [1.24-1.39]**	Additive
rs10958409	9	4[a 44, 46, 162, 169]	**1.20 [1.15-1.26]**	**1.22 [1.1-1.34]**	**1.23 [1.17-1.3]**	Additive

[a] 4 studies including one Caucasian and three Japanese populations, were excluded.

The odds ratios in **bold** show significant association with IA.

Table 3 - 3 - Subgroup ethnicity analysis. The odds ratios in bold show significant association with IA.

SNP	No. of Studies	Ethnicity	FE OR – all ethnicities (all studies)	FE-OR (95%) ethnicity sub-analysis	$P_{corrected}$	p_{HET} (I^2)	Genetic Model
ACE I/D	4/5	Caucasian	**1.39 [1.05-1.84]**	**1.42 [1.06-1.92]**	0.002	0.006 (76%)	Recessive
		Japanese		1.20 [0.56-2.57]	0.54	N/A	Recessive
IL-6 G572C	3/5	Chinese	**0.30 [0.19-0.47]**	**0.41 [0.29-0.58]**	<0.00001	0.0001 (95%)	Recessive
	2/5	Caucasian		4.26 [0.92-19.7]	0.1	0.03 (78%)	Recessive
	3/5	Chinese	**0.58 [0.44-0.75]**	**0.50 [0.39-0.64]**	<0.00001	<0.00001 (97%)	Additive
	2/5	Caucasian		1.38 [0.83-2.29]	0.1	0.19 (42%)	Additive
rs1333040	6/11	Japanese	**1.24 [1.2-1.29]**	**1.15 [1.09-1.22]**	$1.75x10^{-06}$	$3.06x10^{-08}$ (88%)	Additive
	5/11	Caucasian		**1.30 [1.24-1.36]**	$1.91x10^{-06}$	0.78 (0%)	Additive
rs10757278	5/7	Caucasian	**1.29 [1.21-1.38]**	**1.30 [1.2-1.43]**	$1.2x10^{-09}$	0.91 (0%)	Additive
	2/7	Japanese		**1.27 [1.13-1.41]**	$2.6x10^{-05}$	0.22 (33%)	Additive
rs9298506	5/8	Caucasian	**1.21 [1.15-1.27]**	**1.35 [1.27-1.44]**	$2.4x10^{-04}$	0.52 (0%)	Additive
	3/8	Japanese		1.03 [0.95-1.12]	0.45	$6.54x10^{-04}$ (86%)	Additive
rs10958409	5/9	Caucasian	**1.2 [1.15-1.26]**	**1.31 [1.23-1.39]**	$8.2x10^{-02}$	0.052 (62%)	Additive
	4/9	Japanese		**1.09 [1.02-1.17]**	0.009	0.08 (55%)	Additive
rs700651	3/3	Caucasian	**1.11 [1.06-1.18]**	**1.19 [1.09-1.29]**	$4.82x10^{-05}$	0.23 (31%)	Additive
	3/3	Japanese		1.06 [0.94-1.31]	0.08	0.02 (75%)	Additive
rs6841581	4/6	Caucasian	**1.22 [1.14-1.31]**	**1.25 [1.13-1.38]**	$1.21x10^{-05}$	0.18 (38%)	Additive
	2/6	Japanese		**1.2 [1.08-1.32]**	$3.56x10^{-04}$	0.87 (0%)	Additive
rs6538595	4/6	Caucasian	**1.16 [1.10-1.23]**	**1.16 [1.08-1.24]**	$2.13x10^{-05}$	0.91 (0%)	Additive
	2/6	Japanese		**1.17 [1.06-1.28]**	$1.49x10^{-03}$	0.6 (0%)	Additive

Chapter 4 - Genetics and Observational Study on Subarachnoid Haemorrhage (GOSH): study setup and DNA analysis

4.1 Background and rationale

Knowledge about the genetic determinants of intracranial aneurysms remains significantly less compared to other conditions such as; coronary heart disease, diabetes and abdominal aortic aneurysms. Nevertheless, our comprehensive meta-analysis identified 16 candidate SNPs that were associated with intracranial aneurysms. However, these studies were conducted in mostly, Dutch, Finnish, American, Japanese and Chinese populations, and may not be generalizable to other populations. Only 3 SNPs have been analysed in association with a UK population, which include the; ACE insertion deletion polymorphism,[192]APOE gene[103] and the IL-6 G172C SNP.[154] These studies totalled 343 patients. Whether the other genetic variants we identified are linked to intracranial aneurysms in a UK population remains unknown. Although Dutch and Finnish patients were included in some studies, the incidence of intracranial aneurysms is lower in the UK population than in these countries.

Lack of risk factor and detailed phenotype data published in previous genetic studies especially genetic influences different for posterior versus anterior aneurysms, and interaction of genetics with environmental risk factors e.g. smoking, hypertension was not analysed in depth.
We will therefore conduct a comprehensive genetic study on a UK based population, with detailed phenotype data including aneurysm location, clinical outcome and vascular risk factors.

4.2 GOSH Study Research Aims and Objectives

1. To identify genetic variations associated with ruptured intracranial aneurysms compared to unruptured aneurysms and control subjects in a UK population in a large, multi-centre study. We confirmed the strongest previously identified SNP associations found from all genetic association studies (see systematic review chapter 3).
2. We will perform multi-variate analysis to examine the influence of aneurysmal risk factors (e.g. hypertension, smoking) on any positive genetic associations with intracranial aneurysms.
3. To contribute important large-scale UK data to international Genome-Wide Association Studies.

4.3 Methods

Implementing the GOSH study required reaching key milestones, prior to the first patient being recruited. These included; development of the case report form (CRF) which collected detailed

phenotype and outcome data, and a database for storing this information electronically. In addition, large numbers of cases were required; this was anticipated by making the study multi-centre. Once additional sites were opened, recruitment processes were explained and provisions made to ensure efficient operation of the study at each participating centre.

4.4 Data collection and management

4.4.1 Creation of the GOSH Case Report Form (CRF)

The CRF was designed to carefully collect phenotype information in relation to intracranial aneurysms. The form was split into 13 sections (see appendix II, figures 1 and 2). Each section recorded a different facet of the disease such as; symptoms, size and site of aneurysm(s), social history, past medical history, clinical and radiological status upon admission, complications during admission and outcome with the GOS and mRs scales. This information will enable us to later perform genetic analysis in association with many factors associated with the disease. The form was piloted on ten potential participants by research practitioners in the Thames Stroke research Network to maximise user-friendliness and reduce ambiguities in data entry. The feedback was incorporated and it was felt that the proforma should be divided into 2, to reflect rupture status. Following on from this, two separate CRFs were developed; one for ruptured cases and the other for unruptured intracranial aneurysms cases (see appendix II, see figure 2). Confidentiality of patient data was maintained, by not including their name, date of birth and hospital number on the CRFs. Instead, we assigned a unique study ID for each patient recruited.

4.4.2 Creation of the Electronic CRF (e-CRF)

This was created in Microsoft Access 2010 by the author of the thesis. The aim of the electronic version was to be able to record all data recorded on paper CRFs electronically for safe storage in a database and subsequent analysis, ensuring privacy of patient data. In creating the database, knowledge was required on the use of Microsoft Access 2010 and how to implement visual basic programming to create a smooth running and secure database. The author attended various MS-Access courses at UCL and read information gleaned from books about programming in the software package. The database was created and piloted by the GOSH study group. Any issues were noted and later fixed by the author (see Appendix II, figure 3 for image of the e-CRF).

4.4.3 Setting up the GOSH study network at participating centres

The GOSH study was hosted via the UK Stroke Research Network (SRN) which attracted interest from interested centres. Expressions of interest forms were sent to the research fellow from sites wishing to participate in the study. Each site that showed interest in GOSH was checked to see if they had provisions to undertake the study at their centre. The criteria for a participating centre were:

1. Access to participants with intracranial aneurysms – inpatient or outpatient, with at least 100 expected cases of aneurysmal SAH seen per year.
2. Staff available and trained to take blood samples from patients
3. Adequate clinical facilities to interview patients and take blood samples
4. A refrigerator which could freeze between -20 to -80 degrees in order to store blood samples safely for subsequent DNA extraction.
5. Up to date Good Clinical Practice (GCP) training for recruitment staff.
6. A designated principal investigator (PI) for the site.

Once these checks were satisfied, designated PIs of each site were sent Site-specific forms (SSIFs) through the IRAS website to begin the approval process through their local Research and Development (R&D) committees. In the meantime, the sponsor (UCLH) would send out clinical and financial agreements to each site once R&D (research and development) approval was in the later stages.

4.4.4 Site initiation visits

Upon R&D approval, a site-initiation visit (SIV) to open the study at each site was undertaken by the research fellow, where a PowerPoint presentation for the relevant staff was performed about the GOSH study. The SIV covered the background and rationale of the study, the process of recruitment, guidance on filling out the CRF (case report forms) on each patient and how to transport blood samples to the chief site.

A batch of 48 Royal Mail safe boxes designed for transporting biological material was provided to each site so they could send samples to the chief site. Each box could hold 6 blood tubes i.e. 3 patient samples. A site folder was provided by the research fellow with all the necessary official documents pertaining to the study. These documents included the latest study protocol, copies of the R&D and ethics approval locally, copies of the CRF proformas, adverse incident forms and a site operation log. Each research nurse/fellow recruiting at each site, had to provide a copy of their research CV and proof of an up-to-date GCP certification.
The sites participating in the GOSH study is shown in the appendix (See Appendix II, Table 1)

4.4.5 Recruitment Process

The clinical research fellow developed a system in order to recruit as many cases of intracranial aneurysms as possible at the NHNN and all participating sites. At our local site this included, liaising with the Neurovascular nurses at the NHNN about the latest admissions each week to the unit and those patients coming back for elective check angiograms. The research fellow would check all neurosurgical outpatient clinics each week to determine which intracranial aneurysm cases were coming for routine

outpatient appointments with their respective consultants and would contact them beforehand to see if they wished to be recruited.
The research fellow arranged to have weekly clinics at the UCL Hospital Clinical Research Facility in order to recruit patients previously discharged from care in attempt to boost accruals.

The process of recruitment involved the following steps:

1. Provide verbal and written on information about the GOSH study to the patient or next of kin (NOK). Answer any questions, allow sufficient time for them to make an informed decision, provide additional information and reassurance as necessary. Explain they can withdraw at any point without providing a reason.
2. Obtain signed consent on the relevant consent form(s), either from the patient if they had mental capacity or the next of kin (NOK), if they lacked capacity (see appendix II, figures 4 & 5). Explain that one blood sample in 2 bottles will be taken, along with information sought at interview which will be recorded on the CRF proformas.
3. Take one blood sample in 2 purple-top EDTA tubes for DNA extraction and subsequent analysis.
4. Store blood samples in a research freezer ready for DNA extraction.
5. Complete a CRF on each patient, obtaining information from patient notes as required.
6. Store information recorded on CRFs onto the electronic Microsoft Access database 2013 prepared by the research fellow.

4.5 Sample Size calculation

We assume the SNP and disease locus has the same allele frequencies and are in complete linkage disequilibrium (LD). Our estimated prevalence is 0.03 assuming disease loci with an additive affect. Our case-control ratio of 1:1, with 1500 cases and 1500 controls, gives us 80% power to detect a susceptibility locus with a odds ratio of ≥ 1.2 with a type I error of 0.05, for a SNP with a risk allele frequency of ≥ 0.2. The Harvard genetics power calculator was used to perform this power calculation.[159]

Controls

Control DNA was drawn from the Wellcome 1958[2] cohort (n>1500) and our own cohort of Caucasian Parkinson's disease patients, with pathology reports confirming no intracranial aneurysm(s) (n>300). Data on risk factor status; hypertension, sex, smoking and alcohol status was available for the Wellcome controls.

Recruitment

2527 patients were recruited across all 20 centres. The GOSH study closed on 01/01/2014.

DNA extraction

In order to extract DNA for analysis, genetic material has to be isolated from the nucleus of blood cells collected from our patients.

1. The cells were lysed to obtain access to the DNA. This was performed by Sonification. A detergent such as SDS (sodium dodecyl sulfate) is added to remove the lipid membranes of the cell and nucleolus.
2. DNA associated proteins such as histones were degraded by the addition of proteinase K. The proteins are precipitated out by the addition of a salt – ammonium. Once the samples had been vortexed with phenol-chloroform and centrifuged, the proteins fell to the bottom and were drawn off. The DNA will be found at the interface between the 2 phases.
3. Subsequently, the DNA was precipitated by mixing it with cold isopropanol and centrifuging. Due to insolubility of the DNA in alcohol it will come out of solution. The isopropanol serves a dual purpose by washing out the salt added in the previous stage.
4. The resultant DNA pellet is washed again with cold ethanol to form into a pellet, following centrifugation.
5. The ethanol is removed and the pellet dryed. The DNA is then re-suspended in a buffer such as TE (Tris-EDTA).

Candidate gene sequencing

<u>Stage 1: DNA plating and preparation.</u>

DNA was extracted from the blood of all intracranial aneurysm participants to the GOSH study, at the LGC Genomics lab in Hoddesdon, UK. We stored 100-400ul of DNA in a -80°C freezer which was of high quality (A260/280 ratio > 1.8) and stock concentration is between 180 to 1300 ng/ul. DNA was first aliquoted into separate stock tubes and diluted to 100ng/ul. DNA was then plated into deep-well secure sealed plates with known water controls and stored at -20°C ready for analysis. We obtained 1500 control DNA samples from the Wellcome Trust 1958 cohort. These samples were stored in our -80°C freezer in 50ng/ul secure plates.

<u>Stage 2: Targeted amplicon sequencing of SAH cases.</u>

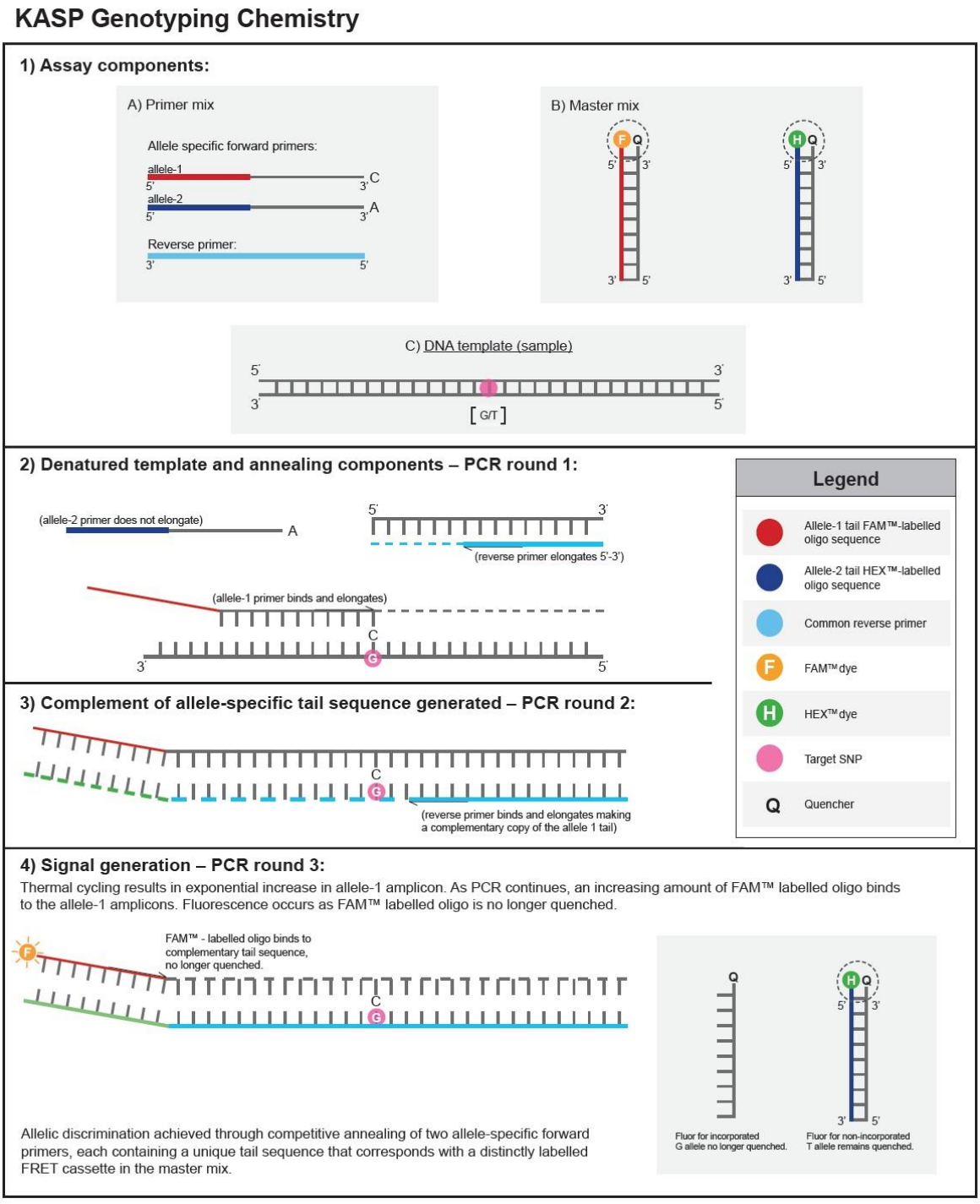
Targeted amplicon sequencing isolates genomic regions of interest through the use of custom primer (KASP primer mix and Universal KASP master mix) sets that define those regions similarly to a

conventional PCR reaction. The KASP Primer mix contains three assay-specific non-labelled oligonucleotides: two allele-specific forward primers and one common reverse primer which allows for a high level of sample multiplexing. The allele-specific primers each harbour a unique tail sequence that corresponds with a universal FRET (fluorescence resonant energy transfer) cassette; one labelled with FAM™ dye and the other with HEX™ dye. The KASP Master Mix contains the universal FRET cassettes, ROXTM passive reference dye, taq polymerase, free nucleotides and $MgCl_2$ in an optimised buffer solution. During thermal cycling, the relevant allele-specific primer binds to the template and elongates, thus attaching the tail sequence to the newly synthesised strand. The complement of the allele-specific tail sequence is then generated during subsequent rounds of PCR, enabling the FRET cassette to bind to the DNA. The FRET cassette is no longer quenched and emits fluorescence. Bi-allelic discrimination is achieved through the competitive binding of the two allele-specific forward primers. If the genotype at a given SNP is homozygous, only one of the two possible fluorescent signals will be detected. If the genotype is heterozygous, a mixed fluorescent signal will be detected (see figure 4-1). This makes possible the systematic detection of common and rare variants at significantly lower cost per sample and in a fraction of the time compared to Sanger sequencing. The technology also allows SNP analysis and sequencing on the same platform. The technology is similar to that used in exome sequencing; however, because relatively few genomic targets are being sequenced, the level of sample multiplexing can be increased with a straightforward analysis protocol.

Following completion of the initial 35 cycles of PCR, all genotyping reaction plates run were read on a BMG PHERA Star plate reader. This initial read data was visually inspected by a member of the genotyping team to assess the progression of the PCR reaction. The plates were then recycled (3 cycles per recycle step) and read after each recycle step. The laboratory operator visually inspected the read data after each recycle step and, once satisfied that the PCR reaction has reached the endpoint, plates were deemed completed. The Kraken software was used to automatically call all genotypes and these were verified by two lab technicians.

Variant files were subject to robust bioinformatics analysis to determine the variants and in each sample an exact report for each amplified region was prepared.
We assessed the association of each variant with the risk of having an intracranial aneurysm compared with controls. We examined variants to assess if risk variant load increases this association with intracranial aneurysms and causes a particular or a more severe phenotype. We have collaborations with other groups to allow the confirmation of variants, including Yale University (Lead: Professor Murat Gunel) and the US National Institutes of Health (Lead: Prof A Singleton) group. The software used to carry out statistical analysis was R[198] and PLINK.[199]

Figure 4- 1 – KASP genotyping illustration



Chapter 5 - Genetics and Observational Study on Subarachnoid Haemorrhage (GOSH): Candidate Gene (CGAS) Association testing on 22 SNPs selected for association analysis with cerebral aneurysms

5.1 Introduction

Numerous candidate gene association studies (CGAS) have been performed to date on intracranial aneurysms. These studies have been performed in different ethnicities ranging from, Caucasian to Japanese and Chinese populations. As described earlier, this approach is hypothesis driven and aims to confirm or refute previous findings of plausible candidate gene SNPs thought to be associated with intracranial aneurysms. A major drawback of this approach is that novel variants are unlikely to be detected as per GWAS. However, with a large sample size of greater than 1600 cases and 1200 controls, our study would remove previous shortcomings in other CGAS of being sufficiently powered to detect a modest effect in a UK Caucasian population (as per power calculations in chapter 4). This has not been previously done. We summarised the results for each candidate gene SNP tested in our UK based cohort. SNPs with greater than or equal to two studies underwent meta-analysis (see Chapter 3). Our meta-analysis guided selection of promising candidate gene SNPs to be analysed in our GOSH cohort, excluding genome-wide association SNPs. The SNPs arised from a variety of backgrounds thought to be possibly involved in aneurysm pathology/pathogenesis. These included genes coding for, vasuclar endothelial functions, sch as ACE I/D SNP, inflammatory mediators, such as IL-6 and TNF-alpha and those genes coding for various components and enzymes involved in extracellular matrix breakdown, for example Collagen or Matrix Metalloproteinase (MMP) genes. Sparse information on the pathobiology of cerebral aneurysm initiation, potentiation and rupture is currently available. Factors such as smoking, hypertension, female gender, alcohol consumption, altered local haemodynamic forces and inherited connective tissue diseases predisposing to cerebral aneurysms have been extensively discussed. Light evidence base exists on the molecular mechanisms of aneurysm formation, propagation and rupture despite numerous attempts to characterise molecular and structural abnormalities within aneurysmal specimens[200] to shed light on possible mechanisms. Several avenues investigating aneurysm pathology have been studied. These include examining those genes/pathways which may contribute to aneurysm formation via; an inflammatory reaction, those affecting extracellular matrix homeostasis and those affecting vascular endothelial function/permability/abnormal angiogenesis,[201] which is the effort, no direct causal pathway for aneurysm formation has yet to be elucidated. In this chapter the results of all 22 candidate gene SNPs tested in our cohort are presented with odds ratios. We also present the observational phenotypic data collected. The following 3 chapters will present results systematically broken down by their plausible function on intracranial aneurysm pathology.

5.2 Methods

2861 samples were processed for the purpose of this study. 1619 were cases of angiographically proven intracranial aneurysms and 1241 controls taken from the Wellcome[2] cohort and our Brain bank of 297 Parkinsons Disease samples, with negative post-mortem analysis for intracranial vascular disease. All samples tested were UK-Caucasian, Irish or White European after exclusion of other ethnicities. We also determined paternal and maternal grandparent ethnicity and only those of pure Caucasian decent were included. The demographic data for the total recruitment of cases following cessation of the study are presented in table 5-1. The total number of cases and controls that were available at the time of anaylsis for each SNP are presented in table 5-2. Exclusion of cases with other genetic disease such as ADPKD, Marfans or non-traumatic SAH was carried out in the recruitment stage.

Therefore, all cases recruited were sporadic intracranial ruptured or unruptured aneurysm(s). Data was recorded on each recruited patient as per the CRF (see appendix II, figures 1 & 2). Data was collected on demographic, past medical and family history and clinical and radiological parameters for each patient.

DNA was extracted from blood samples for each patient as described in chapter 4 at the LGC Genomics lab in Hoddesdon, UK. Extracted DNA was then plated into 96-well master plates in 250 microlitres of sterile RNA/DNAase water at a DNA concentration of 100ng/ul. From these master plates 50ml of solution was sent to LGC Genomics Hoddesdon, UK, to be genotyped for the SNPs selected (see table 5-2) via KASP genotyping (see chapter 4). Quality control was performed on all samples by the LGC and plate maps with all genotype results were available. The genotype results were checked and verified by LGC and sent back to us to be statistically analysed. Statistical analyses as carried out using the software R[198] and PLINK.[199] The additive genetic model was used to detect association of candidate SNPs with disease phenotype. Meta-analysis was restricted Caucasian populations only. Each SNP was checked for Hardy-Weinberg Equilibrium (HWE) in controls, as another potential method for detecting genotyping errors. All 22 SNPs were not significant when conducting this test, which meant genotyping errors were unlikely. Bonferroni correction was applied for multiple testing.

5.3 Results

Twenty two SNPs were analysed in 1619 cases and 1572 controls. However the total number of cases and controls included in each analysis was corrected taking into account bad samples or unknown genotypes, this is reflected in table 5-3 for each SNP. The mean age for cases was 65.4 and controls was 59.8 years. The majority of cases were female in origin, almost double that of men which is typical of intracranial aneurysm disease (see table 5-1). All samples analysed were Caucasian in ethnicity. The baseline characteristics of the date are summarised in table 5-1.

Two SNPs in two genes (ACE I/D, MMP-2) were found to be robustly associated with intracranial aneurysm disease in our UK population.

The ACE (Angiotensin Converting Enzyme) I/D SNP(rs1799752) was analysed in 1572 cases of which 1208 were ruptured and 364 were unruptured aneurysm cases. The number of controls analysed for this SNP was 1193. Under an Additive genetic model the deletion (D) allele of this SNP showed an overall association with intracranial aneurysm occurrence with an odds ratio of 1.14 [1.02-1.28], p=0.02 in a Caucasian population.

For the MMP-2 (Matrix Metalloproteinase) SNP (rs243865) was analysed in 1558 cases, with 1231 ruptured and 364 unruptured aneurysm patients. The number of controls analysed was 1208. This SNP demonstrated an increased risk of aneurysm development conferring an odds ratio of 1.18 [1.04-1.33], p=0.012 in our UK cohort, under an Additive genetic model.

The remaining 20 SNPs shown in table 5-3 did not meet statistical significance in our large UK Caucasian cohort.

Table 5- 1 - Demographic data of total ruptured and unruptured cases recruited into the GOSH study.

Variable	Ruptured cases	Unruptured cases	OR [95%CI]	p-value
Age (mean)	53.2 (12.7 SD)	57.0 (12.2 SD)	0.98 [0.97-0.98	<0.001
Sex			1.0 [0.82-1.22]	0.99
Male	514 (29.7%)	180 (29.8%)		
Female	1215 (70.3%)	425 (70.3%)		
Ethnicity				0.02
Caucasian	1484 (87.9%)	551 (93.4%)		
Mixed	26 (1.54%)	5 (0.85%)	1.93 [0.74-5.05]	
Asian	72 (4.27%)	17 (2.9)	1.57 [0.92-2.69]	
Black	106 (6.28%)	17 (2.89%)	2.32 [1.37-3.90]	
Family History (+ve UIA/SAH)	213 (12.3%)	111 (18.4%)	0.63 [0.49-0.80]	<0.001
Current Smoker	765 (44.3%)	228 (37.7%)	1.31 [1.09-1.59]	0.005
ETOH	1168 (67.6%)	392 (64.8%)	1.13 [0.93-1.37]	0.215
Recreational drug use	99 (5.7%)	20 (3.3%)	1.78 [1.09-2.90]	0.02
Hypertensive	542 (31.4%)	281 (46.5%)	0.53 [0.44-0.64]	<0.001
Aneurysm location				
MCA	341 (21.04%)	194 (34.34%)		
ICA	135 (8.33%)	164 (29.03%)	0.47 [0.35-0.62]	
ACA/ACOM	572 (35.29%)	93 (16.46%)	3.50 [2.64-4.64]	
PCOM		63 (11.15%)	2.39 [1.68-3.40]	
Posterior circulation		51 (9.03%)	0.91 [0.90-0.93]	
Aneurysm size (mean mm)	6.6 (4.16 SD)	9.0 (6.3 SD)	0.91 [0.90-0.93]	<0.001
Multiple aneurysms	447 (25.9%)	161 (26.6%)	0.96 [0.78-1.19]	0.714

Table 5- 2 – Number of cases and controls available for genotype analysis from the GOSH cohort.

Total Cases	Total Controls	Cases Gender (F/M)	Mean Age Cases	Mean Age Controls
1619	1572	1027/592	65.4	59.8

Table 5- 3 – Basic association results for each candidate gene SNP. (Significant results highlighted in bold)

SNP	Gene	Total cases	Cases analysed (After QC)	Total Controls	Controls analysed (After QC)	Ruptured	Unruptured	OR [CI] (corr)	P-value (corr)
I/D rs1799752	ACE	1607	1384	1299	1275 (0.194)	1054	330	**1.14 [1.02-1.28]**	**0.02**
rs1799983 (G>T)	eNOS 894	1619	1405	1241	1199 (0.44)	1067	338	0.95 [0.85-1.07]	0.41
rs2070744 (T>C)	eNOS 786	1619	1416	1241	1200 (0.8)	1077	339	0.99 [0.89-1.11]	0.89
rs1998923 (C>T)	Endoglin	1619	1429	1241	1209 (0.53)	1087	342	0.65 [0.38-1.25]	0.198
rs175646 (C>T)	Jun-2	1619	1430	1241	1209 (0.12)	1088	342	1.4 [0.98-1.98]	0.06
rs429358	APOE –ε4	1619	1413	1241	1207 (0.27)	1077	336	1.71 [0.96-3.04]	0.067
rs7412	APOE- ε2	1619	1409	1241	1221 (0.23)	1081	328	1.15 [0.92-1.43]	0.23
rs243865	MMP-2	1619	1420	1241	1208 (0.58)	1083	337	**1.18 [1.04-1.33]**	**0.012**
rs3025058 (5A/6A)	MMP-3	1619	1389	1241	1170 (0.11)	1057	332	0.94 [0.85-1.05]	0.305
rs1799768 (4G/5G)	SERPINE-1/PAI-1	1619	1414	1241	1201 (0.83)	1075	339	0.96 [0.86-1.07]	0.42
rs4934 (G>A)	SERPINA-3	1619	1410	1241	1200 (0.13)	1073	337	1.00 [0.9-1.12]	0.98

rs3767137 (G>A)	HSPG2	1619	1415	1241	1201 (0.91)	1078	337	1.002 [0.89-1.13]	0.97
rs42524 (G>C)	COL1A2	1619	1400	1241	1209 (0.092)	1065	335	1.04 [0.91 - 1.18]	0.58
rs285678 (G>A)	Elastin	1619	1419	1241	1202 (0.71)	1077	342	1.08 [0.91-1.28]	0.38
rs331079 (G>C)	Fibrillin	1619	1405	1241	1207 (0.31)	1069	336	1.07 (0.91-1.27)	0.431
rs251124 (C>T)	Versican	1619	1406	1241	1202 (0.81)	1068	338	0.997 [0.86-1.15]	0.97
rs2621215 (T>G)	COL1A2	1619	1414	1241	1195 (0.15)	1078	336	1.1 [0.93-1.3]	0.26
rs3783107 (C>T)	COL4A1	1619	1396	1241	1189 (0.49)	1061	335	1.05 [0.94-1.17]	0.403
rs1800255	COL3A1	1619	1403	1241	1203 (0.17)	1066	337	1.03 [0.91-1.17]	0.62
rs1800796 (G>C)	IL-6	1619	1402	1241	1225	1215	366	0.95 [0.73-1.22]	0.667
rs16944 (A>G)	ILβ-1	1619	1424	1241	1203	1084	340	0.96 [0.86-1.08]	0.53
rs1800629 (G>A)	TNFα- 308	1619	1403	1299	1284	1068	335	1.02 [0.88-1.17]	0.83

5.4 Discussion

Our candidate gene association study demonstrated association of 2 SNPs with different functions but plausible effects on intracranial aneurysm pathogenesis. The first being the ACE I/D SNP which demonstrated association of the D allele with IA presence. The first such association of this allele in an intracranial aneurysm population. This is in contrast to previous studies examining this SNP with intracranial aneurysms, including a study performed on a UK Caucasian population.[192] Our study was far more powered to detect modest effects and has the largest sample size to date, examining this SNP. Our results are in accordance with those studies analysing the same SNP with AAA (abdominal aortic aneurysm) disease, where a recent meta-analysis demonstrated D allele carriers to be associated with AAA occurrence.[202] The presence of the D allele or DD genotype has been shown to increase serum

ACE activity which can increase the local RAAS (Renin-Angiotensin-Aldosterone system) activity, causing a rise in blood pressure.[203]

In the next chapter (chapter 6) we analyse in depth all vascular SNPs tested in our cohort and determine if hypertension affects the association with IA by strengthening it, in the presence of this polymorphism. However, this is a promising association with plausible function on IA pathology. This study will need to be repeated in several populations with similar statistical power before robust conclusions can be determined. However, to counter this we will perform a meta-analysis of this SNP with the inclusion of our data. As the previous meta-analysis conducted by our group, demonstrated the I allele to be significantly associated with IA presence.[204]

The second SNP associated with our large UK Caucasian cohort included the MMP-2 rs243865 C>T SNP. This codes for the Specificity factor-1 (SP-1) transcription factor, that can influence signalling of multiple genes including the MMP-2 gene. MMP-2 is an important enzyme involved in the maintenance of extracellular matrix components (ECM) within vascular walls. Unregulated activation of this gene and this enzyme can cause significant damage to vascular walls, as it will digest ECM components. MMPs are counter-regulated by the TIMPs which inhibit their action to maintain vascular homeostasis.[205] Interestingly, MMP-2 and MMP-9 products have shown increased expression in the walls of ruptured compared to unruptured intracranial aneurysms.[147] Whether aberrant MMP-2 expression is a key terminal event in the rupture of IAs, remains to be elucidated. In chapter 7 we will perform a subgroup analysis to determine if this is associated in ruptured cases only. We will also meta-analyse this SNP with the 2 previous studies (One Caucasian[148] and one Japanese[206]) to determine if this SNP is robustly associated in (i) all populations and (ii) Caucasian populations.

The remaining 20 SNPs did not show statistical significance with intracranial aneurysm occurrence in our UK-based cohort. Previous genetic association studies with these SNPs have demonstrated conflicting results regarding their association with intracranial aneurysms. However, these were under-powered small studies in Caucasians and different ethnicities. Our study was sufficiently powered to determine an effect in a European Caucasian population and will significantly add to the existing body of literature, pertaining to the relevant SNPs. This does not rule out meaningful associations within different loci of these genes, with intracranial aneurysms. This highlights the limitations of a candidate-gene approach and that other genomic areas within the same gene could harbour variants that may be associated with intracranial aneurysm presence. To answer this question, repeated studies in different populations will need to be carried out, as well as scanning flanking regions of possible variants to see if there are any SNPs that may not be in LD with the SNPs we examined. Alternatively, obtaining data from previously published GWAS papers could help identify chromosomal loci within areas of these SNPs used to perform candidate-gene studies and see if any strong associations exist, that may not have reached GWAS level of significance, but would still be <0.05. This would require large collaborations and researchers who would be willing to share their data.

In the following 3 chapters we will present further analyses of SNPs based on their potential functional role (vascular endothelial function, extracellular matrix integrity, inflammation).

Chapter 6 - Genetics and Observational Study on Subarachnoid Haemorrhage (GOSH): Vascular Endothelium candidate SNPs and association with intracranial aneurysms

6.1 Abstract

Introduction

Intracranial aneurysm (IA) disease is present in 2-5% of the general population. A small minority of these aneurysm rupture, giving rise to subarachnoid haemorrhage (SAH), which has approximately 50% morbidity and mortality. This form of haemorrhagic stroke is prevalent in younger age groups and usually provides no warning of the presence of an aneurysm until it ruptures. Well associated risk factors have been previously studied in relation to this disease but predicting which patients develop and rupture from their aneurysm(s) is difficult. Research has now focused on genetic factors that could potentially help in predicting these patients, in particular, insult to vascular endothelium and disordered repair mechanisms that could develop intracranial aneurysms.

Methods

We examined the genetic association of multiple candidate gene SNPs thought to be plausibly associated with intracranial aneurysm disease. The only SNP of these 7 that was associated with intracranial aneurysms, was the ACE I/D SNP in our UK-cohort of 1384 ruptured and unruptured intracranial aneurysm patients and 1275 matched-controls. Further analysis was conducted to determine genetic association between ruptured versus unruptured cases. Multiple logistic regression was performed to examine the effects of well-known risk factors; hypertension and smoking on overall genetic association. Meta-analysis was conducted to include our samples in determining robust associations of this SNP with IA disease.

Results

Under an additive genetic model, D allele carriers of the ACE I/D SNP had increased risk of aneurysm rupture (OR 1.14 [1.02-1.28], p=0.02). Multivariable analysis strenthened genetic association when known risk factors; hypertension and smoking were incorporated in to the regression model (OR 1.22 [1.08-1.38], $p_{corr}=3.33x10^{-03}$). Meta-analysis revealed no significant association when all studies were included, however our study was the most powered and only one to demonstrate the D allele to increase the risk for IA.

Conclusions

Our study is the largest candidate-gene association study examining genetic association of the ACE I/D SNP with IA disease. We demonstrated D allele presence to increase risk of IA rupture, contrary to previous findings. Our study suggests a gene-enviornment interaction between hypertension, smoking and the gene variant under a multiple regression model. The ACE gene may confer predisposition to IA through hypertension mediated effects yet to be defined.

6.2 Introduction

Subarachnoid Haemorrhage (SAH) from ruptured intracranial aneurysms (IA) is a devastating form of stroke leading to death or dependency in aprroximately 50% of suffererers.[32] The complex genetic and enviornmental factors in development and rupture of of IA remain poorly understood. Genetic variations in relvant biological pathways show promise to increase understanding of mechanisms (e.g extracellular matrix, endothelial maintenance or inflammation) but most case-control studies have been underpowered or not replicated[204]. Genes involved in the maintenance of vascular endothelium have been examined in relation to IA disease with mixed results. Most of these variants had plausible relationship to aneurysm pathology and have been tested via a candidate-gene approach, the majority prior to the advent of GWAS. Based on our meta-analysis[204] we determined from previous literature which SNPs were associated with IA. We then performed a sub-group analysis based on ethnicity to determine if associations are significant in different populations. We progressed to examine SNPs which could potentially affect vascular endothelial function. These included, the ACE, Jun 2 dimerisation protein, Endoglin, endothelial Nitric Oxide Synthase (eNOS) and Apolipoprotein E (APOE) genes. Despite conflicting results examining these SNPs prior to our study, we aimed to test these genes in the largest UK based Caucasian intracranial aneurysm cohort and determine if these SNPs were robustly associated with intracranial (ruptured or unruptured) aneurysm cases, via a case-control design.

6.2.1 Genes affecting vascular endothelium function

Blood vessels are dynamic organs which respond to acute or chronic changes in blood pressure, flow or metabolism which lead to vascular remodelling.[171] Studying potential genetic variants predisposing to intracranial aneurysm disease with plausible connections to aneurysm development were identified and revealed from our meta-analysis (see chapter 3). Previously, candidate-gene association studies have examined potential credible neurobiological links between aneurysm development and genes involved in regulating vascular endothelial function and tone. Although the exact mechanisms of aneurysm initiation, progression and rupture are yet to be fully delineated, a wide body of evidence exists demonstrating that aneurysm initiation occurs from disruption of vascular endothelial homeostasis and subsequent changes to vessel wall integrity, which will be expanded upon in the forthcoming chapters. Our meta-analysis reviewed the previous candidate-genes that were analysed in association with intracranial aneurysms and replicated these in our large UK cohort, to determine if a robust association is present with intracranial aneurysms.

6.2.2 Endothelial structure and function

The endothelial layer is a monolayer, structurally supported and anchored by subendothelial connective tissue to the *internal elastic lamina*. This layer forms part of the intima, crucial in monitoring and maintenance of vascular homeostasis.[77, 201] There are multiple facets that contribute to endothelial layer breakdown, these may include factors related to haemodynamic force transmission, infiltration and

destruction of the layer by inflammatory cells, or disruption of extracellular matrix components or altered vasomotor tone, such as that caused by the effects of chronic hypertension.

Morphological changes causing damage to the endothelium include; endothelial cell loss, adhesion of inflammatory cells and widening of junctions between cells causing gap formation. Other changes noted in the context of patients suffering with chronic hypertension include; endothelial cell swelling, intimal oedema with subendothelial accumulation of fibrin and infiltration of inflammatory cells. Collectively these changes lead to characteristic prominent intimal thickening and is an attempt of the endothelium to repair itself from haemodynamic related damage. These changes are well characterised in the walls of cerebral aneurysms.[207, 208]

Endothelial cells are responsible for regulating vascular tone, principally through the mediator, nitric oxide (NO) and have important anti-thrombotic and atherogenic properties, as well as responding dynamically to alterations in vascular flow on the luminal surface. Nitric Oxide is produced by endothelial nitric oxide synthase (eNOS) and is protective against arterial disease such as abdominal aortic aneurysms (AAA).[208] Reduced levels of eNOS have been show to correlate with endothelial dysfunction, with increased incidence of intracranial aneurysms in eNOS deficient female mice.[209] However, contrasting results have been demonstrated with a similar incidence of cerebral aneurysms in male mutant and wild type mice,[210] but interestingly, neuronal NOS (nNOS) was upregulated within the walls of intracranial aneurysms in response to reduced levels of eNOS, possibly contributing to the similar incidence of intracranial aneurysms in both sets of mice. This was further tested by determining incidence of intracranial aneurysms in mice that were both eNOS and nNOS knockout, subsequently showing increased incidence of intracranial aneurysms, as well as macrophage infiltration within aneurysm walls, once the nNOS compensatory effect was removed. Whether NO stabilises endothelial dysfunction remains to be determined, these animal studies, demonstrate nitric oxide may have a significant role in intracranial aneurysm pathogenesis.

In response to mechanical insult or haemodynamic stress, the endothelium undergoes an adaptive response whereby smooth muscle cells (SMC) migrate and proliferate at the site of endothelial injury, in a process known as myointimal hyperplasia.[211] This is seen within the walls of unruptured cerebral aneurysms and it is unclear whether this is an adaptive change in response to haemodynamic insult or true weakening of the arterial wall. It is known that high wall shear-stress can cause activation of major inflammatory mediator NF-κB, thereby initiating an inflammatory reaction, involving pro-inflammatory cytokines/genes, including iNOS[212] and MMP[213] as well as release of various chemo-attractants, hence, summoning various cells of the immune system to the site of vascular injury. [214, 215]

Several studies have examined expression of vascular growth factors and their effect on cerebral vessel/aneurysmal walls. In particular, immunostaining of cerebral aneurysm walls for vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).[200] VEGF promotes

vascular bed permeability and has a crucial role in the initiation of angiogenesis and neo-vessel recruitment. Cells including astrocytes, myocytes and fibroblasts can secrete VEGF but its unique target, is the endothelial cell. VEGFs actions include increasing permeability at intercellular junctions resulting in the secondary activation of enzymes involved in ECM breakdown. [200] Other actions are thought to involve microvascular sprouting and arborisation with subsequent maturation of neo-vessels and formation of new capillary tubes,[200] as seen in early atherogenesis and tumour proliferation. Interestingly, several studies have demonstrated increased VEGF expression in haemodynamically stressed AVM vessels.[200] Also, under haemodynamic stress, VEGF has been shown to activate angiogenesis. [200]

6.2.3 Phenotypic modulation

Cerebral vascular smooth muscle cells (SMCs) reside within the tunica media layer of the vasculature, responsible primarily, for contraction, pressure maintenance and regulation of blood flow.[216] SMCs provide structural integrity to the vessel wall, they secrete collagen under normal physiological haemodynamic load, to maintain vascular integrity. Unlike cardiac and skeletal muscle which are terminally differentiated, vascular smooth muscle cells are able to de-differentiate their function from predominantly being contractile in nature to a synthetic, pro-inflammatory phenotype, under certain environmental or genetic triggers.[217-219] These triggers include; local inflammatory factors/cytokines such as TNF-α and IL-1 or as a result of local haemodynamic stress, causing vascular endothelial injury.[220] Recent literature has implicated SMCs phenotypic modulation as contributory in aneurysm initiation, progression and rupture. Areas of myointimal hyperplasia with aggregation of SMCs have been characterised in samples of human of unruptured cerebral aneurysms. This has been shown in experimentally created aneurysms by Frosen and colleagues,[201, 211] where luminal pads of neointimal hyperplasia were present within aneurysm walls, similar to those seen in atherosclerotic lesions.[211] Nakajima and colleagues compared ruptured and unruptured aneurysm walls finding significantly reduced expression of contractile proteins such as smooth muscle-α-actin and myosin heavy chains in the wall of cerebral aneurysms when compared to control cerebral arteries.[217] This group managed to demonstrate a difference in phenotypic expressions between the contractile and synthetic types of SMCs. The contractile vascular SMCs expressed SM1 and SM2 isoforms of myosin heavy chains (MHCs) compared to SMemb produced by synthetic, pro-matrix remodelling SMCs. Interestingly of note, they revealed ruptured aneurysms to have reduced expression of SM1 and SM2 within aneurysm walls, suggesting the phenotypic switch of vascular SMCs to allow for re-modelling of the vessel/aneurysmal wall, in the face of haemodynamic stress. In the same study, they noted Desmin, which is a major intermediate filament, lining Z-lines to the cell nucleus within muscles to be significantly reduced within the walls of ruptured aneurysms compared to non-ruptured. Since Desmin is a crucial component of the cytoskeleton architecture, its reduced presence in aneurysm walls could plausibly result in weakness and predispose to rupture of intracranial aneurysms.

In normal vessel walls, vascular SMCs are arranged in tight compacted parallel formation which are spindle-like, however, SMCs can dissociate from each other and resemble spider like cells, within aneurysmal walls, further proof of SMC de-differentiation.[221]

Kilic[219] *et al* examined expression of structural proteins within ruptured and unruptured aneurysmal walls and noted decreasing staining intensity of contractile proteins (α-actin) in ruptured aneurysms compared to unruptured. In addition, they also noticed angioarchitectural disruption where SMCs were scattered in patchy zones and not in their usual tight compact bands, within the aneurysm walls, possibly reflecting phenotypic modulation.

6.2.4 Apoptosis

The role of apoptosis or programmed cell death is relevant in the context of aneurysmal sac weakening and rupture. It has been suggested that once VSMCs switch their phenotype, this may lead to a terminal event, whereby they completely lose their phenotype resulting in apoptosis and reduced density of VSMCs. This would entail the aneurysmal wall becoming thinner and precariously susceptible to sudden changes in haemodynamic force, subsequently leading to aneurysm rupture. In one study, it has been demonstrated that ruptured aneurysms contained raised numbers of degenerated SMCs and a greater degree of apoptotic bodies, when compared with unruptured aneurysms. Interestingly, apoptotic bodies presence has been noted very close to the point of aneurysmal rupture within the thin wall of the dome. Guo and colleagues[222] found reduced SMC density and increased caspase activity of more than 6-fold, when comparing ruptured aneurysms to control vessels. Caspases are effector enzymes which are markers of apoptosis. This demonstrated apoptosis has a significant involvement in aneurysmal rupture and may represent a pre-terminal event.

6.3 Genetic evidence for dysfunction of vascular endothelium genes

Several association studies examining plausible genes with credible links to vascular endothelial function have been performed with mixed results. Genes examined in our study included, ACE, eNOS, Endoglin, Jun-2 (JDP2) and APOE.

6.3.1 ACE (Angiotensin Converting Enzyme)

Polymorphisms in the ACE (Angiotensin Converting Enzyme) I/D (Insertion/Deletion) SNP have been investigated in six studies[191-195, 223] to date in relation to IA with varying results. Serum ACE is released from the lungs and converts Angiotensin I to Angiotensin II as part of the RAAS cascade, leading to arteriolar constriction and subsequent rise in blood pressure.[194] The ACE I/D SNP affects circulating levels of ACE[224] and has been associated with hypertension.[225] In the latest meta-analysis conducted by the author,[204] the ACE I allele was significantly associated with intracranial aneurysms, with an odds ratio 1.23[1.07-1.4], p=0.003, in five studies. However, another study from China by Liu[223] *et al* showed the D allele to be protective. We sought to determine association between the I/D SNP and

intracranial aneurysms in a large UK Caucasian population. We also meta-analysed, incorporating our data to determine if any robust association exists with cerebral aneurysms.

6.3.2 Endothelial Nitric Oxide Synthase (eNOS/NOS3)

Endothelial (eNOS) Nitric Oxide synthase, also known as NOS3, catalyses L-Arginine to Citrulline, producing Nitric Oxide (NO),[226] which is an important vascular relaxing factor in response to shear stress, inhibiting platelet and monocyte adhesion, as well as preventing SMC proliferation.[227, 228] Adversely affected levels of NO caused by genetic polymorphisms could promote endothelial dysfunction and herald development of intracranial aneurysms. The eNOS gene is located on chromosome 7q35-36. Multiple polymorphisms exist within this gene, with 3 in particular that have been tested in association with intracranial aneurysms. These include the T786C (rs2070744) SNP present in the 5' flanking region of the eNOS gene, potentially reducing promoter activity and hence NO production.[229] The second being G894T (rs1799983) SNP present in exon 7, thought to reduce eNOS activity.[230] The last being a 27- basepair VNTR (Variable number Tandem Repeat) at intron 4 which affects basal NO generation.[231] Accounting for all three SNPs, between 7-10 articles have been published examining this SNP in association with IA, with mixed and conflicting results. This may reflect that studies had been poorly designed, with limited numbers and were not replicated in similar populations and that multiple ethnicities were tested, including US Caucasian, European, Indian and Chinese. Our meta-analysis also did not demonstrate any significant association either, when combining all studies for each SNP, or when stratifying them by ethnicity. In our study, we examined 2 SNPs (T786C and G894T) in association with a UK Caucasian cohort, the first to be done, in order to determine if an association existed or not, in a well-powered study.

6.3.3 Endoglin

The Endoglin gene codes for a transmembrane glycoprotein that is mostly present on vascular endothelial cells. It is present within chromosome 9q34 and mutations within it cause Hereditary Haemorrhagic Telangiectasia (HHT) and Arteriovenous Malformations (AVMs). Endoglin helps maintain vascular integrity by acting as a co-receptor to TGF-β. The first association found with intracranial aneurysms was with the 6 basepair insertion (6bINS) polymorphism in a small Japanese population.[232] However, subsequent studies in German[233], Japanese,[234] US Caucasian,[235] Polish[236] and Korean[237] populations did not find association with intracranial aneurysms. Onda *et al*[234] further examined 4 SNPs in close proximity to the Insertion polymorphism of the Endoglin gene, including the rs1998923 (T>C) SNP in Intron 1 of the Endoglin gene in 104 affected sibling pairs and 179 sporadic cases of IA. This was to confirm if any other SNPs elsewhere within the gene were associated with intracranial aneurysms. This also, demonstrated no association, confirming robustly no association of this gene with intracranial aneurysms. Our meta-analysis[204] also demonstrated no association of this

SNP with intracranial aneurysms. However, we examined the rs1998923 (T>C) SNP in a UK Caucasian population for the first time, based upon its function.

6.3.4 Jun-2 Dimerization Protein (JDP2)

Chromosomal loci 14q22 was found to be associated in 104 Japanese affected sibling pair intracranial aneurysm cases in a previous genome-wide linkage study.[126] This prompted further investigation into plausible candidate genes in this chromosomal region, with possible effect on aneurysm development.[120] From this screening, the Jun dimerization protein 2 gene (JDP2), present on Chromosome 14q24 was identified. The JDP2 gene represses the transcriptional activator protein 1 (AP-1), by dimerising a component of AP-1, the c-Jun protein, thereby preventing its transcriptional activation.[238] AP-1 affects vascular remodelling via regulation of gene expression of downstream molecules and influencing apoptosis. Loss of smooth muscle cells from apoptotic pathways have been shown in cerebral aneurysm specimens previously.[239] Expression of MMPs, nitric oxide synthase (NOS) and adhesion molecules are affected by AP-1, which are known to impact aneurysm development.[240-242] Moreover, stimulation of AP-1 by pro-inflammatory mediators leads to increased expression of MMPs which can influence aneurysm development. SNP variations within the JDP2 gene can cause aberrant signalling with increased AP-1 activation, possibly linking to intracranial aneurysm development.[120]

The study by Krischek[120] examined the rs175646 (C>T) SNP with intracranial aneurysm association in Dutch, Japanese and Korean populations. They demonstrated this SNP to be associated with Japanese and Korean populations but not in the Dutch populations. When meta-analysing this SNP in these 3 populations, there was an overall significant association with intracranial aneurysms (OR 1.26 [1.09-1.44], P<0.001), with moderate statistical heterogeneity. Subgroup analyses incorporating only the Japanese and Korean populations demonstrated a stronger association with intracranial aneurysms (OR 1.46 [1.214-1.761], p<0.0001), with no significant statistical heterogeneity. These varying results reflect the differing risk allele frequencies between populations for this SNP and many others, hence, why we replicated this study in a large UK Caucasian population.

6.3.5 Apolipoprotein E (APOE)

APOE is thought to play an important role in maintaining lipid homeostasis within the CNS[243, 244] and resisting oxidative stress form free radicals on mitochondria.[245, 246] The APOE gene is present on chromosome 19q13 and has been implicated in many diseases, in particular, Alzheimer's disease and cardiovascular disease. [247-250] The direct relation to IA disease remains unclear but it is thought that lipid dysregulation and presence of oxidative species could potentially damage vascular endothelium, predisposing to intracranial aneurysms.[250] The APOE gene has 3 alleles – ε2, ε3 and ε4 which encode three different isoforms of the protein, each differing by a single amino acid.

Inconclusive results of APOE as a genetic risk factor exist, with initial reports of an association by Kokubo and colleagues in a small Japanese rural population, carrying the e4 allele.[250] Subsequent studies, did not demonstrate an association of APOE with occurrence of IA.[109, 251, 252]

Kaushal and colleagues did not find association of any of these APOE alleles with SAH occurrence but they demonstrated association at the haplotype level in the 5' promoter and 3' region of the APOE gene, in a US Caucasian population.[139] This highlighted, the flaws with candidate-gene association studies, in not being able to scan large parts of a gene, which may harbour causal variants to the condition in question. We sought to demonstrate if an association of the APOE alleles existed in our UK Caucasian cohort of intracranial aneurysm patients and adding our data to the previous meta-analysis to see if any robust conclusions could be made.

6.4 Methods

The study was conducted following aprroval of our local ethics committee (REC 3). All cases of angiographically-proven intracranial aneurysm (IA), whether ruptured or unruptured were prospectively recruited into the study from 20 neurosurgical centres across England. Those patients with non-aneurysmal SAH, such as trauma, peri-mesencephalic SAH, mycotic aneurysms and arterio-venous malformations (AVM) were excluded from the study. In addition, patients with known inherited connective tissue disease, such as Marfan's, Ehlers-Danlos syndrome and adult polycystic kidney disease (ADPKD) were also excluded. We estimated those patients suffering from hypertension who were on >1 anti-hypertensive agent at time of initial presentation. Smoking status was confirmed whether patients were; current, ex or non-smokers. We also determined the number of pack years for each subject recruited into the study. Control DNA were utilised from the 1958 Wellcome cohort[2] and 297 Multiple-System Atrophy (MSA) patients with autopsy reports confirming no cerebral aneurysms from our biobank. Polymerase Chain Reaction (PCR) was under taken using KASP (KBioscience Competitive Allele-Specific) genotyping assays at LGC Genomics bio-laboratories. The KASP primer mix which is assay-specific for each SNP was mixed with a universal KASP master mix, then added to DNA samples. Following PCR thermal cycling, an end-point fluorescent read is conducted using labelled allele-specific forward primer dyes, to perform bi-allelic discrimination.

To confirm whether genotyping methods were accurate, we compared results of genotyping of 91 samples that were previously analysed by Morgan[154] *et al* and included and re-genotyped in our study. The genotypes were identical for all 91 samples, confirming a high level of accuracy.

Statistical analysis was conducted on European Caucasian samples, using R[198] and PLINK[199] software. Chi-sqaured tests, with odds ratio calculations were performed for each SNP, under an additive genetic model. Controls were also checked for Hardy-Weinberg Equilibrium (HWE).

Further analysis was conducted to determine if each SNP was differentially associated with cases based upon rupture risk. Smoking and hypertension status were also tested in association with those SNPs that

reached statistical significance. Following this, a meta-analysis was conducted using REVMAN[167] and Comprehensive Meta-analysis (CMA) software.

6.5 Results

1619 cases from the total 2523 recruited were available for genetic analysis. Demographic data are presented in table 6-1, with a highly significant proportion of sufferers of cerebral aneurysms being female (OR 2.32 [1.996-2.705], $p=1.59x10^{-27}$). The total number of controls was 1299. Following quality control (QC) for genotyping errors (<1%) and removal of non-Caucasian samples, the total number of cases and controls available for statistical analysis are presented in table 6-2 for each SNP. Controls were confirmed to be in Hardy-Weinberg Equilibrium (HWE), with p-values also presented in table 6-2. The ACE D allele was significantly associated with cerebral aneurysm presence under an additive (see table 6-2 and 6-3) genetic model (OR 1.14 [1.02-1.28], p=0.02). The genotype counts for the ACE SNP are presented in table 6-3 with results of the subgroup analysis, based on rupture status. Association testing of the remaining six SNPs in this category; eNOS 894 (rs1799983), eNOS T786C (rs2070744), Endoglin (rs1998923), JDP2 (rs175646), APOE-ε4 (rs429358) and APOE-ε2 (rs7412) did not demonstrate any significant association with intracranial aneurysms (see table 6-2), even when breaking down analysis into ruptured and ruptured cases.

Table 6- 1 – Patient demographic data

	Cases	Controls
Number	1384	1275
Female (%)	948 (68.5%)	624 (48.9%)
Mean Age (years)	54.08	57.58
Current smokers (%)	626 (45.2%)	258 (20.2.%
Hypertensive (%)	433 (31.3%)	190 (14.9%)
Ruptured aneurysms (%)	1054 (76.2%)	

Table 6- 2 – Association testing for each SNP involved in Vascular endothelial maintenance

SNP	Gene	Total cases	Cases analysed **(Caucasian + after QC)**	**Total Controls**	**Controls analysed (HWE P-value)**	**Ruptured**	**Unruptured**	**OR [CI]**	**P-value**
I/D rs1799752	ACE	1607	1384	1299	1275 (0.194)	1054	330	**1.14 [1.02-1.28]**	**0.02**
rs1799983 (G>T)	eNOS 894	1619	1405	1241	1199 (0.44)	1067	338	0.95 [0.85-1.07]	0.41
rs2070744 (T>C)	eNOS 786	1619	1597	1241	1200 (0.8)	1077	339	0.99 [0.89-1.11]	0.89
rs1998923 (C>T)	Endoglin	1619	1429	1241	1209 (0.53)	1087	342	0.65 [0.38-1.25]	0.198
rs175646 (C>T)	Jun-2	1619	1430	1241	1209 (0.12)	1088	342	1.4 [0.98-1.98]	0.06
rs429358	APOE – e4	1619	1413	1241	1207 (0.27)	1077	336	1.71 [0.96-3.04]	0.067
rs7412	APOE- e2	1619	1409	1241	1221 (0.23)	1081	328	1.15 [0.92-1.43]	0.23

Table 6- 3 – Genotype and allele counts and association of the ACE I/D SNP according to IA rupture status.

	Cases	Controls
DD	421	336
DI	690	656
II	273	283
D Allele	1532	1328
I Allele	1236	1222
	Association tests (Additive model)	
	OR [95%CI]	**p-value**
All aneurysms	1.14 [1.02-1.28]	0.02
Ruptured aneurysms	1.15 [1.02-1.29]	0.02
Unruptured aneurysms	1.14 [0.95-1.35]	0.15

Subsequent sub-phenotype analysis demonstrated the ACE I/D SNP to be significantly associated with ruptured (1.15 [1.02-1.29], p=0.02) but not unruptured (1.14 [0.95-1.35], p=0.15) aneurysms (see table 6-3).

Following adjutment, regression analysis demonstrated independent (without genotype) associations of known risk factors with IA presence; hypertension (OR 2.08 [1.71-2.52], $p=1.89x10^{-13}$) and smoking (OR 2.44 [2.02-2.88], $p=2.16x10^{-22}$) repectively. Neither hypertension (p=0.8) or current smoking (p=0.61) status confounded the genetic association of the ACE I/D SNP with intracranial aneurysm presence significantly (see table 6-4). Interaction between these risk factors and the genetic variant were tested for, demonstrating minimal increase in the additive association of the ACE I/D SNP, (OR 1.23 [1.08-1.40], $p=1.55x10^{-03}$), with ruptured intracranial aneurysms (see table 6-4).

Table 6- 4 – Multivariable logistic regression and interaction terms for ruptured intracranial aneurysms

Variable	OR [CI]	p-value	Interaction p-value
Hypertension*	1.64 [1.12-2.4]	$1.08x10^{-02}$	0.8
Smoking status*	2.53 [1.79-3.56]	$1.13x10^{-07}$	0.61
ACE I/D SNP	1.23 [1.08-1.40]	$1.55x10^{-03}$	-

*Interaction terms between risk factor and ACE I/D SNP under additive genetic effects.

Meta-analysis was conducted on all studies investigating the ACE I/D SNP with intracranial aneurysms. In total 7 studies (including this one) were initially included, five of which were in Caucasian populations, with the other 2 studies conducted in Japanese and Chinese populations. The overall meta-

analysis results (figure 6-1) and those of caucasian studies only are presented below (figure 6-2). The remaining SNPs in this category were meta-analysed with the exception of Endoglin as the genotype counts nor odds ratiors in sporadic cases examined were available to pool into our anlysis. All meta-analyses were conducted with studies that had Caucasian participants, with incorporation of our data. All 6 SNPs in this category did not demonstrate significant association with IA following meta-analysis (See table 6-5).

Table 6- 5 – Meta-analyses of all 6 SNPs analysed in the vascular endothelial category including our study.

SNP	Gene	Meta-analysis*	P-value	Studies	Cases/Controls
I/D rs1799752	ACE	1.01 [0.90-1.13]	0.31	5/7	4742/5334 **(4136/4686)**
rs1799983 (G>T)	eNOS 894	0.93 [0.81-1.06]	0.16	3/7	1598/1479 **(1405/1199)**
rs2070744 (T>C)	eNOS 786	1.01 [0.88-1.15]	0.89	3/9	1603/1474 **(1416/1200)**
rs175646 (C>T)	Jun-2	1.124 [0.94-1.34]	0.194	2/4	2329/2448 **(1796/1881)**
rs429358	APOE –e4	1.18 – [0.8-1.44]	0.254	4/6	1890/3343 **(1749/2094)**
rs7412	APOE- e2	1.12 [0.9-1.31]	0.13	4/6	1886/3343 **(1745/2094)**

Number in bold represent meta-analysis numbers of cases and controls in Caucasian studies only.

Figure 6- 1 – ACE I/D meta-analysis with all 7 studies

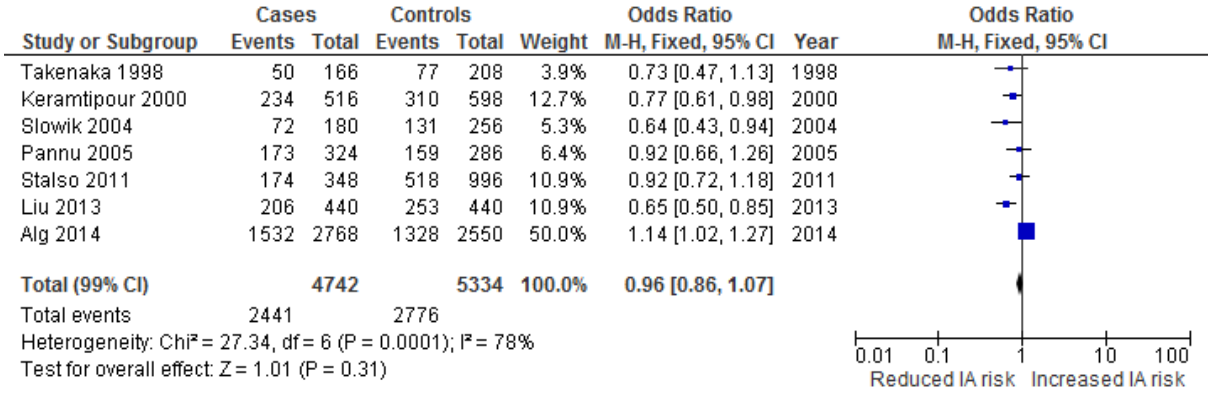
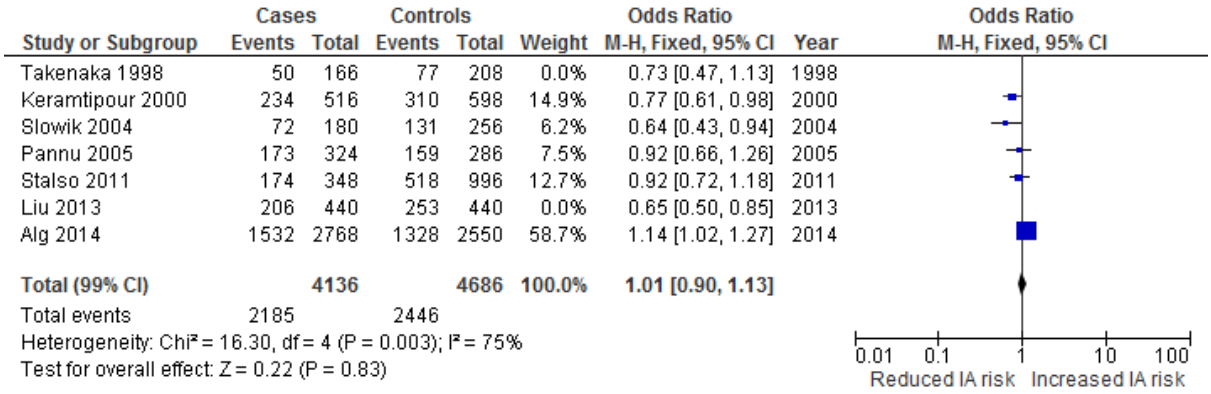


Figure 6- 2 - ACE I/D meta-analysis with studies with Caucasian subjects



With all seven studies analysed, the results are suggestive but not significant of a protective effect of the D allele under the additive (figure 1) model (OR 0.96 [0.86-1.02], p=0.31). However, once both the Chinese[223] and Japanese[191] studies were removed (see figure 2), the results suggested an increased although not significant risk for cerebral aneurysm formation (OR 1.01 [0.90-1.13], p=0.83). However significant statistical heterogeneity exists (p=0.003, I^2=75%). On subsequent sensitivity analysis by removing our study, there was an overal significant protective association with cerebral aneurysms and the remaining 6 studies on the ACE I/D SNP (OR 0.78 [0.67-0.91], p=0.0001). There was no significant heterogeneity (p=0.33, I^2=13%).

6.6 Discussion

Our results revealed an association of the ACE I/D - D allele with intracranial aneurysm risk under an additive genetic model in a large prospective UK multi-centre Caucasian population. Our results are in contrast to a much smaller UK based study including 234 cases[192] which described an association of the I allele with intracranial aneurysms. Moreover, all six previous studies on the I/D SNP have shown the D allele or DD+DI genotypes to be protective for intracranial aneurysms. However, our meta-analysis

overall and including only Caucasian cohorts, did not confirm a statistically significant association of the ACE I/D allele with IA. This may reflect differences in the minor allele frequencies (MAFs) between studies and comparibility of different populations. Of note, the previous UK[192] study demonstrated a MAF in cases of 0.45 for the D allele compared to 0.55 in the present study. From reviewing all the previous ACE I/D studies in our meta-analysis, heterogeneity exists in MAF for this SNP, but interestingly the Chinese[223]and Japanese[191] studies demonstrated a reduced MAF for the D allele in cases ranging from 0.3 – 0.47, as did three[192, 193, 195] of the four Caucasian studies. The study[194] conducted on Caucasians in the USA, demonstrated a smilar AF for the D allele – 0.54 as our study. This may reflect closer genetic sharing between US and UK populations, despite no association of this SNP with intracranial aneurysm presence in the US study. However, previous studies may have been underpowered to detect association of intracranial aneurysm with the D allele. This is highlighted when comparing our study with our counterparts in Cambridge.[192]

The (rs1799752) SNP is characterised by the Insetion/Deletion of a 287-bp sequence present within intron 16 of the ACE gene, which lies on chromosome 17q23.[253] The ACE I/D polymorphism was initially detected by restriction fragment length polymorphism (RFLP) analysis.[203] The first polymerase chain reaction (PCR)-based detection of this polymorphism was reported by Rigat *et al,*[254] who used a set of primers flanking the insertion sequence.

Mean ACE activity levels in DD carriers were approximately twice that found in II genotype individuals.[203] The ACE I/D SNP is in high linkage disequilibrium with other SNPs in the same region that may also have an affect on serum ACE activity.[253] Limited knowledge exists regarding how the ACE gene can influence aneurysm pathology directly, but local renin-angiotensin systems (RAS) have been shown to influence vascular remodelling. Ohkuma *et al*[53] demonstrated reduced local expression of RAS (measured by mRNA expression of the ACE gene and angiotensin I products) in the aneurysmal walls of ruptured and unruptured cases compared to cortical arteries of control patients. Continuous haemodynamic stress could reduce vascular remodelling, thereby preventing reactionary thickening of arterial walls in response. This could partly explain the thinning of the medial layer present in cerebral aneurysms, due to loss of smooth muscle cells as observed by this study.[53] Lesser vascular remodelling capacity of the I versus the D allele may cause the arterial wall to protectively thicken under increased haemodynamic stress.[193] The pathophysiology behind the ACE I/D SNP and aneurysm formation or rupture is unclear and yet to be fully delineated. However, angiotensin II is known to act as a potent pro-inflammatory mediator[202, 255] and this could provide an alternate pathway in aneurysm formation, possibly leading to local inflammation and destruction of arteries at sites of highest haemodynamic stress, leading to abnormal repair mechanisms.

Despite the D allele or DD genotype not being associated with intracranial aneurysms prior to our study, work carried out on abdominal aortic aneurysms (AAA) have shown the D allele to be significantly

associated with aneurysm risk. A recent meta-analysis conducted by Song[202] *et al*, showed that in under 7000 subjects the D allele was indeed associated with all types of aortic aneurysm in European Caucasians but not Asian populations. Of note, the D allele has also been associated with stiffer abdominal aortas in men between the age of 70-88 years compared to I allele carriers.[256] This may lead to aneurysmal disease, but caution needs to be drawn as there was no information on risk factor profiles to check if there was a gene-environment interaction. One study on patients with aortic dissection demonstrated significant association with D allele carriers (DD or DI) of this SNP.[257] Slowik[193] *et al* also demonstrated the DD genotype to be a significant risk factor in the development of intracerebral haemorrhage, of which hypertension is known to be well associated with.

ACE converts the inactive angiotensin I to angiotensin II, which promotes vasoconstriction and stimulates smooth muscle growth.[258] Angiotensin II also causes release of Aldosterone from the adrenal cortex, promoting water retention and subsequent blood pressure rise. ACE metabolises the potent vasodilator Bradykinin, which is part of the kinin-kallikrein cascade, thereby further raising blood pressure.[253, 259] Our results suggested an association of hypertension with D allele carriers (OR 1.64 [1.12-2.4]) under a multiple logistic regression model, but the interaction with genotype was non-significant (p=0.8). Despite these findings, no linkage[260] or genome-wide association studies have implicated the ACE locus in hypertension. Three meta-analysis[224, 261, 262] were conducted to determine association of the I/D SNP and hypertension but none showed significant findings. The 1st meta-analysis[261] did however, demonstrate a 10% increase risk of hypertension in those who were of DD genotype, although not significant in 6923 subjects, with significant heterogeneity.

6.6.1 Other vascular endothelial SNPs examined

Despite the significant association shown of the ACE I/D SNP with our UK cohort, the other SNPs in this category (See table 6-2) did not show significance with intracranial aneurysm patients in our population. Despite these negative findings, our study provides important insight and statistical power to investigate previous associations with Caucasian subjects. When comparing our study on each of these SNPs, our study is overpowered to detect an effect, meaning more robust conclusions can be made whether these SNPs are indeed associated with intracranial aneurysms. Previous studies may not have been powered enough in Caucasian populations to determine a robust effect. This does not mean that studies in non-Caucasian intracranial aneurysm sufferers can be ignored either, as the different associations may reflect differing minor allele frequencies. However, our study and subsequent meta-analysis has not demonstrated significant associations for these SNPs, making them less likely to be involved in intracranial aneurysm pathology. In order to further strengthen these findings, similarly designed studies, with similar power, will be required in Caucasian and non-Caucasian populations to effectively determine if a true association exists. It can be argued that our approach is a hypothesis driven approach based on anecdotal evidence or previous chance associations, and that next-generation

sequencing, such genome-wide association studies or Exome sequencing may be required to detect as yet undiscovered novel variants, in a completely non-hypothesis driven approach.

6.6.2 Conclusions

Our study demonstrates an overall association of the D allele with cerebral aneurysms. This is in contrast to previous studies examining this SNP with intracranial aneurysm presence, which have demonstrated the I allele to be associated with intracranial aneurysms. This may be related to factors such as; sample size or different MAFs between populations. The strength of this study is the large numbers of cases and controls analysed compared to previous studies. Our meta-analysis[204] subsequently demonstrated that this SNP was not robustly associated with intracranial aneurysm presence but this may reflect the addition of our study in demonstrating the D allele to be associated with intracranial aneurysms compared to all other studies. To better address the robustness of our findings, similarly powered studies will need to be conducted to allow direct comparison.

Future studies should aim to look at serum co-markers of ACE gene activity, such as ACE and Bradykinin levels to see if these correlate with genotype. It would be plausible to sequence the entire ACE gene or flanking regions of the I/D variant, to determine if other unknown variants are in linkage disequilibrium or associated with intracranial aneurysms. Indeed, this data may already be present from previous GWAS studies conducted on intracranial aneurysm disease and it may prove useful to enter into collaborations. In addition, we would aim to determine ACE gene expression and local RAS activity by examining mRNA profiles from aneurysmal tissue. Further studies in different ethnicities would be required of similar power to ours, in order to determine robust associations between this SNP and intracranial aneurysm disease.

Chapter 7 - Genetics and Observational Study on Subarachnoid Haemorrhage (GOSH): Extracellular matrix (ECM) candidate SNPs associations with intracranial aneurysms

7.1 Abstract

Introduction

Intracranial aneurysm (IA) are prevalent in 2-6% in the general population.[1] The development, progression and subsequent rupture of some of these aneurysms are yet to be fully understood, with many plausible pathways postulated. One particular pathway of aneurysm development gaining interest, is that of enzymes involved in extracellular matrix (ECM) remodelling. If balance is not present than enzymes favouring destruction of components could outweigh those that inhibit destruction, predisposing one to aneurysm formation or rupture. Increasingly more genetic studies are aiming to determine if variants in extracellular matrix genes favour aneurysm formation.

Methods

We examined the genetic association of 12 SNPs involved in coding for various extracellular matrix (ECM) genes. The rs243865 C>T SNP in the MMP-2 gene of our UK-based aneurysm cohort of 1409 cases and 1290 matched controls was the only SNP associated with intracranial aneurysms. We determined if this variant was differentially associated with ruptured versus unruptured aneurysm cases. Synergy between well-known risk factors; hypertension and smoking with this variant was determined to assess if there was an overall risk increase of intracranial aneurysms. We also carried out a meta-analysis on previous studies examining these SNPs to confirm or refute previous associations and determine if any difference existed between ethnicities.

Results

Under an additive genetic model, those with the T allele for the MMP-2 rs243865 SNP had increased prevalence of cerebral aneurysm (OR 1.18 [1.04-1.33], p_{corr}=0.0168). Multi-variable analysis did not strengthen any association. Meta-analysis revealed this SNP to be significantly associated (OR 1.174 [1.042-1.323], p=0.008) in Caucasian populations only. The remaining 11 ECM SNPs tested in our UK cohort were not significantly associated with intracranial aneurysms.

Conclusions

Our study is the 1st Caucasian based cohort to determine an association between this functional ECM gene variant and aneurysm rupture. This leads on from previously described reports of increased MMP-2 expression in the walls of human cerebral aneurysms. Future mapping of the entire MMP-2 gene would be beneficial to determine if other unknown variants within the gene contribute to disease.

7.2 Introduction

Approximately 2-5% of the general population harbour an intracranial aneurysm[1]. Predicting rupture risk remains challenging for clinicians treating the disease, in an attempt to offset subarachnoid haemorrhage and its potentially devastating sequelae. Trials have previously been conducted to determine rupture risk based upon aneurysm size and location,[72, 263] but with no real consensus developed as when to treat aneurysms. In addition to size, risk factor profiles have been studied in patients who are hypertensive, smokers, female gender or have positive family history to determine whether these better predict rupture risk.[35] However, despite increased prevalence of aneurysms in patients exhibiting these risk factors[1], it is still unclear which individuals would benefit from early screening of aneurysms.

The pathogenesis of intracranial aneurysms remains poorly understood and in order to gain a better insight into the process of aneurysm formation, focus has shifted to possible genetic risk factors of aneurysms. Certain approaches include determining genetic association of candidate-genes of known biological function with a plausible relationship to aneurysm formation. Genes which effect vascular remodelling, inflammatory processes and extracellular matrix components (ECM) have been extensively studied in relation to cerebral aneurysm formation. We investigated the association of the 12 ECM gene single nucleotide polymorphisms (SNPs) in ruptured and unruptured aneurysms in a large UK Caucasian population.

7.2.1 ECM constituents with intracranial arteries and normal histology

Cerebral arteries consist of 3 layers: intima (innermost layer), Tunica media and Tunica adventitia (outermost layer). A single stratum of endothelial cells lines the intimal layer, directly in contact with the vessel lumen, which is separated from the Tunica media layer by subendothelial connective tissue and the internal elastic laminae (IEL). Concentric sheets of smooth muscle cells (SMCs) and collagen fibres (mostly Type III) define the medial layer. A key difference between cerebral and normal vasculature is the lack of external elastic laminae in the former when proceeding to the adventitial layer. The Adventitia is composed of organised network of Elastin, fibroblasts, nerves, Vasa Vasorum and Type I collagen which is arranged circumferentially towards the media and graduates to a longitudinal orientation on the outer aspects.

The ECM within the arterial wall is a dynamic and complex meshwork of proteoglycans and proteins, designed to provide structural support and aid activities such as cell differentiation, proliferation and migration. Components of the ECM include; elastin, collagen, fibronectin, laminin, nidogen, perlecan and syndecans. Collagens Type 1 and III account for 80-90% of total arterial collagen, providing tensile strength the blood vessel. The remaining 10-20% of collagen within the vessel wall include; Types IV-VIII.

Elasticity and stretching ability of vessels is provided by Elastin which provide 90% and microfibrillar glycoproteins provide 10% of elastic fibres.

The Glycoproteins Fibronectin and Laminins make up the rest of the ECM. These glycoproteins serve to connect collagen fibres to cells to allow transmigration of cells within the ECM. They also assist in cell adhesion, signalling and binding to platelets at the site of vascular injury.

7.2.2 Intracranial aneurysm histology

Compared with normal intracranial arterial walls, ECM structures show a typically disorganised structure with, decreased cellular components, myointimal hyperplasia, loss of endothelial cell layer integrity. [207, 264, 265] One study, demonstrated admixture of Fibronectin and type I collagen within IA walls, when they are normally limited to the media and adventitia respectively. [266] Also noted, is reduction in collagen content within aneurysms.

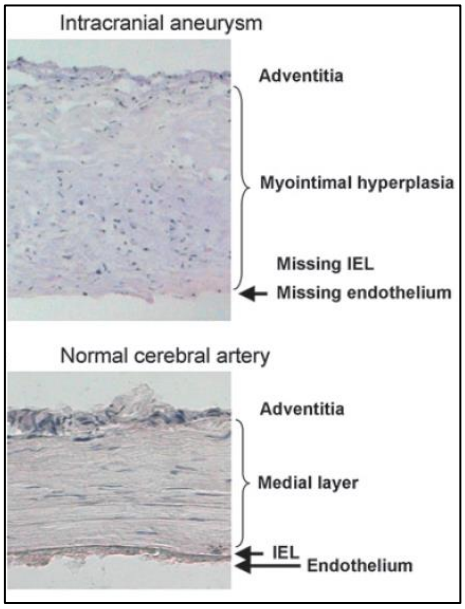
Importantly, smooth muscle cells (SMCs) are principally associated with contraction but can be affected by environmental stimuli, which can trigger phenotypic switching from a contractile to pro-inflammatory synthetic state. This switch is associated with vascular remodelling, where there is decreased expression of contractile proteins. [201, 211, 217]

7.2.3 Differences between ruptured and unruptured

Imbalance between synthesis and degradation of ECM and various elements within the vascular wall are responsible for IA formation. Medial thinning, attenuated biosynthesis of ECM and exaggerated degradation are hallmarks of IA development (see figure 7-1).

Key pathological hallmarks described in ruptured aneurysms include; pronounced endothelial damage, thin hyalinsed and hypocellular walls. [264] Frosen *et al*, demonstrated 4 histological entities that correlated with aneurysm rupture. Type 1 – Endothelialised walls with linearly organised smooth muscles – which 42% ruptured. Tube B – Thickened wall with disorganised SMCs – 55% ruptured. Type C – Hypocellular wall with myointimal hyperplasia – 64% ruptured. Type D – Thin, hyalinised and hypocellular wall – 100% ruptured (See figure 7-1).[201, 211]

Figure 7- 1 - Adapted from Tulamo et al: Differences between normal cerebral vessel wall and aneurysm walls: a review.[267]



7.2.4 Mechanisms of IA development and key mediators

A balance between the synthesis and degradation of ECM products is vital for vessel wall structural integrity. However, it remains unclear how certain risk factors such as, age, female gender, smoking, hypertension and previous history of SAH increase the risk of IA disease directly. Current knowledge suggests abnormal vascular homeostatic mechanisms, in combination with predisposing risk factors and environmental stimuli can influence IA development. Coupled to this, genetic studies in large family pedigrees with IAs and recent GWAS and candidate gene studies have suggested genetic factors in addition can increase risk of IA. How all these factors interplay and lead to the overall IA phenotype is yet to be determined.

From a molecular viewpoint, dysregulation of proteolysis, apoptosis, inflammation and haemodynamic stress have been shown to contribute to vessel wall degermation and aneurysm formation and subsequent progression and rupture. [216, 268]

Matrix metalloproteinases (MMPs) are proteinases that are responsible for breakdown of ECM components, such as Elastin and Collagen. They are important mediators in vascular cell re-modelling, they can be released and activated by various inflammatory cells/molecules, fibroblasts and smooth muscle cells in response to vascular insult. They are opposed by tissue metalloproteinase inhibitors (TIMPs). Bruno *et al*, demonstrated increased areas of focal expression of MMP-2 and MMP-9 in both ruptured and unruptured aneurysms. Also noted by the same group, was the presence of Cathepsin-D cells at areas of where collagen had been degraded. Cathepsin D is an endopeptidase which also has ECM degrading properties. [205]

7.2.5 Genetic evidence in relation to genes involved ECM maintenance

Multiple SNPs within genes coding for ECM components has been tested in association with intracranial aneurysms. Mixed results were obtained in individual populations when looking at plausible associations of these SNPs. These could be attributed to underpowered studies or differences in risk allele frequencies in different ethnicities, or different SNPs within those genes that are as yet untested. Based on this, we performed a meta-analysis (Chapter 3),[204] combining all results from each study to determine which SNPs coding for ECM components were associated with IA. The results and functional significance of these results are presented in Chapter 3 of this thesis. In summary, our meta-analysis demonstrated six ECM SNPs to be associated with IAs, including COL1A2 (rs42525), COL3A1 (rs1800255) encoding genes within type I and III collagen respectively, HSPG2 – perlecan gene (rs3767137), CSPG2 (rs251124 & rs173686) – Versican and SEPRINA3 (rs4934) genes. These SNPs were examined in a limited number of populations, with varying outcomes, consisting of Japanese, Dutch and Chinese. Our study examined these SNPs for the first time in a large UK population, excluding the CSPG2 (Chondroitin Sulphate Proteoglycan 2) SNP rs173686. The HSPG2 (Heparan Sulphate Proteoglycan 2) gene is located on chromosome loci 1p34.3 which was previously implicated in a linkage association study in a single large North American family with multiple intracranial aneurysm members.[269] Plausible genes associated with intracranial aneurysm pathology within this locus included Perlecan gene. This gene encodes a large basement membrane proteoglycan – Heparin Sulphate proteoglycan, which is thought to strengthen cell adhesion by interacting with other ECM components like, collagen type IV, laminin and fibronectin. [270, 271] The Versican gene also encodes for proteoglycans that strengthen cross-linking of molecules in the basement membrane of intracranial vessels in particular fibronectin. Mutations in both perlecan and versican genes could predispose one to intracranial aneurysms and has been previously associated with Dutch and Japanese intracranial aneurysm populations.[150, 151] Another plausible candidate gene examined previously included the SERPINA3 (Serpin Family A Member 3, otherwise known as α1-antichymotrypsin) rs4934 A>T SNP, which can effect expression of this gene, where TT carriers are thought to have increased risk of aneurysmal SAH. This is due to SERPINA3 being an inhibitor of Cathepsin G, specifically from neutrophils. Cathepsin G degrades ECM, increasing vessel wall fragility and potentially ripening conditions for aneurysm development. Therefore, mutations within this gene could predispose to intracranial aneurysm formation or rupture. Previously a positive association of this SNP with intracranial aneurysms was found in a Polish population,[183] however, this was not replicated in Japanese or Chinese ethnicities.[184, 185]

In addition to these SNPs, we examined the following genes; Fibrillin, Elastin, COL4A1, SERPINE1 and two SNPs from the MMP (matrix metalloproteinase) class; MMP-2 and MMP3 were tested in our study (see table 7-2). Fibrillins are macromolecules which assemble around Elastin providing structural

support. Mutations in this gene may affect assembly of ECM molecules around Elastin and weaken arterial walls, by affecting the Internal elastic lamina (IEL) which is lost within cerebral aneurysms. The Fibrillin gene is located on chromosomal loci 5q23-q31 which overlaps a previously associated loci 5q22-31 that was found in a linkage study, examining a large Japanese family pedigree affected by intracranial aneurysms.[126] The Fibrillin rs33107 SNP has been previously associated with intracranial aneurysm patients in a Dutch population[150], hence, making this a plausible candidate gene.

Intracranial aneurysm association with SNPs within the Elastin gene have generally shown conflicting results. Linkage analysis in 84 sibling pairs with intracranial aneurysms in a Japanese population demonstrated association with the 7q locus, which harbours the Elastin gene.[126] Two Elastin SNPs were subsequently tested for in a Dutch population and association was found with sporadic cases of intracranial aneurysms.[129] However, Hofer and colleagues[138] demonstrated no such association of these Elastin SNPs with their central Caucasian European intracranial aneurysm populations. These discrepancies could be explained in part due to genetic heterogeneity between Japanese and European populations. The Japanese study had significant family history in their study and that overall too few numbers were tested, limiting the statistical power to draw meaningful conclusions.

SERPINE1 inhibits MMPs, thereby limiting the degradation of collagen and other ECM molecules, as well as repressing plasmin, which converts the inactive pro-MMP to active MMP.[272] In patients with AAA, the 4G allele of the SERPINE1 SNP was underrepresented, causing the effect of lower levels of SERPINE1 to inhibit MMPs.[273, 274] The only study to date examining this SNP in an intracranial aneurysm population was conducted by Ruigrok and colleagues who demonstrated an association of this SNP, in 382 Dutch Caucasian patients.[150]

MMPs are zinc and calcium dependent proteinases responsible for breakdown of extracellular matrix proteins like, elastin, collagen and laminin, as part of vascular remodelling.[205] They are controlled by tissue inhibitor matrix metalloproteinase (TIMPs) which inhibit the MMPs [205]. Imbalance between these two enzyme systems could hypothetically predispose one to cerebral aneurysm formation or rupture, whether this is the case is yet to be defined.[275] MMP-2 and 9 have been consistently shown to be greatly expressed within cerebral aneurysmal tissue samples[147, 205, 276-279], significantly MMP-2 more so in those that are ruptured.[277, 280] It is unknown if haemodynamic forces within the cerebral vasculature affect local vascular remodelling by increasing expression of the MMPs thereby predisposing to aneurysm formation. However, wall shear stress (WSS) as a result of change in vascular tone does affect vascular remodelling. [279]

The rs243865 C>T MMP-2 SNP has been previously studied in relation to cerebral aneurysms in US Caucasian[148] and Japanese[206] populations, with conflicting results. A recent meta-analysis[204] demonstrated no significance of this SNP with cerebral aneurysm presence. Despite this, MMP-2 has been implicated in several reports, to play a role in vascular remodelling leading to cerebral aneurysm

formation. We determined for the first time, if an association of this SNP existed in a large UK-based intracranial aneurysm population.

As can be appreciated, all these SNPs show mixed results in association with intracranial aneurysms or indeed other conditions, in general. This could be due to, (i) Genuine findings, (ii) underpowered studies, (iii) different risk allele frequencies between different ethnicities, (iv) controls not being in HWE or representative of the population being studied, (v) different genotyping technologies, (vi) mutations being related to other co-morbidities that participants may have, such as hypertension or diabetes, (vii) the tested SNPs not being representative of true polymorphisms elsewhere within the same gene, either in the untranslated regions, intronic or exonic portions of the gene in question, i.e. these SNPs not being in strong linkage disequilibrium with other as yet unknown variants.
Our study aimed to confirm or refute previous findings from examination of these 12 SNPs for the first-time a in a large UK Caucasian cohort. Following this we meta-analysed each SNP with results from existing data to determine the strength of any association.

7.3 Methods

Cases with sporadic angiographically-proven ruptured or unruptured intracranial aneurysms were included in this study over a 3-year period. Patients were recruited from 20 Neurosurgical centres treating aneurysm patients, excluding non-aneurysmal causes for SAH, such as AVMs (arterio-venous malformations), trauma, peri-mesencephalic SAH or mycotic aneurysms and those with known inherited connective tissue disorders, such as Marfan's, Ehlers-Danlos syndrome and adult polycystic kidney disease (ADPKD). Control subjects DNA was drawn from the 1958 Wellcome cohort[2] and 297 Multi-System Atrophy (MSA) patients with no cerebral aneurysms as confirmed from post-mortem reports. To minimise population stratification, cases which were Caucasian only were included in the analysis.
Polymerase Chain Reaction (PCR) was under taken using KASP (KBioscience Competitive Allele-Specific) genotyping assays at LGC Genomics bio-laboratories, Hoddesdon, UK. The KASP primer mix which is assay-specific for the each of the 12 SNPs being tested, was mixed with a universal KASP master mix, then added to DNA samples. Following PCR thermal cycling, an end-point fluorescent read is conducted using labelled allele-specific forward primer dyes, to perform bi-allelic discrimination.
Statistical analysis was conducted on European Caucasian samples, using R[198] and PLINK[199] software. Chi-sqaured tests, with odds ratio calculations were performed for this SNP, under an additive model. Further analysis was conducted to determine if SNPs was differentially associated with cases based upon rupture risk. Smoking and hypertension status were also tested in association with associated SNPs. P-values were corrected for multiple testing via Bonferroni correction. Following this, a meta-analysis was conducted using REVMAN[167] and CMA software. Controls were also checked for Hardy-Weinberg Equilibrium (HWE).

7.4 Results

A total of 1609 cases and 1299 controls were recruited for this study. Quality control for genotyping errors was less than 1% and subsequent removal of non-Caucasian samples left a total of, 1409 cases and 1290 controls available for statistical analysis. Controls were in HWE (p=0.5004). Demographic data are presented in table 7-1. Twelve candidate-gene SNPs thought to be linked with ECM dysfunction were examined in our cohort. Table 7-2 provides a summary of basic genetic association data for each of the 12 SNPs analysed. Only one SNP was associated with intracranial aneurysm presence in our UK cohort out of these 12 SNPs.

The rs243865 C>T SNP was significantly associated (OR 1.18 [1.04-1.33], p_{corr}=0.0168) in our UK aneurysm cohort under an additive genetic model (see table 7-2 and 7-3). The results of the other SNPs tested are summarised in table 7-2.

Table 7- 1 – Demographic data

	Cases	**Controls**
F:M	962:447	636:654
Mean Age	54.14	57.66
Current smokers	621	258
Hypertensive	448	191
Ruptured aneurysms	1074	
Unruptured Aneurysms	335	

Table 7- 2 – ECM candidate SNPs investigated in our cohort

SNP	Gene	Total cases	Cases analysed (Caucasian + after QC)	**Total Controls**	**Controls analysed (HWE p-value)**	**Ruptured**	**Unruptured**	**OR [CI] (corr)**	**P-value (corr)**
rs243865	MMP-2	1619	1420	1241	1208 (0.58)	1083	337	**1.18 [1.04-1.33]**	**0.012**
rs3025058 (5A/6A)	MMP-3	1619	1389	1241	1170 (0.11)	1057	332	0.94 [0.85-1.05]	0.305
rs1799768 (4G/5G)	SERPINE-1/PAI-1	1619	1414	1241	1201 (0.83)	1075	339	0.96 [0.86-1.07]	0.42
rs4934 (G>A)	SERPINA-3	1619	1410	1241	1200 (0.13)	1073	337	1.00 [0.9-1.12]	0.98
rs3767137 (G>A)	HSPG2	1619	1415	1241	1201 (0.91)	1078	337	1.002 [0.89-1.13]	0.97
rs42524 (G>C)	COL1A2	1619	1400	1241	1209 (0.092)	1065	335	1.04 [0.91 - 1.18]	0.58
rs285678 (G>A)	Elastin	1619	1419	1241	1202 (0.71)	1077	342	1.08 [0.91-1.28]	0.38
rs331079 (G>C)	Fibrillin	1619	1405	1241	1207 (0.31)	1069	336	1.07 (0.91-1.27)	0.431
rs251124 (C>T)	Versican	1619	1406	1241	1202 (0.81)	1068	338	0.997 [0.86-1.15]	0.97
rs2621215 (T>G)	COL1A2	1619	1414	1241	1195 (0.15)	1078	336	1.1 [0.93-1.3]	0.26
rs3783107 (C>T)	COL4A1	1619	1396	1241	1189 (0.49)	1061	335	1.05 [0.94-1.17]	0.403
rs1800255	COL3A1	1619	1403	1241	1203 (0.17)	1066	337	1.03 [0.91-1.17]	0.62

Table 7- 3 – Genotype counts and calculated odds ratios and significance for the MMP-2 SNP

	Cases	**Controls**
CC	748	758
CT	573	456
TT	88	76
C Allele	2069 (0.73)	1972 (0.76)
T Allele	749 (0.27)	608 (0.24)
	Basic Association tests (Additive)	
	OR [95%CI]	**Corrected p-value (BH FDR)**
All aneurysms	1.18 [1.04-1.332]	0.0168
Ruptured aneurysms	1.18 [1.03-1.34]	0.02
Unruptured aneurysms	1.17 [0.97-1.61]	0.106

Sub-group analysis, demonstrated significant (1.18 [1.03-1.34), p_{corr}=0.02) association of the MMP-2 SNP with ruptured aneurysms only (see table 7-3).

Despite highly significant independent (without genotype) association of hypertension and smoking risk factors with cerebral aneurysms, when fitted into a multiple logistic regression with the MMP-2 genotype, the risk factors did not confound the basic genetic association with ruptured aneurysm cases (see table 7-4). When testing for statistical interaction between the risk factors; hypertension (p=0.5) and smoking (p=0.2) did not modify genetic association for the MMP-2 SNP. Despite unruptured aneurysms having similar odds ratios to ruptured cases, the result was non-significant, probably as a result of fewer numbers of unruptured compared to ruptured cases (1074 Vs 335) recruited.

Table 7- 4 – Multivariable logistic regression and interaction terms

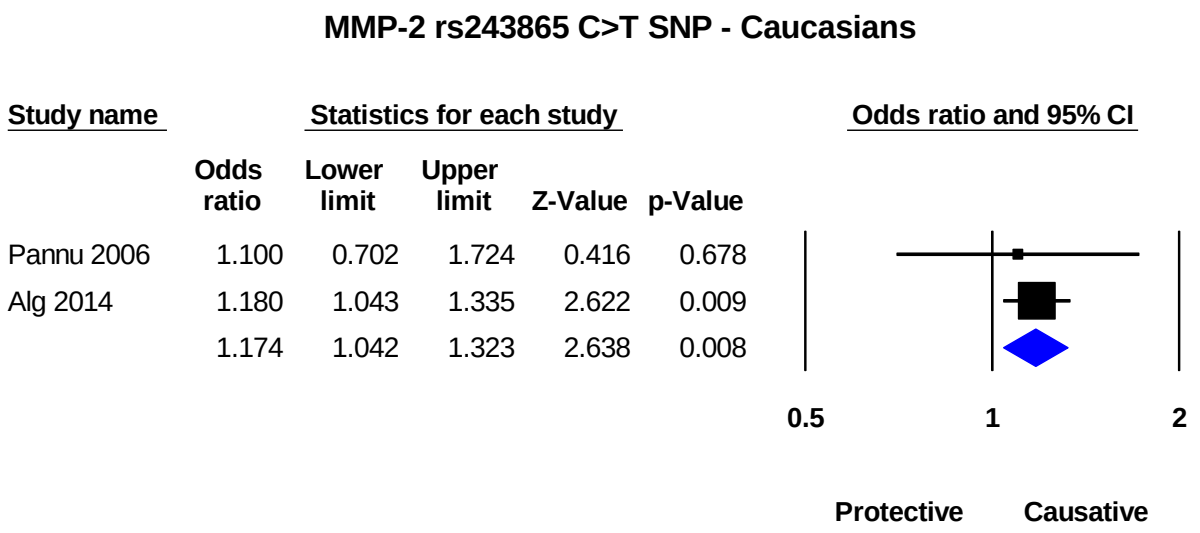
Variable	**OR [CI]**	**p-value corrected**	**Interaction p-value**
Hypertension	1.74 [1.41-2.15]	$5.145x10^{-07}$	0.50
Smoking status	2.76 [2.29-3.32]	$5.31x10^{-26}$	0.2
MMP-2 SNP	1.16 [1.01-1.35]	$4.20x10^{-02}$	-

Meta-analysis of all SNPs

We added the previous two studies examining this SNP with our data and carried out a meta-analysis. The study by Pannu[148] was conducted on a US Caucasian, and the study by Low[206] was in a Japanese population. In a previous meta-analysis of the 2 earlier studies,[204] the rs243865 SNP was not

significantly associated with aneurysm presence. With addition of our study there was a significant association of this SNP although marginal (1.12 [1.01-1.23], p=0.03). We further meta-analysed factoring ethnicity, and included both Caucasian studies (see figure 7-2).[148] Overall association increased (OR 1.174 [1.042-1.323], p=0.008), suggesting a stronger link of this SNP in Caucasian populations. The Japanese study far outweighs both Caucasian studies in numbers of cases and controls to make this a good comparison between the ethnicities. There was no significant publication bias (Egger p-value=0.78) nor statistical heterogeneity (p=0.768, I^2=0%).

Figure 7- 2 – Meta-analysis of Caucasian studies on the MMP-2 rs243865 SNP



Despite only 1 out of the 12 SNPs analysed being associated with intracranial aneurysm presence in our UK cohort. Six additional SNPs (3 collagen SNPs, one perlecan, one versican and one fibrillin gene) that were subsequently meta-analysed demonstrated significant association with intracranial aneurysm presence in Caucasian subjects (See table 7-5). These significant associations were only significant when taking into account European Caucasian studies only and not when combing all population from different backgrounds. For each SNP significantly associated from the meta-analysis, by inclusion of our data, there was no statistical heterogeneity or publication bias.

Table 7- 5 – Meta-analysis of ECM candidate gene SNPs.

SNP	Gene	Meta-analysis*	P-value	**Studies**	**Cases/Controls**
rs243865	MMP-2	**1.12 [1.01-1.23]**	**0.038**	3	1545/1442 **(1420/1208)**
rs3025058 (5A/6A)	MMP-3	0.95 [0.83-1.09]	0.33	3	1535/1493 **(1389/1170)**
rs1799768 (4G/5G)	SERPINE-1/PAI-1	0.98 [0.87-1.12]	0.74	4	1654/1740 **(1414/1201)**
rs4934 (G>A)	SERPINA-3	0.98 [0.86-1.12]	0.69	4	1590/1463 **(1410/1200)**
rs3767137 (G>A)	HSPG2	**1.1 [1.003-1.22]**	**0.043**	4	2099/2135 **(1415/1201)**
rs42524 (G>C)	COL1A2	**1.19 [0.99-1.44]**	**0.01**	4	1726/1398 **(1400/1209)**
rs2856728 (G>A)	Elastin	1.50 [0.90 – 1.22]	0.52	3	1763/1672
rs331079 (G>C)	Fibrillin	**1.16 [1.008-1.33]**	**0.039**	3	2089/2141 **(1405/1207)**
rs251124 (C>T)	Versican	**1.14 [1.03 – 1.26]**	**0.014**	4	2022/1841 **(1405/1202)**
rs2621215 (T>G)	COL1A2	1.11 [0.95-1.30]	0.08	4	1726/1398 **(1400/1209)**
rs3783107 (C>T)	COL4A1	**1.11 [1.012 – 1.21]**	**0.026**	3	2080/2123 **(1396/1189)**
rs1800255	COL3A1	**1.16 [1.02-1.32]**	**0.004**	3	**1949/3438**

* Caucasian studies result only. (Caucasian study numbers in bold)

7.5 Discussion

Our results demonstrate increased risk of cerebral aneurysms in those which are T allele carriers of the MMP-2 rs243865 SNP. Our study is the 1st study to show an association between this SNP and intracranial aneurysms under an additive genetic model. Meta-analysis revealed this SNP to be significantly associated in Caucasian populations, despite our study being significantly larger than that of Pannu and colleagues.[148] Compared with the Japanese study,[206] which had a greater number of cases and controls, our study demonstrated a significant Caucasian association with this SNP. Despite this, the remaining 11 SNPs (see table 7-2) did not show significant association with intracranial aneurysms in our UK cohort. This may represent a true effect, represented by the size of our study compared to previous ones and also different ethnicities were some of these SNPs, were previously associated.

Interestingly, following our meta-analysis, six further SNPs (see table 7-5) were associated with IA presence in Caucasian populations following the addition of our study. As can be seen from table 5 the numbers in bold represent, the number of Caucasian subjects analysed which is significantly higher and more powered to determine an effect than a single study alone. This is despite those 6 SNPs not being associated with our UK cohort, in isolation. This highlights the problem with testing common variants as higher numbers of cases are required to robustly determine a modest association with intracranial aneurysms. Caution will need to be drawn in interpreting this result. This may represent an overall significant association in Caucasians but will need replication in further cohorts and with similar numbers to our study for these SNPs to be robustly associated with intracranial aneurysms. Also, as of yet we do not know if these SNPs tested are in strong linkage disequilibrium with other as yet unknown variants that could be functional in nature. Therefore, full sequencing of these genes in large intracranial aneurysm cohorts would be beneficial in the future and help address these questions.

The rs243865 C>T MMP-2 SNP is within an intergenic segment of the MMP-2 gene present on chromosome 16. The family of 20 MMPs are calcium and zinc dependent enzymes capable of ECM breakdown as part of vascular remodelling.[205] MMP-2 is thought to be the major gelatinase responsible for the breakdown of various other components of the extracellular matrix (ECM), such as laminin, fibronectin and elastin.[205, 279] MMP-2 and MMP-9 have been implicated in vascular remodelling on human aneurysm and animal model samples.[276, 277, 279, 281] MMPs are known to be released by vascular endothelial, smooth muscle and inflammatory cells, particularly macrophages.[148] Bruno[205] *et al* demonstrated increased MMP-2 and 9 expression within human cerebral aneurysm walls taken from clipping compared to control basilar artery tissue taken from autopsy specimens. Unruptured aneurysms also had increased MMP-2 activation with ubiquitous remodelling present, suggesting dynamic changes in otherwise previously thought static lesions. In the same study, Plasmin was also observed to be overexpressed in aneurysm tissue and it is known that plasmin can activate various MMPs including MMP-2. Caird *et al*[278] also demonstrated increased MMP-2 levels within non-atherosclerotic aneurysm tissue. In a series by Jin[147] *et al*, overexpression of MMP-2 and MMP-9 genes in ruptured versus unruptured aneurysms was demonstrated, suggesting they may have a pivotal role in aneurysm rupture. Jin[147] also showed there was an imbalance between these enzymes and TIMPs (tissue inhibitor matrix metalloproteinases) –which inhibit MMPs. TIMP-2 specifically inhibits MMP-2 and it was found that the ratio between them was in disequilibrium, in favour of MMP-2 expression, which was more predominant in ruptured tissue than MMP-9. These findings may indicate that TIMPs are adaptive in their response to MMPs in limiting deleterious vascular remodelling, it may be plausible that genetic factors such as the rs243865 SNP variant or others associated with both enzyme systems may lead to under/overexpression, predisposing to aneurysm formation. This remains to be elucidated. Anecdotal evidence also exists of MMP-2 and other MMPs being upregulated in human aneurysm tissue on a non-branching segment of an artery but not the parent artery.[276] Again, this provides strong evidence in

implicating MMPs in vascular remodelling and subsequent aneurysm formation. To further support the role of MMPs in aneurysm development or rupture, MMP-2 levels in the serum of aneurysm patients have been significantly elevated compared to controls. Todor[277] *et al* in following up the work of Bruno[205] *et al* demonstrated pro-MMP-2 – the inactive version of MMP-2 being raised and they further confirmed that the gelatinolytic activity observed *in vitro* was indeed due to MMP-2 activity. This finding has been later supported by Francis[282] *et a*l, who also found significantly higher serum concentrations of MMPs and other proteolytic enzymes in cerebral aneurysm patients.

Despite various studies implicating MMPs and in particular MMP-2 in vascular remodelling and aneurysm formation. It remains unclear the instigating trigger leading to activation of MMPs and TIMPs in abnormal vascular remodelling, giving rise to cerebral aneurysms. Some reports suggest changes in local haemodynamic forces may cause increased wall shear stress (WSS), as dynamic vascular remodelling takes place to adapt the vessels in the face of pressure changes, as plausible in hypertensives. This could activate the MMPs and if unregulated – due to as yet to be identified factor(s), may predispose one to aneurysm formation.[279] Another theory is if local inflammation occurs within vessel walls in response to release of inflammatory cytokines such as TNF-α, in the face of increased haemodynamic forces.[282] It has been shown that various inflammatory cytokines and white cells, particular macrophages are expressed within the walls of aneurysm tissue.[201] TNF-alpha can activate MMP-2 further compounding the effect on the vessel wall. Recently, an important intracellular secondary messenger, NF-κB, has been shown to be differentially expressed in aneurysm tissue. This messenger signals pro-inflammatory cytokine cascades and subsequent activation of the gelatinases involved in ECM breakdown.[279, 280]

A mouse model of intracranial aneurysms has been previously developed closely mimicking histopathological features of sporadic human aneurysms. By combining angiotensin II infusions to cause hypertension and a single elastase injection, aneurysms were formed in the mouse vasculature in a dose-dependent manner. Our study attempted to determine if hypertension or smoking present in aneurysm cases could magnify the overall the genetic association seen with the MMP-2 SNP, but this was not demonstrated, as the odds ratio hardly changed. It is unclear whether hypertension could trigger aneurysm formation in our population based upon direct activation of MMP cascades, or if there are separate non-haemodynamic effects, which may trigger inflammation and leukocyte migration in aneurysm tissue, as a result of smoking or not.[281] Interestingly from this study, Doxycyline- an MMP inhibitor, reduced activity of MMPs and led to a reduction in the incidence of intracranial aneurysms, potentially providing a future pharmacological target.

Despite our finding, it is not clear whether this variant or others as yet unknown with strong linkage disequilibrium within the MMP-2 gene increase the risk of intracranial aneurysm development. This is a limitation to our study and future research might be better served if the entire MMP-2 gene is sequenced as well as the promoter regions, to determine if a number of variants exist predisposing to

intracranial aneurysm development or rupture. Pannu and colleagues[148] demonstrated MMP-9 gene variants to be significantly associated with cerebral aneurysms and not MMP-2, which may reflect a difference in allele frequencies of these variants between US and UK Caucasian populations. However, larger studies with greater statistical power would need to be conducted to confirm this. The Japanese study did not demonstrate an association with this SNP and intracranial aneurysms, and due to the comparable sizes of studies, this may reflect different genetic aetiologies within different ethnic groups, and that the rs243865 SNP may be more prevalent in Caucasian populations.

Future studies in addition to looking for genetic variants, could perform microarray expression studies on human aneurysm tissue harvested from neurosurgical clipping to determine the expression levels of various MMPs, TIMPs and inflammatory cytokines within the aneurysmal tissue. In addition to this, it may be prudent to test for serum markers of these enzymes in order to provide a clearer picture on pathobiology of intracranial aneurysm disease and correlate this with genetic variants observed in a particular population.

7.5.1 Conclusion

We have demonstrated the 1st genetic association of the MMP-2 SNP variant rs243865 in a UK Caucasian population. This supports various biochemical and histopathological research in determining increased expression of MMPs, in particular MMP-2 levels in cerebral aneurysms. Despite no evidence of a significant gene-environment interaction between this variant and known risk factors in our study, this does not rule out the possibility of unknown pathways or mechanisms linking smoking or hypertension to trigger a signalling cascade, causing an excessive breakdown in ECM components, as caused by MMP-2 activation. Future studies investigating the MMP genes need to conducted in more ethnicities to determine if there are heterogeneous variants leading to overall aneurysm disease presentation.

Chapter 8 - Inflammatory candidate genes and association with intracranial aneurysms in our UK GOSH cohort

8.1 Abstract

8.2 Introduction

2-5% of the general population harbour an intracranial aneurysm. A small proportion rupture developing SAH, a potentially devastating outcome for people in their middle ages. Therefore, recognising which patients are likely to harbour an aneurysm or develop aneurysmal SAH becomes critical. The development of intracranial aneurysms is poorly understood but molecular and gene expression studies have demonstrated the presence of various inflammatory cells within the walls of cerebral aneurysms, particularly ruptured. A question arises, what could cause such an inflammatory reaction within aneurysm walls and does it lead to rupture? Our study examined 3 candidate gene inflammatory SNPs in a large UK intracranial aneurysm cohort to determine if association existed.

8.3 Methods

We examined three SNPs, from the TNFα, IL-1β and IL-6 genes to determine if a significant association was present in our large UK cohort of >1600 cases and 1200 controls. We also conducted a meta-analysis to determine if our data combined with previous studies could demonstrate any robust association with intracranial aneurysms.

8.4 Results

All the SNPs were not associated with intracranial aneurysms in our cohort (see table 8-2). They did not reach statistical significance either when conducting the meta-analysis (see table 8-5).

8.5 Conclusions

Despite our study not demonstrating association with these 3 inflammatory gene variants, this does not exclude other unknown variants in the same genes, or other inflammatory genes being associated with cerebral aneurysms. We have added a significant contribution to the existing body of evidence in refuting association of these particular SNPs with intracranial aneurysms. However, more studies examining these SNPs and sequencing large portions of inflammatory genes will be required to draw firm conclusions of inflammatory gene involvement with cerebral aneurysm development or rupture.

8.6 Introduction

In many acute and chronic inflammatory diseases, the inflammatory reaction is aseptic, involving no pathogen. Moreover, inflammation occurs in many diseases as a result of tissue injury caused by a variety of triggers/insults, and as part of wound healing and scar formation.

Inflammation was first suggested to occur in cerebral aneurysms by Virchow in 1847.[283] Further evidence comes from the 1930s when Maass[133,134] described round cell infiltration, most likely lymphocytic, predominantly in the intracranial aneurysm neck. Later studies have confirmed presence of inflammatory cell infiltrates and mediators within the intracranial aneurysm, that can contribute to vessel wall remodelling and possible rupture. In recent years, chronic inflammation has been increasingly implicated as contributing to intracranial aneurysmal development and rupture. This evidence has stemmed from molecular, biochemical, expression profiling and genetic analysis of excised aneurysm tissue.

Evidence of activation of the innate and humoral immunological responses within human intracranial aneurysms exists by the presence of; macrophages, T and B cells, immunoglobulins and complement with membrane attack complexes (MACs).[201, 211]

Despite the presence of inflammatory cells within aneurysmal walls, the trigger and co-ordination of an inflammatory response is not fully understood. Commonly accepted stages in aneurysm formation include; initial endothelial injury/dysfunction as a result of haemodynamic forces and shear stress, followed by an inflammatory cell response and VSMC phenotypic modulation to a pro-inflammatory state, resulting in ECM remodelling, cell death and subsequent vessel wall degeneration. [171, 284]

Wall shear stress modulates pro-inflammatory transcription factor NF-κB activity, leading to activation of and up-regulation of monocyte chemotactic protein – 1 (MCP-1) expressed on endothelial cells, which is pivotal in recruiting; monocytes, macrophages, T-cells, basophils, Natural Killer (NK) cells as well as vascular cell adhesions molecule-1 (VCAM-1), to the site of vascular injury/insult.[267, 285] Interestingly, MCP-1 and VCAM-1 are not expressed in normal control arteries.[286, 287]

Other inflammatory mediators up-regulated by NF-κB include; TNFα and COX-2. NF-κB has a prominent role in regulating the immune response, particularly in response to stress, free radicals, cytokines and oxidatively modified low-density lipoprotein (oxLDL), by transactivation of genes related to endothelial function. Aoki[288] *et al* demonstrated by blocking NF-κB in rats who underwent experimentally induced intracranial aneurysms, the incidence significantly reduced. They also noted reduced macrophage infiltration and down-regulation of inflammatory genes.

8.6.1 Inflammatory cells present within intracranial aneurysms

Histopathological analysis has demonstrated multiple inflammatory cells present within aneurysmal tissue, ruptured or unruptured. Analysing ruptured aneurysms, it is unclear whether inflammatory infiltrate presence is due to the aneurysm developmental process or as a consequence of rupture. Macrophages and neutrophils infiltrate vascular endothelium at the site of injury very early and co-ordinate the immune response.[289] Previously one study examining 23 unruptured and 2 ruptured aneurysm walls, demonstrated presence of monocytes/macrophages and T-cells, scattered throughout the aneurysmal wall, rather than at a specific focal point.[286] Frosen and colleagues demonstrated

evidence of macrophage and leukocyte infiltration in 24 ruptured and 42 unruptured cerebral aneurysms. Ruptured aneurysms showed prominent areas of leukocyte infiltration compared to unruptured specimens, who also did not demonstrate any myointimal hyperplasia or thrombosis.[211] The presence of leukocytes has been shown to be associated with damage of smooth muscle cells and collagen fibres, predominantly in ruptured aneurysms (40/40 specimens) and partly in unruptured specimens (10/20). This strongly suggests that Leukocytes and their mediators contribute significantly to aneurysm growth and rupture.[264] Following rupture, thrombus within the aneurysmal sac can stimulate inflammatory cell migration to aid vessel wall repair, therefore a direct causal relationship between aneurysm rupture and inflammation would be difficult to ascertain in this situation. However, inflammatory cells are present within unruptured aneurysms and it is yet to be fully determined if chronic inflammation plays a key role in aneurysm development, or subsequent rupture. Macrophages can programme cell-death via Fas ligand activation through TNFα mediation, which could signal a pre-terminal event for aneurysm rupture.[290] This has yet to be proved conclusively within aneurysmal tissue, but it is known that macrophages secrete ECM degrading proteinases and they can induce fibrosis via release of TGF-β, TNFα and superoxides.[290, 291] In one study, macrophage depleted mice displayed a lower incidence of intracranial aneurysm suggesting a crucial role of these cells in aneurysmal development.[292]

Other inflammatory cells noted within aneurysmal tissue include; neutrophils, mast cells, lymphocytes and thrombocytes. Neutrophils migrate to the site vascular injury and secrete pro-inflammatory mediators such as; MMPs, peroxidases and cytokines: IL-1β and TNFα. These all serve to promote inflammation within the aneurysmal sac by reducing collagen biosynthesis.[285, 293] Walls of ruptured aneurysms have a significant population of mast cells.[294, 295] Mast cell involvement in cerebral aneurysm development is based upon release of pro-inflammatory cytokines and expression and activation of MMPs following degranulation.

As demonstrated by Frosen *et al*, thrombus formation is present within intracranial aneurysms and contributes to wall degeneration by possible oxidative stress due to the release of peroxidases by trapped neutrophils within the fibrin meshwork.[201] Intact vascular endothelium prevents formation of thrombosis on the luminal surface through the actions of NO (nitric oxide) and prostacyclin. Thrombocytes are attracted by exposed collagen if there is breach of an intact endothelium. Angiogenic and vascular growth factors such as, platelet-derived growth factor (PDGF), VEGF and TGF-β are released, thrombocytes degranulate, which in turn affect SMC growth, ECM integrity and increases permeability of the endothelial layer. As a result, plasma proteins (complement and antibodies) and lipids diffuse into the aneurysm wall. This can potentially trigger apoptosis, further weakening the aneurysmal wall. Platelets and neutrophils release MMPs and elastase which cause further degradation of the aneurysmal wall and further invade the vascular wall, till they are indistinguishable (see figure 8-1). [267, 296]

8.6.2 Inflammatory mediators/growth factors released by cells within aneurysmal tissue

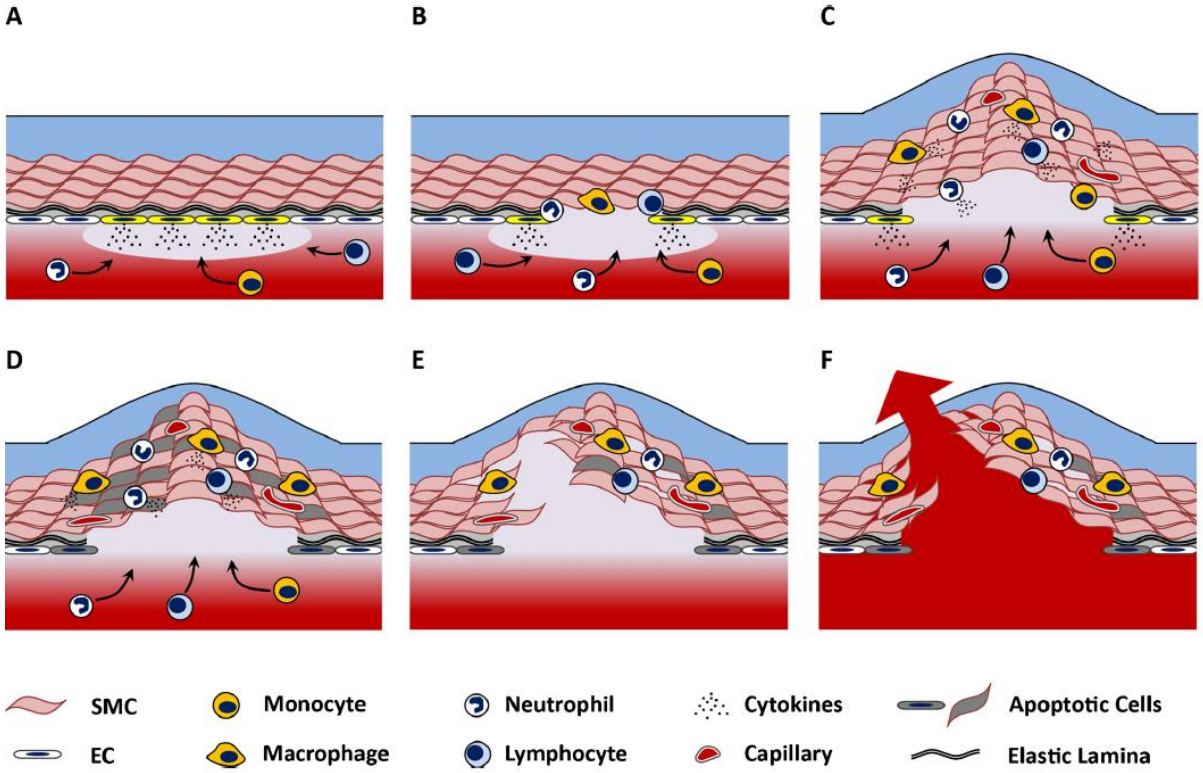
Multiple inflammatory mediators and molecules are expressed from various cell types involved in intracranial aneurysm disease. Some of these molecules are secreted by multiple different cell lines and can potentially accentuate the inflammatory reaction within aneurysmal tissue. It is still unclear how the complex interplay of these factors along with their cell lines are co-ordinated to eventually determine aneurysmal phenotype.

Inflammatory cytokines such as, IL-1β, IL-6 and TNFα have been implicated in aneurysmal pathology. These cytokines can be produced by endothelial, vascular smooth muscle cells and Leukocytes in response to vascular insult or as a direct propagation of the inflammatory response. These cytokines may promote increased blood brain barrier permeability into the aneurysm wall by upregulating acute phase protein synthesis as part of the systemic inflammatory response. IL-1β reduces collagen biosynthesis and inhibits lysl oxidase – the enzyme which crosslinks collagen, thereby affecting aneurysm initiation and development at both transcriptional and post-transcriptional levels.[293, 297] IL-1β knockout mice demonstrated lower percentage of advanced experimentally induced cerebral aneurysms but the incidence remained unchanged, suggesting that IL-1β may affect aneurysm progression but not initiation.[298]

TNFα, another pro-inflammatory cytokine released by macrophages has been shown to trigger apoptosis via the Fas-associated death domain protein pathway within cerebral aneurysms. The same group demonstrated that raised TNF levels within human intracranial aneurysms correlated with increased expression of Toll like receptors that lead to reduced expression of TIMP-1 – which inhibit MMPs and also affect innate immunity.[299, 300] TNFα also activates MMP potentially contributing to cerebral aneurysm development. Well known risk factors associated with intracranial aneurysms; gender, smoking, alcohol, age and hypertension have been linked with TNFα induction.[301-305]

Importantly, direct causal link between these well-known risk factors and intracranial aneurysms is also not fully understood but there have been several hypotheses regarding their effects on inflammation within aneurysm walls.

Figure 8- 1 – Inflammatory reaction, progression and rupture of intracranial aneurysms.



A – Upregulation of pro-inflammatory genes and cytokine expression from Endothelial cells (EC) following haemodynamic stress/triggers to recruit inflammatory cells. **B** – Internal Elastic Laminae (IEL) degeneration and endothelial damage caused by shear stress and infiltration of inflammatory cells. **C** – Smooth muscle cell (SMC) proliferation caused by increased level of cytokines and matrix-degrading collagenases and proteinases promoting aneurysm formation. **D** – further aneurysm progression from increased inflammatory cells and mediators. **E** – Apoptosis of SMCs causing structural weakness within aneurysm walls. **F** – imbalance of remodelling forces and inflammatory responses, leads to aneurysm rupture. *Adapted from Hosaka et al*[285]

8.6.3 Genetic evidence of inflammatory genes involvement in IAs

The genetic basis for inflammatory mediator involvement in the development of IAs has stemmed from human and animal histopathological, biochemical and molecular studies, where plausible relationships between dysfunction in certain inflammatory mediator genes could potentially lead to the development or rupture of cerebral aneurysms, through aberrant mediator signalling. Gene expression profile studies have shown upregulation of mRNA coding for TNFα, INL-1β, IL-6 cytokines and chemokines IL-8, MCP-1 within intracranial aneurysmal tissue when compared to normal cerebral vessels.[298, 299] It is not clear in what order these cyto/chemokines are activated but it has been suggested they are activated in a hierarchical manner, with TNFα being the central co-ordinator of the immune response.[300]

Specifically, candidate-gene analysis has examined polymorphisms within TNFα, IL-6 and IL-1β genes to detect if variants within these genes confer a greater risk of IA disease, possible aneurysmal rupture and provide a link to previous biochemical and histopathological research implicating the inflammatory process in intracranial aneurysm disease.

8.6.3.1 TNFα

Results from genetic association studies of these cytokines has been conflicting. The polymorphism TNFα 308 G>A polymorphism (rs1800629) present on the promotor region of chromosome 6p21.3 has been associated with higher levels of TNFα expression in multiple autoimmune disease states.[306, 307] Therefore in line with previous knowledge of TNFα being a pro-inflammatory cytokine, which is present in aneurysmal tissue and the potential deleterious effects, make it a plausible candidate SNP for aneurysm pathogenesis. Indeed, if intracranial aneurysm patients are subject to higher levels of TNFα as determined by the A allele of this SNP, this may predispose sufferers to greater risk of aneurysm development or rupture. The only previous study was by Fontanella and colleagues, who examined this SNP and risk of aneurysmal SAH in 171 cases and 144 controls. They demonstrated that the GG genotype was more prevalent in aneurysmal SAH cases compared to being heterozygous or AA. They concluded that the GG phenotype was associated with SAH, despite the minor allele A being associated with higher TNFα expression.[308] Firm conclusions on association of this SNP could not be made due to this being a single study, in Italian subjects without replication in other populations and the relatively small number of cases and controls. The same result was seen when analysing the same SNP in relation to abdominal aortic aneurysms (AAA) patients.[309]

8.6.3.2 IL-1 and IL-6

Interleukins – 1 and 6 gene polymorphisms have been studied in intracranial aneurysm patients, with conflicting results. Both cytokines are pro-inflammatory and have been implicated in intracranial aneurysm pathology. IL-1 can reduce collagen biosynthesis and prevent crosslinking leading to a weakened vessel wall. This cytokine can also affect vessel wall remodelling by inducing MMPs and promoting vascular smooth muscle cell apoptosis.[310, 311] Two previous studies[312, 313] examined the IL-1β C511T gene polymorphism in relation intracranial aneurysms. Slowik[312] *et al* demonstrated an association of this SNP with intracranial aneurysms in 231 cases and 231 controls in a Polish Caucasian population.

Fontanella[313] attempted to replicate these findings but no association was seen with intracranial aneurysms in an Italian Caucasian population. Our meta-analysis confirmed no association with intracranial aneurysms, however both studies were relatively small and there was a difference between the minor allele frequencies (MAF) between both populations studied.

IL-6 can affect the acute phase response and can lead to endothelial dysfunction by releasing adhesion and chemokine molecules, such as MCP-1, affecting vascular wall integrity.[314] Two IL-6 SNPs (572 G>C and 174G>C) have been studied in association with intracranial aneurysms. These two variants

are present on chromosome 7p21 within the IL-6 promoter region and are thought to increase levels of IL-6. One study also demonstrated an association of the IL-6 G572C SNP and abdominal aortic aneurysms.[315] Our meta-analysis[204] demonstrated a protective effect of the IL-6 G572C SNP and risk of cerebral aneurysms (See chapter 3). However, there was significant statistical heterogeneity and when the only study[155] which demonstrated a protective association was removed in a Chinese Han population, the effect was no longer present. The only study in a western population which demonstrated significant association of both IL-6 SNPs with IA, was that conducted by Morgan *et al*[154], however this study was relatively small and cases were all male which is not representative of an SAH population. Subsequent studies in European populations did not replicate this finding. This does not discount IL-6 as a significant mediator in intracranial aneurysm pathology. It has been shown that IL-6 levels are raised in the CSF of SAH patients, 4 hours post-ictus[181] but this may be a reflection of an inflammatory response post-SAH, rather than chronic elevation as part of aneurysmal development or acute elevation heralding imminent aneurysmal rupture. This is difficult to quantify; hence it would be interesting if this SNP was associated in unruptured intracranial aneurysms.

Due to the limited number of studies, small numbers of cases, controls and inconclusive results regarding association of IA with all three SNPs linked with inflammatory mediators, we sought to undertake replication of these studies in the first, largest UK Caucasian case-control genetic association study.

Our study aimed to investigate whether these 3 SNPs were associated with intracranial aneuryms with sub-group analysis between rupture and unruptured cases.

8.7 Methods

Cases with sporadic angiographically-proven ruptured or unruptured intracranial aneurysms were included in this study over a 3-year period. Patients were recruited from 20 Neurosurgical centres treating intracranial aneurysm patients, excluding non-aneurysmal causes for SAH, such as AVMs (arterio-venous malformations), trauma, peri-mesencephalic SAH, fusiform, dissecting or mycotic aneurysms and those with known inherited connective tissue disorders, such as Marfans, Ehlers-Danlos syndrome and adult polycystic kidney disease (ADPKD). Control subjects DNA was drawn from the 1958 Wellcome cohort[2] and 297 Multi-System Atrophy (MSA) patients with no cerebral aneurysms as confirmed from post-mortem reports. To minimise population stratification, cases which were European Caucasian only were included in the analysis.

Polymerase Chain Reaction (PCR) was undertaken using KASP (KBioscience Competitive Allele-Specific) genotyping assays at LGC Genomics bio-laboratories. The KASP primer mix which is assay-specific for the IL-1,6 and TNF-alpha SNPs was mixed with a universal KASP master mix, then added to DNA samples. Following PCR thermal cycling, an end-point fluorescent read is conducted using labelled allele-specific forward primer dyes, to perform bi-allelic discrimination.

Statistical analysis was conducted using R[198] and PLINK[199] statistical software packages. Chi-sqaured tests, with odds ratio calculations were performed for these SNPs, under an additive genetic model. Further analysis was conducted to determine if these SNPs were differentially associated with cases based upon rupture status. Smoking and hypertension status were also to be tested in association with each SNP significantly associated with IA disease. P-values were corrected for multiple testing by Bonferroni correction. Following this, a meta-analysis was conducted using REVMAN[167] and CMA software. Controls were also checked for Hardy-Weinberg Equilibrium (HWE).

8.8 Results

For each of the three SNPs analysed, a total of 1619 cases and 1241 controls were sequenced for each respective variant. Following quality control, <1% of samples could not be genotyped, which is an accepted rate for genetic association studies.[165] The mean age of cases was 53.61 and controls were 57.69 years. More cases of ruptured compared to unruptured aneurysms were recruited and analysed (1068 Vs 335). A significantly increased proportion of cases were current smokers with a 20-pack year history when compared to controls (876 vs 258) and the same was true with those being currently treated hypertensives (633 Vs 56, see table 8-1).

For each SNP the genotype and allelic counts along with the association tests for each SNP studied are shown in tables 8-3 to 8-5. The controls for each SNP were in Hardy-Weinberg Equilibrium as shown. Each of the inflammatory gene SNPs examined in our cohort did not show association with our intracranial aneurysm (ruptured and unruptured) UK Caucasian population. The association testing was also split by rupture status of intracranial aneurysm patients and as shown in tables 8-3 to 8-5, there was no significant difference demonstrated between ruptured and unruptured cases when comparing to matched controls.

Meta-analysis conducted on all 3 SNPs including our data, as presented in table 8-6, did not demonstrate any new association. When performing the meta-analysis, we analysed Caucasian studies only to minimise the effect of population stratification.

Table 8- 1 -Demographic data

	Cases	**Controls**
F:M	961:442	636:648
Mean Age	53.61	57.69
Current smokers	876	258
Hypertensive	633	56
Family History	206 (rupt)	
Ruptured aneurysms	1068	
Unruptured Aneurysms	335	

Table 8- 2 - Basic association results for inflammatory SNPs analysed in Caucasians

SNP	Gene	Total cases	Cases analysed (After QC)	Total Controls	Controls analysed (After QC)	Ruptured	Unruptured	OR [CI] (corr)	P-value (corr)
rs1800796 (572G>C)	IL-6	1619	1402	1241	1225	1215	366	0.95 [0.73-1.22]	0.667
rs16944 (511AC>T)	ILβ-1	1619	1424	1241	1203	1084	340	0.96 [0.86-1.08]	0.53
rs1800629 (G>A)	TNFα-308	1619	1403	1299	1284	1068	335	1.02 [0.88-1.17]	0.83

Table 8- 3 – Genotype counts and association testing for TNFα rs1800629 SNP

rs1800629 (G>A) – TNFα	**Cases**	**Controls**
GG	930	859
GA	431	384
AA	42	41
G Allele	2291	2102
A Allele	515	466
HWE p-value		0.758
	Basic Association tests (Additive)	
	OR [95% CI]	**p-value**
All aneurysms	1.02 [0.88-1.17]	0.83
Ruptured aneurysms	0.94 [0.58-1.52]	0.8
Unruptured aneurysms	1.22 [0.94-1.58]	0.13

Table 8- 4 - Genotype counts and association testing for IL-1β rs16944 SNP

rs16944 (C>T) – IL-1β	**Cases**	**Controls**
CC	612	532
CT	657	545
TT	155	126
G Allele	1881	1609
A Allele	967	797
HWE p-value		0.43
	Basic Association tests (Additive)	
	OR [95% CI]	**p-value**
All aneurysms	0.96 [0.86-1.08]	0.53
Ruptured aneurysms	0.89 [0.67 – 1.17]	0.41
Unruptured aneurysms	1.1 [0.72 – 1.66]	0.66

rs1800796 (G>C) – IL-6	**Cases**	**Controls**
GG	1276	1113
GA	124	106
AA	2	6
G Allele	2676	2332
A Allele	128	118
HWE p-value		0.05
	Basic Association tests (Additive)	
	OR [95% CI]	**p-value**
All aneurysms	0.95 [0.73 – 1.22]	0.667
Ruptured aneurysms	0.37 [0.07 – 1.88]	0.23
Unruptured aneurysms	1.34 [0.94 – 1.26]	0.97

Table 8- 5 – Meta-analysis of all 3 SNPs.

SNP	Gene	Meta-analysis	P-value	**Studies**	**Cases/Controls**
rs16944	**IL-1**	1.01 [0.88 - 1.15]	0.92	3/3	1870/1589 **(1424/1203)**
rs1800796	**IL-6 G572C**	1.06 [0.80-1.40]	0.60	3*/6	1672/3993 **(1402/1225)**
rs1800629 (G>A)	**TNF-308**	0.96 [0.84-1.10]	0.59	2/2	1585/1350 **(1414/1206)**

*****number of Caucasian studies included in meta-analysis. Numbers in bold are cases/controls from this study included in meta-analysis.

8.9 Discussion

Analysis of the three inflammatory gene SNPs (rs16944, rs1800796 & rs1800629) in our large UK Caucasian cohort of ruptured and unruptured aneurysms, did not demonstrate any statistically significant association. Meta-analysis of all three SNPs with the addition of our UK Caucasian cohort did not demonstrate any statistical significance (See table 8-6) with intracranial aneurysm incidence.
Our study was sufficiently powered (>80%) to detect a modest association [159] with intracranial aneurysm phenotype and aimed to confirm or refute previous associations.

As outlined previously in this chapter, a growing body of evidence has demonstrated inflammatory reactions within aneurysm tissue. Questions remain as to which time point the inflammatory reaction occurs and how it contributes to aneurysm development or rupture. Whether the inflammatory reaction is induced as a result of haemodynamic stress or endothelial dysfunction which could lead to influx of inflammatory cells into abnormal vessel walls, or both, remains to be elucidated. Chronic inflammation may ripen conditions for aneurysmal development and progression, and changes in haemodynamic forces or stress may lead to aneurysm rupture at a weak point within the aneurysm wall. What is not known is whether an acute inflammatory reaction within an already developed intracranial aneurysm could/does cause rupture and cause subsequent, aneurysmal SAH. These questions remain to be answered, but our study aimed to shed light on possible links of known genetic polymorphisms within inflammatory genes and the occurrence of intracranial aneurysms.
Prior studies had demonstrated a significant association of; IL-6 G572C [154, 156], IL-1B C5111T[312] and TNF alpha A308G[308] respective SNPs with intracranial aneurysms. These SNPs were in promoter regions of each cytokine and were hypothesised to upregulate the inflammatory cytokine profile and possibly contribute to rupture or progression of cerebral aneurysm(s) development. It was not clear whether these cytokines were upregulated as a result of the inflammatory reaction from aneurysmal SAH or were a direct consequence of cerebral aneurysm rupture. What is clear, are each of these cytokines act as influential mediators in the inflammatory response and are secreted by various cell types which could compound the pro-inflammatory effect and cause release of other molecules, such as MMP, which lead to digestion of extracellular matrix components, thereby leading to weakening of vessel walls or worsening aneurysm progression. TNFα can initiate apoptosis, by causing binding of FasL ligand to Fas receptors on smooth muscle cells, inadvertently reducing SMC volume. TNFα also upregulates adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, which in turn attract leukocytes, [316, 317] at sites of vascular injury. Interestingly TNFα can upregulate MMP activity[285] via release from macrophages. Neutrophils are usually the primary responding inflammatory cells at site of injury but are important in releasing TNFα and IL-1β. In the context of vascular injury, these cytokines affect cell proliferation, differentiation and apoptosis, as well as affecting collagen biosynthesis within aneurysm walls. [293, 297]

TNFα can also cause release of IL-6, which can to affect collagen crosslinking and expression from inflammatory cells, which can potentially destabilise the aneurysm wall, and affect vascular wall tensile strength. IL-6 can be released from a variety of cells including; fibroblasts, monocytes/macrophages, endothelial and smooth muscle cells. Polymorphisms of the IL-6 gene have been associated with coronary artery and abdominal aortic aneurysms.[318, 319] Conflicting evidence exists in the association of IL-6 G572C polymorphism and intracranial aneurysms, with only 2 studies out of 6 previously demonstrating positive association[154, 156] in a UK Caucasian and Chinese Cantonese populations respectively.

In isolation, these studies were underpowered, our meta-analysis aimed to increase the power by combining studies, to determine if a significant association of the IL-6 gene with cerebral aneurysms was present. Following this, no significant association for the IL-6 G572C, nor, IL-1β SNPs was seen. [204]

Large numbers of cases and controls would be required in any further genetic association study, for more robust conclusions to be determined.

Our study was sufficiently powered (>80%) to make robust conclusions in a UK Caucasian population, with over 1400 cases and 1200 controls examining all 3 SNPs, not demonstrating an association with intracranial aneurysm incidence. Sub-group analysis splitting into ruptured and unruptured aneurysms did not show association either, in our cohort.

Based on our findings, our data does not support genetic association of intracranial aneurysms with these 3 inflammatory SNPs. However, this does not mean that, (1) these SNPs are not associated with other ethnic/Caucasian groups with intracranial aneurysms or (2) there is no genetic role or mutations(s) that could be responsible for the inflammatory response seen within intracranial aneurysms. These SNPs will need to be examined in more populations with similar power to our study. Also, other unknown genetic loci with SNPs in linkage disequilibrium with these inflammatory SNPs, could be associated with intracranial aneurysms and should be sought.

Mutations in other inflammatory genes should also be sought, such as, NF-κB, TGF-β and IFN-γ, if a hypothesis driven approach is used. Data from Translational microarray analyses which determine gene expression profiles in ruptured and unruptured IA samples comparing them to Superficial Temporal and Middle Meningeal artery sample controls, has implicated genes coding for inflammatory, immune and extracellular matrix maintenance mediators. In a recent meta-analysis by Xu *et al*, [320] they found 512 upregulated genes mainly associated with inflammatory and immune processes in intracranial aneurysms. These genes encoded IL-6 production, wound healing, chemotaxis, cytokine-cytokine receptor interaction, Toll-like receptors and chemokine signalling pathways, providing evidence of inflammatory processes being upregulated within intracranial aneurysms.

Chapter 9 – Final conclusions

The aim of this study was to investigate whether plausible candidate genes relevant to intracranial aneurysm pathology were associated with a large UK Caucasian cohort. This was achieved by initially, performing a meta-analysis of all previous candidate gene association studies and positively associated SNPs, replicated in multiple genome-wide association studies (GWAS). This directed choosing which SNPs to examine in our own cohort, based on (i) strength of association from our meta-analysis, (ii) plausibility to aneurysm pathogenesis. Following this, only those SNPs confirmed to significantly associated with intracranial aneurysms in our study were assessed for interaction with known risk factors for aneurysms. In a multivariate logistic regression model, we assessed if there was interaction between environmental and genetic risk factors, thereby increasing the risk of aneurysm development or rupture. We meta-analysed all SNPs examined in our cohort to vastly increase the numbers available to existing studies, in an attempt to provide level 1 evidence, and confirm and refute previous association studies.

9.1 Genetics of intracranial aneurysms

Initial investigations into intracranial aneurysm genetics came in the form of Linkage association studies. These studies examined family pedigrees with a large aneurysm burden or multiple families being affected. Linkage studies implemented microsatellite markers and SNPs scattered across the entire genome to localise specific areas or loci which could harbour potential intracranial aneurysm risk genes, using a logarithm of odds (LOD) score. Fifteen such risk loci were detected in affected families with 5 loci being replicated in more than one study. These included; 1p34.3-36.13,[125, 130] 4q32,[114, 115] 7q11,[113, 126] 19q13[122, 132, 135, 321] and Xp22.[130, 135, 321]

From these studies, candidate genes such as Elastin, Perlecan and Versican with plausible relationship to aneurysm pathogenesis were located in these chromosomal loci or were close to them in strong linkage disequilibrium. These spawned studies utilising a candidate gene approach to magnify these regional loci closely and determine if plausible genes, indeed had variants that could cause aberrant signalling and lead to aneurysm development. As increasing knowledge of molecular pathways and examination of aneurysm specimens revealing plausible mediators came to light, so did the increase in candidate gene studies to investigate imbalances seen at the molecular level. The candidate-gene approach was a hypothesis driven one, where previously associated genes in other diseases, including abdominal aortic aneurysms were investigated in intracranial aneurysms. This strategy has led to conflicting results with multiple SNPs being tested in numerous underpowered studies, leading to a lot of false positive results. Although attempts were made to determine genetic risk factors plausible to intracranial aneurysm pathology, the majority of studies significantly differed in their methodology and populations. As outlined in chapter 2, there are fundamental principles in designing a genetic study, these include correct power calculations, ensuring controls meet with HWE expectations, careful

choosing of population substructure to minimise populations stratification, admixture and ensuring robust phenotypic information is collected.

Despite the limitations of a CGAS approach, promising insights into aneurysm formation can be gained. Our study employed this approach to confirm or refute previous findings of strong candidate genes associated with intracranial aneurysms. The choice of SNPs was dependent upon our meta-analysis of all previous CGAS and GWAS studies. We were not able to choose SNPs that had been associated with intracranial aneurysms from the GWA studies, as we had entered into a collaboration with Yale University and they would replicate these SNPs in our cohort, due to having the funding and resources to perform this. Therefore, we continued to perform a candidate gene analysis permitted by our funding. Twenty-two SNPs were examined in total in our cohort. We split SNPs examined based on category of plausible function, into those involved in vascular endothelium function – 7 SNPs (see chapter 6), 12 SNPs involved with ECM constituents (see chapter 7) and those involved with inflammatory processes (3 SNPs) inside aneurysm walls (see Chapter 8).

Our results demonstrated association of the insertion/deletion SNP within the ACE gene and the MMP-2 SNP within our UK Caucasian cohort.

The remainder of the SNPs did not meet statistical significance with our large IA cohort. These negative findings may reflect two reasons in our study, (i) they are truly not associated in our UK population or (ii) as yet unknown variants within those genes could still lead to predisposition to intracranial aneurysms, but not these particular SNPs, as they are hypothesis driven. This may represent a design flaw in our study and future studies may need to take into account sequencing large areas of one particular gene in order to find novel variants. There is more chance in determining multiple hits within a gene that may have spurious associations. Nevertheless, it would provide good insight into large areas of a particular gene of interest and help develop a haplotype map of genes in strong linkage disequilibrium with causal variants. This has been shown previously by Kaushal and colleagues who did not demonstrate association of the APOE ε2, ε3, ε4 alleles with intracranial aneurysm presence but scanned other areas of the APOE gene and demonstrated previously unknown associations with cerebral aneurysms.[139]

However, the negative associations found in our study significantly contribute to the body of knowledge currently present in intracranial aneurysm genetics. Our study was well powered to confirm or refute previous associations, but this was the first time these SNPs, with the exception of IL-6 G572C[154] and ACE insertion/deletion polymorphism,[192] were examined in a UK Caucasian cohort. The fact that the majority of the SNPs examined in our cohort were not associated should not dissuade future investigators from examining them in different populations/ethnicities.

Our study design attempted to control for population stratification by ensuring we had information on 2 generations of ancestors (i.e. up to the origins of parents and grandparents) to ensure comparability

of results. However, this does not completely rule out admixing and as such future similarly designed case-control studies will be required in Caucasian and non-Caucasian populations to determine the robustness of our findings.

9.1.1 ACE Insertion/Deletion Polymorphism

The first SNP significantly associated with intracranial aneurysms in our UK-based Caucasian cohort was the insertion/deletion SNP within the ACE gene. This gene is characterised by either an insertion or deletion of a 287-bp sequence, present within intron 16 of the ACE gene.

This SNP has been examined extensively in multiple studies with relation to intracranial aneurysm association. However conflicting results are present, in part, due to underpowered studies and differing risk allele frequencies in different populations.

It is unclear how the ACE gene influences aneurysm formation or rupture, but it has been postulated, it mediates its effects via activation of local Renin-Angiotensin System (RAS) within aneurysm walls, which effects vascular remodelling. Angiotensin II is a potent pro-inflammatory mediator, and may attract inflammatory cells and signal inflammatory cascades within the aneurysm walls, at sites of vascular stress. Our study was the only one to demonstrate an association between intracranial aneurysm risk and presence of the D allele. This is in stark contrast to all other studies examining this SNP in relation to intracranial aneurysm occurrence. A previous study of UK subjects demonstrated the I allele to be associated with cerebral aneurysms.[192]

Our results are similar to those shown for association of this SNP with abdominal aortic aneurysms, confirmed in a meta-analysis,[202] on 7000 European Caucasian subjects. Those with DD genotype have been shown to have increased serum ACE activity,[203] which could predispose to hypertension, a known risk factor for cerebral aneurysms. Indeed, logistic regression analysis in our study cohort demonstrated increased association of the ACE I/D SNP with intracranial aneurysms, with the OR rising from 1.14 [1.02-1.28] to 1.64 [1.12-2.4]. $p=1.08x10^{-02}$. Caution must be drawn to this however, as association of this SNP with hypertension is mixed, but a recent meta-analysis, suggested a 10% increased risk of hypertension in those with the DD genotype in 6923 subjects, but not reaching statistical significance, with significant statistical heterogeneity.

Meta-analysis if this SNP did not show association with IA (see chapter 6), but this was not the case when we performed our meta-analysis (see chapter 3).[204] This most definitely relates to the addition of our study with a far larger sample size, and the fact we were the only study out of the 7, which demonstrated the D allele to be associated with intracranial aneurysm occurrence.

Whether the ACE I/D polymorphism directly influences aneurysm pathology remains to be elucidated, however, there may be other causal variants in strong LD, as yet unknown that could be contributing to intracranial aneurysm phenotype. To determine this, sequencing of the flanking regions of this SNP and large portions of the ACE gene, in similarly powered studies to ours, in multiple populations would

have to be carried out. In future study designs, it may be prudent in addition to determining association with intracranial aneurysms of the ACE I/D SNP, to also measure serum ACE levels and see if a correlation exists, that may predispose to aneurysm presence or rupture.

9.1.2 MMP-2 C>T SNP

The MMP-2 rs243865 C>T functional variant was a plausible gene associated with aneurysm pathology. This variant affects transcription factor SP-1, which can bend DNA and prevent activation of other transcription factors, possibly leading to unregulated MMP-2 activity. This potential imbalance of matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs) could predispose to aneurysm formation. Molecular and microarray studies have implicated MMP-2 and MMP-9 as being highly expressed within aneurysm tissue.[205, 277, 286 147]

In our study, association was confined to ruptured aneurysm cases only. This may have reflected the difference in numbers (1074 ruptured vs 335 unruptured cases), or may signal that the MMP-2 variant is more prevalent in ruptured cases, as it may hasten rupture in an already developed aneurysm. We tested for interaction between known modifiable risk factors, such as hypertension (p=0.5) and smoking (p=0.2), but this was non-significant for any confounding effect. However, both risk factors under a multi-variable logistic regression analysis strengthened the association of the MMP-2 SNP with IA; hypertension (OR 1.74 [1.41-2.15]) and smoking (OR 2.76 [2.29-3.32]). This would suggest a gene-environment interaction whereby the effects of the MMP-2 SNP are potentiated by the presence of each risk factor. Despite this SNP being associated in our cohort, it is isolated in association and may be in linkage disequilibrium (LD) with one or many other SNPs that could be contributing to intracranial aneurysm phenotype. This can only be determined if flanking sequences to this SNP are tested by sequencing large portions of the MMP-2 gene.

Meta-analysis of this SNP with 2 previous studies[148, 206] still demonstrated significant association although not as robust as our single study, this was partly due to our study being significantly larger compared to the study by Pannu *et al*[148] in a US Caucasian population. However, the study on the Japanese study by Low *et al*[206] was significantly larger than ours with 2050 cases and 1835 controls genotyped. This highlights the fact of different risk allele frequencies and different genetic aetiologies between different ethnicities, leading to contrasting results in genetic association studies.

9.1.3 GWAS

Genome-wide association studies have taken genetic association studies onto a higher level. These studies utilise dense SNP arrays between 500 000 to 1000000 examining the entire genome, detecting novel variants in significantly large numbers of cases and controls. This is a non-hypothesis driven approach and significance level is very high usually set at $5x10^{-08}$. SNPs that achieve this level of significance in discovery and replication cohorts are said to be associated with disease occurrence. The first GWAS on intracranial aneurysms was performed by Bilguvar and colleagues in 2008, in 2100

cases and 8000 controls of Finnish, Dutch ethnicity and replication was performed in a Japanese cohort.[45] This study provided 3 new loci that were significantly associated with intracranial aneurysms, these included Chromosome 9p21.3 (CDKN2B-AS1), 8q11.23-12.1 (SOX-17) and 2q32.1 (PLCL1). It was unclear what the function of these genes were in relation to intracranial aneurysm pathology, but subsequent GWAS went on to confirm previous associations from the first GWAS and also detected further new variants. In particular the chromosome 9p21.3 loci, was replicated in a further 6 GWAS studies.[44, 46, 47, 163, 164, 322-324] Interestingly this loci has been identified in GWAS studies examining association with coronary heart disease, basal cell carcinomas, gliomas and type 2 diabetes mellitus.[325] Across all intracranial aneurysms GWAS, greater than 20 novel risk loci have been discovered in different populations with some replicating in different populations, and others that did not. This reflects underpowered studies in some cases and different populations that were studied.[326] Although GWAS has provided new insight into aneurysm genetics, it has not answered, what are the robust genetic risk factors associated with intracranial aneurysms. The degree of heritability from the GWAS risk loci is very low, explaining only 2.5% of the heritability in a Finnish population, for example.[323] This again highlights the complex interplay of genetic and environmental risk factors that predispose one to sporadic forms of intracranial aneurysms. Questions still remain to be answered, as to what triggers aneurysm development and progression and what set of circumstances trigger some to rupture and others to not. Our study has added to existing body of evidence currently present but we are still very distant in fully understanding aneurysmal genetics and what risk factor profiles would predispose one to develop an intracranial aneurysm and those which would rupture. Our study was not sufficiently funded to perform a GWAS on our UK cohort, but the results would have been interesting to see, if previously discovered loci or novel variants were associated in the first GWAS examining a UK Caucasian population. We entered into a collaboration with Yale University (Professor Murat Gunel), whose group performed the first two GWAS studies on intracranial aneurysms. They are planning to perform another study and will be including our samples to confirm or refute previous findings and detect novel variants, the first of its kind in a UK based Caucasian intracranial aneurysm population.

9.2 Future work

9.2.1 Microarray Analysis

Understanding the genetics behind intracranial aneurysms pathogenesis is important but future work would aim to look at the field of translational research, in particular gene expression studies within aneurysm tissue itself and comparing it to matched controls. This method has been gaining prominence and multiple studies have used microarray-based mRNA chipsets to further understand intracranial aneurysms genetics from a tissue level. Multiple plausible pathways to intracranial aneurysm development and rupture have been found, these include gene products involved in; adhesions and

migration, cell proliferation, extracellular matrix components interaction, apoptosis of smooth muscle cells and inflammatory processes.

In one such study, ruptured intracranial aneurysms showed upregulation of NF-κB, hypoxia-inducible factor and toll like receptors involved in ECM environment regulation and signalling of inflammatory cells.[327] Overexpression of genes encoding MMP, including genes involved with apoptosis were upregulated in ruptured aneurysms compared to unruptured aneurysms in one study.[328]

RNA sequencing employs parallel sequencing technologies to provide a transcription map via transcriptome profiling (where cDNA fragments are converted from RNA), this visualises the extent of gene expression within tissues. In one study, this technology identified 229 differentially expressed genes within intracranial aneurysms when comparing them to matched control cortical arteries. The genes affected in aneurysm tissue, were found to be associated with blood vessel homeostasis, transmembrane transported activity and deposition of ECM components. The same study also demonstrated 1489 genes that had modified function in ruptured aneurysm samples compared to unruptured aneurysm samples.[329]

9.2.2 Whole Exome Sequencing (WES)

WES has led to major discoveries of rare Mendelian disorders. Developed in 2010, its high-throughput sequencing has enabled vast progress to be made in the genetic research field, by looking at coding portions (Exons) of the genome. Only 4 studies[330-333] using WES technology have been utilised in studying intracranial aneurysms. However, these studies have exclusively been performed in families where the incidence of intracranial aneurysms is higher, compared with sporadic cases. Multiple genes have been discovered, such as ADAMST15, MLL2, IL10RA, PAFAH2, THBD, IL1RA, FILIPIL and ZNF222 but only ADMAST15 (rs185269810) was replicated in a familial cohort.[330]

THSD1[332] and RNF213[333] were 2 new genes recently identified in familial cases and replicated successfully in sporadic cases of intracranial aneurysms, using WES. Both these genes are thought to contribute to intracranial aneurysms, by developing angiogenic processes and impaired endothelial cell focal adhesion. These studies will need to be replicated in large sporadic cases of intracranial aneurysms to confirm the robustness of their findings.

9.3 Conclusions

Our study has demonstrated 2 associated SNPs with intracranial aneurysms, that have plausible relationship to the pathogenesis of aneurysm development/rupture in a large UK based cohort. No specific gene has been found to predispose one to intracranial aneurysms currently, but previously associated variants and interaction with known environmental stimuli, such as smoking and hypertension, or vascular shear stress can trigger certain genes which maintain vascular homeostasis, and if aberrant, due to abnormal variants, this could lead towards the path of intracranial aneurysm development or rupture. We have also added to the existing body of literature on previous studies

examining various SNPs that did not show significant association in our cohort. We have meta-analysed these SNPs and demonstrated which genes are robustly associated with intracranial aneurysms. Our study goes some way to confirm or refute previous associations but there are shortcomings as well.

Limitations include, not being able to perform a GWAS on our samples to detect novel variants in our UK population. However, we entered in a collaboration with Yale University and are expecting results of this work very soon.

Despite the two SNP associations, we recognise that as yet unknown variants with LD may be causal to intracranial aneurysms and this would require sequencing of flanking regions in each gene to determine functional variants that would predispose to intracranial aneurysm development.

Microarray analysis has demonstrated upregulation of plausible genes involved in vascular maintenance but future studies would need to test these hypotheses on aneurysm samples of patients that have been genotyped, to determine if correlation exists.

As increasing knowledge of molecular and genetic influences of intracranial aneurysms increases, there could be a potential for pharmacological targets, that could slow progression or arrest aneurysm development. In a mouse model of intracranial aneurysms, where mice were given a 2-week infusion of angiotensin II to raised blood pressure and an injection of elastase into their CSF to induce cerebral aneurysms. This was to investigate the effect of MMP on aneurysm development in these artificial settings. This group utilised Doxycycline, a broad-spectrum inhibitor of MMP and demonstrated a 10% reduction in the incidence of cerebral aneurysms. Interestingly, MMP-9 but not MMP-2 knockout mice had reduced incidence of intracranial aneurysms.[281] Therapeutic intervention for aneurysms is very far off current management, but modifiable risk factors are encouraged to be controlled in aneurysm patients. It would be useful to follow-up those patients harbouring an unruptured aneurysm with hypertension, managed with an ACE inhibitor. Comparing them to those patients with hypertension and not being managed with an ACE inhibitor and determining their aneurysm progression and morphology over time.

The aim of genetic association studies has been to provide genetic risk factors that are robust and plausible in determining a patient's risk of cerebral aneurysm rupture in an already discovered aneurysm and how effectively they are managed. Currently, the evidence of genetic risk factors is small and is most likely a combination of multiple common variants with small effects interacting with environmental influences to produce the sporadic intracranial aneurysm phenotype. An increasing number of well-powered studies in multiple populations, examining multiple candidate and novel variants in the form of a GWAS are required before these can be translated into clinical practice.

Appendices

Appendix I for Chapter 3

Table 1 Risk allele frequencies and control Hardy-Weinberg equilibrium status for each study for all SNP meta-analysed.

GENE	SNP	STUDY	ETHNICITY	RAF	HWE (p>0.05)
SERPINA-3	A>G rs4934	Slowik	Polish	0.5	Yes
		Krischek	Japanese	0.58	Yes
		Liu	Chinese	0.59	Yes
Collagen 1A2	G>C rs42524	Yoneyama	Japanese	0.03	Yes
		Zhu	Chinese	0.04	Yes
		Joo	Korean	0.06	Yes
ACE	I/D	Takenaka	Japanese	0.63	Yes
		Keramtipour	UK-Caucasian	0.48	Yes
		Slowik	Polish	0.49	Yes
		Pannu	USA- Caucasian	0.44	Yes
		Stalso	Danish Caucasian	0.48	Yes
eNOS	T786C	Akagawa	Japanese & Korean	0.1	Yes
		Song	Korean	0.06	Yes
		Krischek	Japanese	0.11	No
		Krex	Germany	0.38	Yes
		Koshy	South Indian	0.21	Yes
		Kim	Korean	0.09	Yes
		Khurana	USA - Caucasian	0.43	Yes
IL-6	G572C	Morgan	UK-Caucasian	0.05	Yes
		Fontanella	Italian Caucasian	0.09	Yes
		Sun	Chinese Han	0.79	Yes
		Zhang	Chinese Cantonese	0.05	Yes
		Liu	Chinese Cantonese	0.78	Yes
Perlecan HSPG2	A>G rs3767137	Ruigrok	Dutch	0.801	Yes
		Ruigrok	Japanese	0.715	Yes
Versican CSPG2	A>G rs173686	Ruigrok	Dutch	0.322	Yes
		Sun	Chinese Han	0.842	Yes
Versican CSPG2	C>T rs251124	Ruigrok	Dutch	0.13	Yes
		Sun	Chinese Han	0.25	Yes
		Ruigrok	Japanese	0.21	Yes
9p21.3	C>T rs1333040	Bilguvar	Finnish	0.47	Yes
			Dutch	0.55	Yes
			Japanese	0.65	Yes
		Yasuno	Finnish	0.56	Yes
			Combined European	0.76	Yes
			Japanese	0.79	Yes
		Akiyama	Japanese	N/A	Yes
		Deka	European	0.58	Yes
		Hashikata	Japanese	0.65	Yes
		Nakaoka	Japanese	0.64	Yes
		Low	Japanese	0.317	Yes
		Foroud	Caucasian	N/A	Yes
9p21.3	A>G rs10757278	Deka	Caucasian	0.48	Yes
		Hashikata	Japanese	0.24	Yes
		Helgadottir	Iceland	0.437	Yes
			Dutch	0.461	Yes
			Finnish	0.4	Yes

		Olsson	Swedish	N/A	Yes
		Nakaoka	Japanese	0.467	Yes
9p21.3	C>G (rs2891168)	Nakaoka	Japanese	0.45	Yes
		Foroud	Caucasian	0.48	Yes
9p21.3	C>T (rs10757272)	Low	Japanese	0.365	Yes
		Foroud	Caucasian	0.48	Yes
2q33	G>A (rs1429412)	Bilguvar	Finnish	0.42	Yes
			Dutch	0.34	Yes
			Japanese	0.29	Yes
		Deka	Caucasian	0.66	Yes
		Akiyama	Japanese	N/A	Yes
2q33	G>A (rs700651)	Bilguvar	Finnish	0.39	Yes
			Dutch	0.35	Yes
			Japanese	0.45	Yes
		Deka	Caucasian	0.66	Yes
		Hashikata	Japanese	0.46	Yes
		Low	Japanese	0.49	Yes
7p13	G>T (rs4628172)	Akiyama	Japanese	0.52	Yes
		Low	Japanese	0.44	Yes
8q11	A>G (rs10958409)	Bilguvar	Finnish	0.18	Yes
			Dutch	0.15	Yes
			Japanese	0.25	Yes
		Yasuno	Finnish	0.19	Yes
			Combined European	0.15	Yes
			Japanese	0.28	Yes
		Deka	Caucasian	0.88	Yes
		Hashikata	Japanese	0.28	Yes
		Akiyama	Japanese	N/A	Yes
		Low	Japanese	0.275	Yes
8q11	A>G (rs9298506)	Bilguvar	Finnish	0.73	Yes
			Dutch	0.81	Yes
			Japanese	0.81	Yes
		Yasuno	Finnish	0.76	Yes
			Combined European	0.81	Yes
			Japanese	0.79	Yes
		Deka	Caucasian	0.17	Yes
		Low	Japanese	0.20	
4q31.23	A>G (rs6841581)	Yasuno	Finnish	>0.01	Yes
			Dutch	>0.01	Yes
			German	>0.01	Yes
			NeurIST (pan-European)	>0.01	Yes
			1st Japanese	>0.01	Yes
			2nd Japanese	>0.01	Yes
18q11.2	A>C (rs11661542)	Yasuno	Combined European	0.49	Yes
			Japanese	0.61	Yes
		Low	Japanese	0.382	Yes
12q22	G>A (rs6538595)	Yasuno	Finnish	>0.01	Yes
			Dutch	>0.01	Yes
			German	>0.01	Yes
			NeurIST (pan-European)	>0.01	Yes
			1st Japanese	>0.01	Yes
			2nd Japanese	>0.01	Yes
20p12.1	G>A (rs1132274)	Yasuno	Finnish	>0.01	Yes
			Dutch	>0.01	Yes
			German	>0.01	Yes
			NeurIST (pan-European)	>0.01	Yes
			1st Japanese	>0.01	Yes
			2nd Japanese	>0.01	Yes

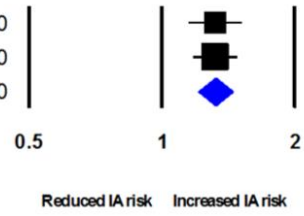
eNOS	VNTR 4a/4b	Khurana	USA – Caucasian	0.87	No
		Krex	German – Caucasian	0.81	Yes
		Krischek	Japanese	0.9	Yes
		Koshy	South Indian	0.81	Yes
		Kim	South Korean	0.9	Yes
Endoglin Intron 7	Wt/INS	Takenaka	Japanese	0.28	Yes
		Krex	German – Caucasian	0.14	Yes
		Onda	Japanese	0.32	Yes
		Peters	USA – Caucasian	0.18	Yes
		Pera	Polish	0.16	Yes
		Joo	Korean	0.3	Yes
eNOS	G894T	Khurana	Caucasian	0.47	No
		Krex	German – Caucasian	0.29	Yes
		Krischek	Japanese	0.11	No
		Ozum	Turkish	0.33	No
		Koshy	South Indian	0.15	Yes
		Kim	Korean	0.1	Yes
IL-6	G174C	Morgan	UK- Caucasian	0.43	Yes
		Fontanella	Italian	0.35	Yes
		Pera	Polish	0.44	Yes
IL-1β	C5111T	Slowik	Polish	0.29	Yes
		Fontanella	Italian	0.37	Yes
MMP-3	5A/6A	Yoon	Finnish	0.65	Yes
		Zhang	UK- Caucasian	0.5	Yes
Elastin	Intron 20 T>C	Hofer	Central European	0.16	Yes
		Ruigrok	Dutch	0.19	Yes
Elastin	Intron 23 C>T	Hofer	Central European	0.47	Yes
		Ruigrok	Dutch	0.43	Yes
PAI-1	4G/5G	Yoon	Finnish	0.49	Yes
		Ladenvall	Sweden	0.52	No
GP IIIa A2	A1/A2	Iniesta	Spanish	0.17	Yes
		Adamski	Polish	0.15	Yes
Factor XIII	Val/Leucine	Corral	Spanish	0.16	Yes
		Ladenvall	Swedish	0.24	Yes
		Admaski	Polish	0.3	Yes
APOE	ε2	McCarron	Scottish	0.09	Yes
		Kokubo	Rural Japanese	0.05	Yes
		Tang	Chinese	0.19	Yes
		Kaushal	USA-Caucasian	0.058	Yes
		Fontanella	Italian	0.1	Yes
APOE	ε4	McCarron	Scottish	0.22	Yes
		Kokubo	Rural Japanese	0.79	Yes
		Tang	Chinese	0.091	Yes
		Kaushal	USA-Caucasian	0.132	Yes
		Fontanella	Italian	0.06	Yes
COL41A	C>T (rs3783107)	Ruigrok 2006	Dutch	0.354	Yes
		Ruigrok 2009	Japanese	0.432	Yes
FBN2	C>G (rs331079)	Ruigrok 2006	Dutch	0.08	Yes
		Ruigrok 2009	Japanese	0.096	Yes
MMP-2	C1306T (rs243865)	Pannu	USA – Caucasian	N/A	Yes
		Low	Japanese	0.07	Yes
MMP-9	C1562T (rs3918242)	Zhang	UK – Caucasian	0.17	Yes
		Pannu	USA – Caucasian	N/A	Yes
		Szczudlik	Polish	0.15	Yes
COL 3A1	G>A (rs1800255)	Hua	Chinese	0.14	Yes
		Chen	Chinese	0.2	Yes
COL 3A1	G>A (rs2138533)	Zhu	Chinese	0.3	Yes
		Chen	Chinese	0.36	Yes

COL 3A1	A>G (rs11887092)	Zhu	Chinese	0.13	Yes
		Chen	Chinese	0.11	Yes

Figure 1

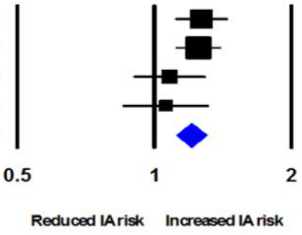
Chr 9p21.3 (rs2891168) C>G SNP - All populations

Study name	Odds ratio	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Nakaoka - Japanese	1.320	1.148	1.518	3.901	0.000	40.74
6th GWAS Foroud - Caucasian	1.320	1.176	1.482	4.705	0.000	59.26
	1.320	1.208	1.443	6.112	0.000	



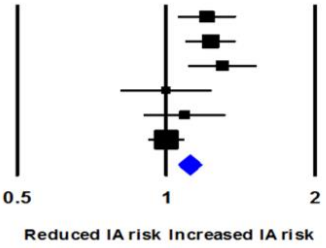
Chr 2q33 (rs1429412) G>A SNP - All populations

Study name	Odds ratio	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
1st GWAS - Finnish	1.270	1.111	1.452	3.501	0.000462921	30.98
1st GWAS - Dutch	1.250	1.113	1.404	3.766	0.000165907	41.11
1st GWAS - Japanese	1.080	0.899	1.298	0.821	0.411443122	16.44
Deka - Caucasian	1.060	0.851	1.321	0.519	0.603461171	11.47
	1.203	1.117	1.296	4.872	0.000001103	



Chr 2q33 (rs700651) G>A SNP - All populations

Study name	Odds ratio	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
1st GWAS - Finnish	1.210	1.057	1.386	2.757	0.006	15.54
1st GWAS - Dutch	1.230	1.093	1.384	3.440	0.001	20.51
1st GWAS - Japanese	1.300	1.107	1.526	3.205	0.001	11.09
Deka - Caucasian	1.000	0.808	1.237	0.000	1.000	6.30
Hashikata - Japanese	1.090	0.900	1.320	0.882	0.378	7.78
5th GWAS Low - Japanese	1.001	0.919	1.091	0.023	0.982	38.78
	1.114	1.056	1.175	3.972	0.000	



Chr 7q13 (rs4628172) G>T SNP - All populations

Study name	Odds ratio	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Akiyama - Japanese	1.300	1.141	1.481	3.940	0.00008	30.09
5th GWAS - Japanese	1.038	0.953	1.131	0.854	0.39325	69.91
	1.111	1.034	1.193	2.875	0.00404	

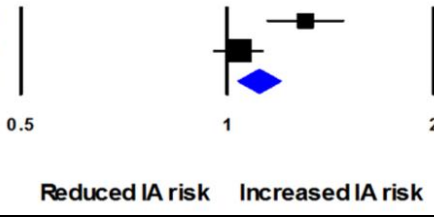


Figure 2

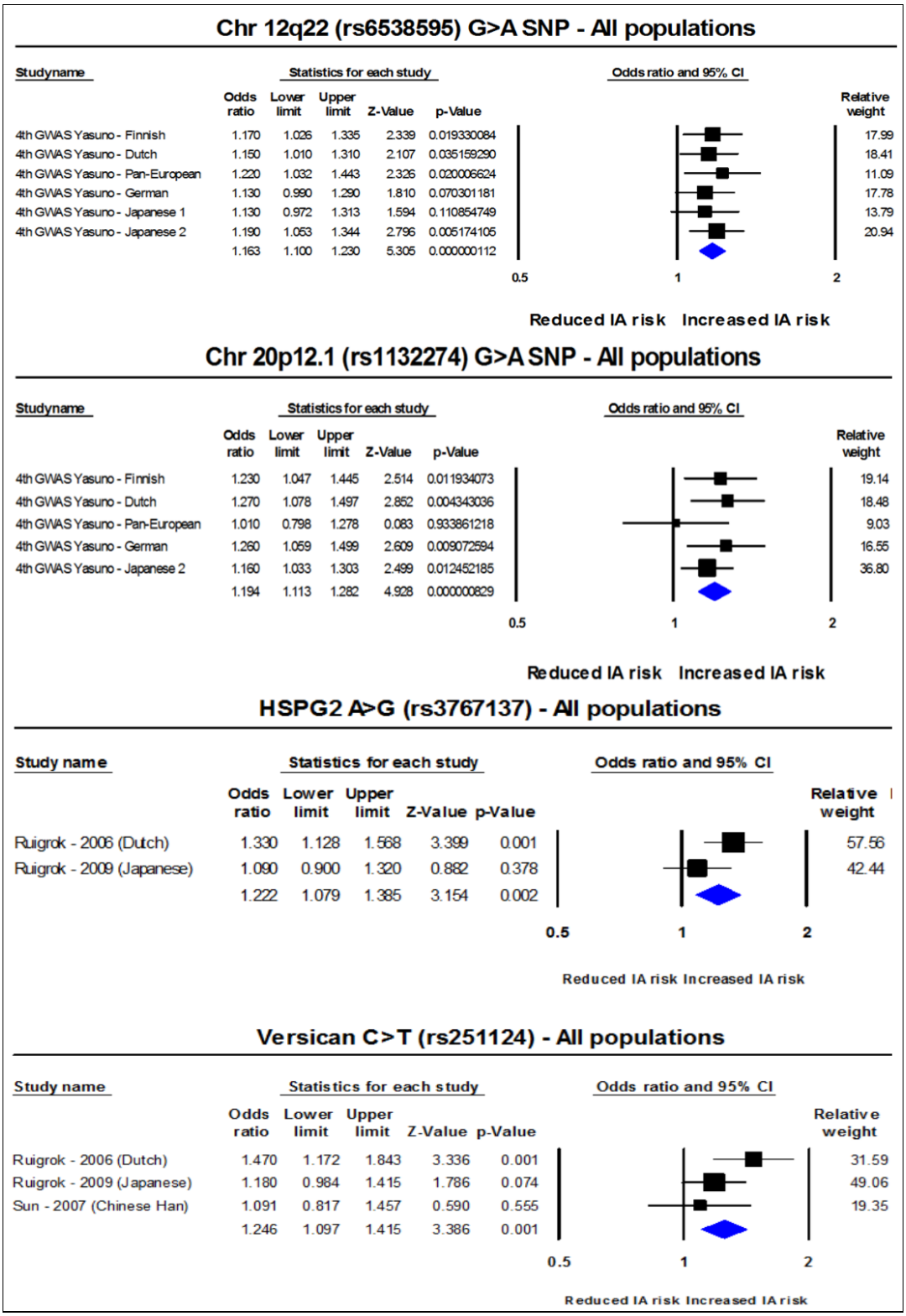

Chr 12q22 (rs6538595) G>A SNP - All populations
Studyname
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
4th GWAS Yasuno - Finnish 1.170 1.026 1.335 2.339 0.019330084 17.99
4th GWAS Yasuno - Dutch 1.150 1.010 1.310 2.107 0.035159290 18.41
4th GWAS Yasuno - Pan-European 1.220 1.032 1.443 2.326 0.020006624 11.09
4th GWAS Yasuno - German 1.130 0.990 1.290 1.810 0.070301181 17.78
4th GWAS Yasuno - Japanese 1 1.130 0.972 1.313 1.594 0.110854749 13.79
4th GWAS Yasuno - Japanese 2 1.190 1.053 1.344 2.796 0.005174105 20.94
1.163 1.100 1.230 5.305 0.000000112
0.5
1
2
Reduced IA risk
Increased IA risk
Chr 20p12.1 (rs1132274) G>A SNP - All populations
Studyname
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
4th GWAS Yasuno - Finnish 1.230 1.047 1.445 2.514 0.011934073 19.14
4th GWAS Yasuno - Dutch 1.270 1.078 1.497 2.852 0.004343036 18.48
4th GWAS Yasuno - Pan-European 1.010 0.798 1.278 0.083 0.933861218 9.03
4th GWAS Yasuno - German 1.260 1.059 1.499 2.609 0.009072594 16.55
4th GWAS Yasuno - Japanese 2 1.160 1.033 1.303 2.499 0.012452185 36.80
1.194 1.113 1.282 4.928 0.000000829
0.5
1
2
Reduced IA risk
Increased IA risk
HSPG2 A>G (rs3767137) - All populations
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
Ruigrok - 2006 (Dutch) 1.330 1.128 1.568 3.399 0.001 57.56
Ruigrok - 2009 (Japanese) 1.090 0.900 1.320 0.882 0.378 42.44
1.222 1.079 1.385 3.154 0.002
0.5
1
2
Reduced IA risk
Increased IA risk
Versican C>T (rs251124) - All populations
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
Ruigrok - 2006 (Dutch) 1.470 1.172 1.843 3.336 0.001 31.59
Ruigrok - 2009 (Japanese) 1.180 0.984 1.415 1.786 0.074 49.06
Sun - 2007 (Chinese Han) 1.091 0.817 1.457 0.590 0.555 19.35
1.246 1.097 1.415 3.386 0.001
0.5
1
2
Reduced IA risk
Increased IA risk

Figure 3

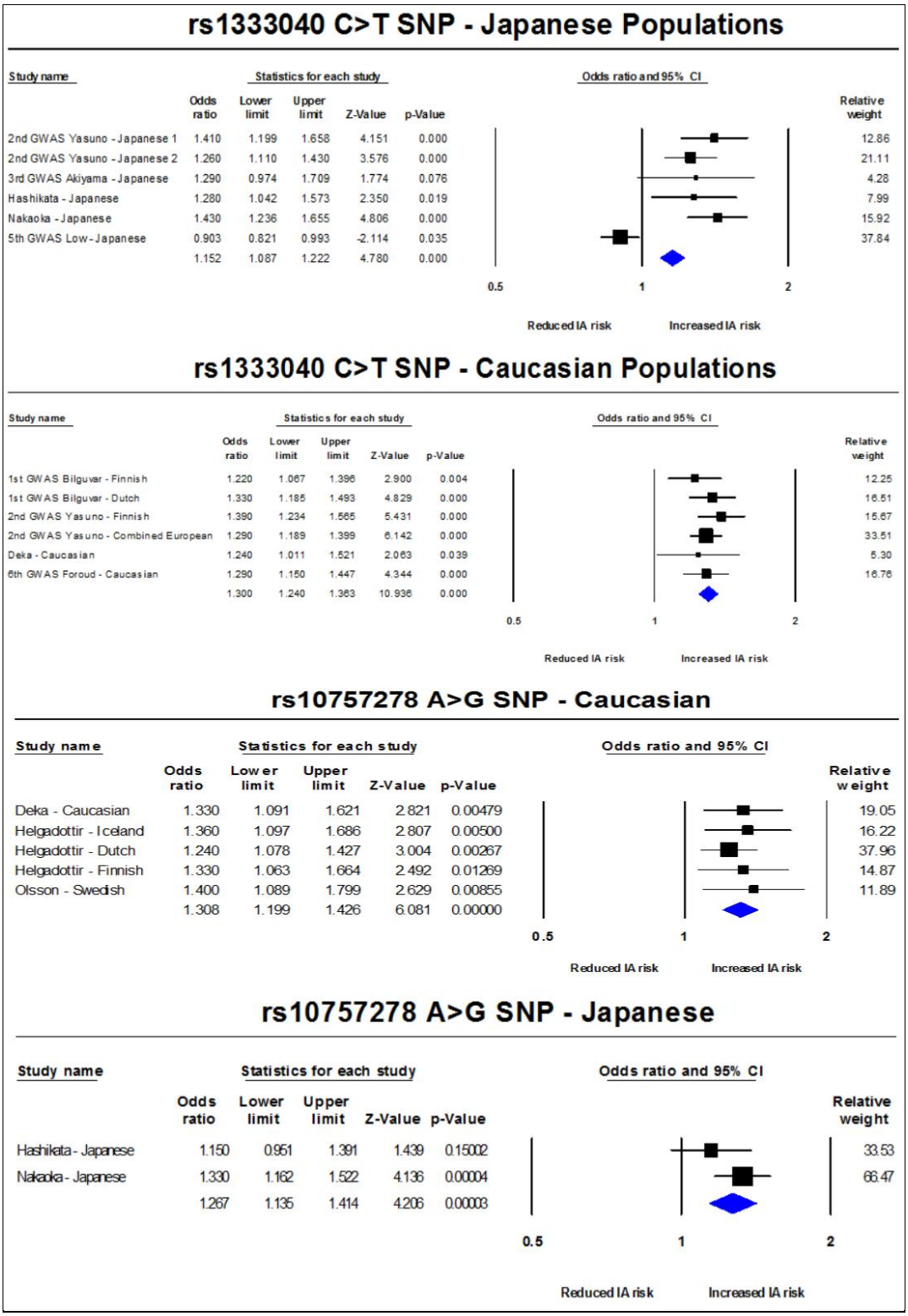

rs1333040 C>T SNP - Japanese Populations
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
2nd GWAS Yasuno - Japanese 1 1.410 1.199 1.658 4.151 0.000 12.86
2nd GWAS Yasuno - Japanese 2 1.260 1.110 1.430 3.576 0.000 21.11
3rd GWAS Akiyama - Japanese 1.290 0.974 1.709 1.774 0.076 4.28
Hashikata - Japanese 1.280 1.042 1.573 2.350 0.019 7.99
Nakaoka - Japanese 1.430 1.236 1.655 4.806 0.000 15.92
5th GWAS Low - Japanese 0.903 0.821 0.993 -2.114 0.035 37.84
1.152 1.087 1.222 4.780 0.000
0.5
1
2
Reduced IA risk
Increased IA risk
rs1333040 C>T SNP - Caucasian Populations
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
1st GWAS Bilguvar - Finnish 1.220 1.067 1.396 2.900 0.004 12.25
1st GWAS Bilguvar - Dutch 1.330 1.185 1.493 4.829 0.000 16.51
2nd GWAS Yasuno - Finnish 1.390 1.234 1.565 5.431 0.000 15.67
2nd GWAS Yasuno - Combined European 1.290 1.189 1.399 6.142 0.000 33.51
Deka - Caucasian 1.240 1.011 1.521 2.063 0.039 5.30
6th GWAS Foroud - Caucasian 1.290 1.150 1.447 4.344 0.000 16.76
1.300 1.240 1.363 10.936 0.000
0.5
1
2
Reduced IA risk
Increased IA risk
rs10757278 A>G SNP - Caucasian
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
Deka - Caucasian 1.330 1.091 1.621 2.821 0.00479 19.05
Helgadottir - Iceland 1.360 1.097 1.686 2.807 0.00500 16.22
Helgadottir - Dutch 1.240 1.078 1.427 3.004 0.00267 37.96
Helgadottir - Finnish 1.330 1.063 1.664 2.492 0.01269 14.87
Olsson - Swedish 1.400 1.089 1.799 2.629 0.00855 11.89
1.308 1.199 1.426 6.081 0.00000
0.5
1
2
Reduced IA risk
Increased IA risk
rs10757278 A>G SNP - Japanese
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
Hashikata - Japanese 1.150 0.951 1.391 1.439 0.15002 33.53
Nakaoka - Japanese 1.330 1.162 1.522 4.136 0.00004 66.47
1.267 1.135 1.414 4.206 0.00003
0.5
1
2
Reduced IA risk
Increased IA risk

Figure 4

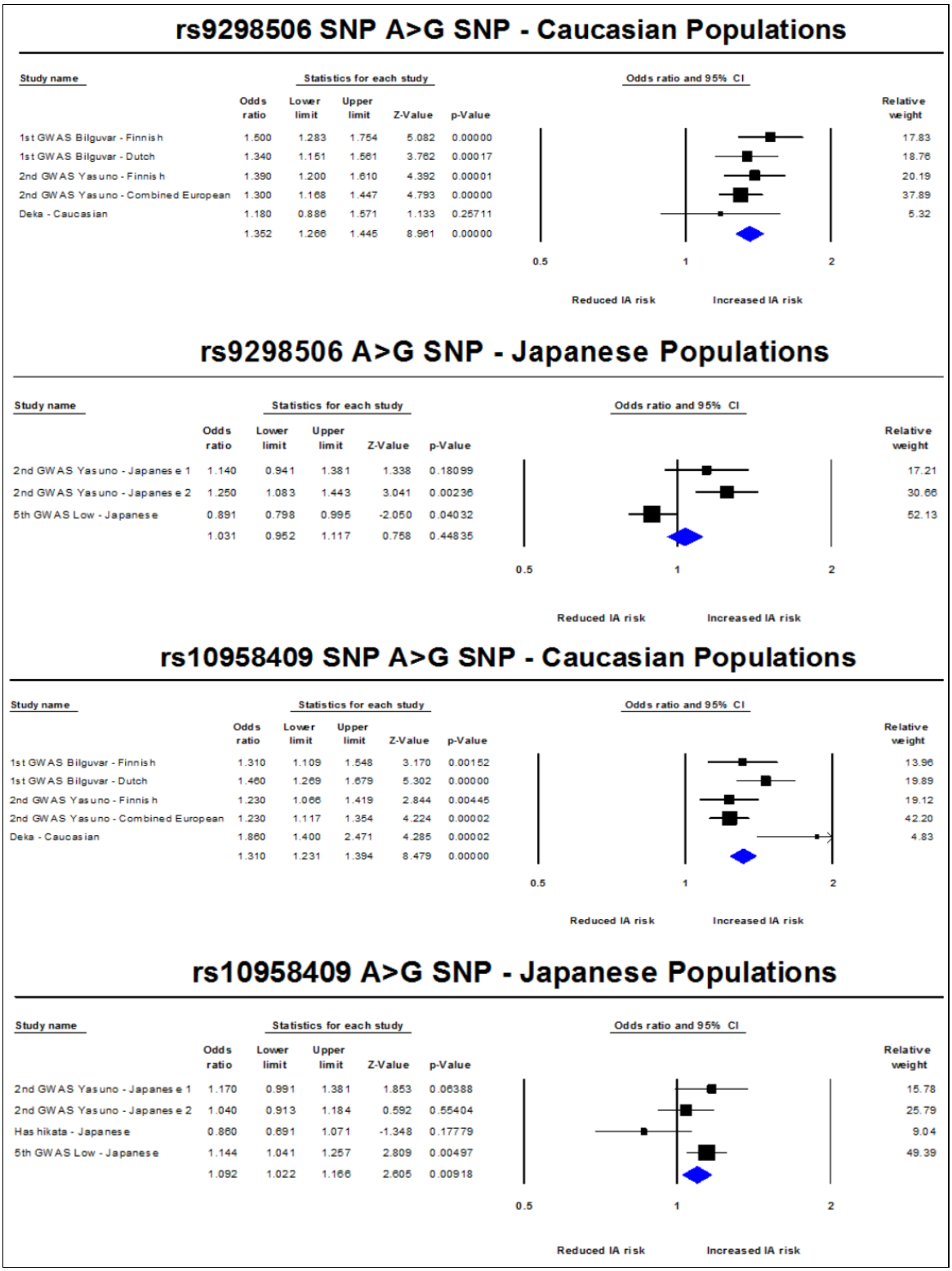

rs9298506 SNP A>G SNP - Caucasian Populations
Study name
Statistics for each study
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Odds ratio and 95% CI
Relative weight
1st GWAS Bilguvar - Finnish 1.500 1.283 1.754 5.082 0.00000 17.83
1st GWAS Bilguvar - Dutch 1.340 1.151 1.561 3.762 0.00017 18.76
2nd GWAS Yasuno - Finnish 1.390 1.200 1.610 4.392 0.00001 20.19
2nd GWAS Yasuno - Combined European 1.300 1.168 1.447 4.793 0.00000 37.89
Deka - Caucasian 1.180 0.886 1.571 1.133 0.25711 5.32
1.352 1.266 1.445 8.961 0.00000
0.5
1
2
Reduced IA risk
Increased IA risk
rs9298506 A>G SNP - Japanese Populations
Study name
Statistics for each study
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Odds ratio and 95% CI
Relative weight
2nd GWAS Yasuno - Japanese 1 1.140 0.941 1.381 1.338 0.18099 17.21
2nd GWAS Yasuno - Japanese 2 1.250 1.083 1.443 3.041 0.00236 30.66
5th GWAS Low - Japanese 0.891 0.798 0.995 -2.050 0.04032 52.13
1.031 0.952 1.117 0.758 0.44835
0.5
1
2
Reduced IA risk
Increased IA risk
rs10958409 SNP A>G SNP - Caucasian Populations
Study name
Statistics for each study
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Odds ratio and 95% CI
Relative weight
1st GWAS Bilguvar - Finnish 1.310 1.109 1.548 3.170 0.00152 13.96
1st GWAS Bilguvar - Dutch 1.460 1.269 1.679 5.302 0.00000 19.89
2nd GWAS Yasuno - Finnish 1.230 1.066 1.419 2.844 0.00445 19.12
2nd GWAS Yasuno - Combined European 1.230 1.117 1.354 4.224 0.00002 42.20
Deka - Caucasian 1.860 1.400 2.471 4.285 0.00002 4.83
1.310 1.231 1.394 8.479 0.00000
0.5
1
2
Reduced IA risk
Increased IA risk
rs10958409 A>G SNP - Japanese Populations
Study name
Statistics for each study
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Odds ratio and 95% CI
Relative weight
2nd GWAS Yasuno - Japanese 1 1.170 0.991 1.381 1.853 0.06388 15.78
2nd GWAS Yasuno - Japanese 2 1.040 0.913 1.184 0.592 0.55404 25.79
Hashikata - Japanese 0.860 0.691 1.071 -1.348 0.17779 9.04
5th GWAS Low - Japanese 1.144 1.041 1.257 2.809 0.00497 49.39
1.092 1.022 1.166 2.605 0.00918
0.5
1
2
Reduced IA risk
Increased IA risk

Figure 5

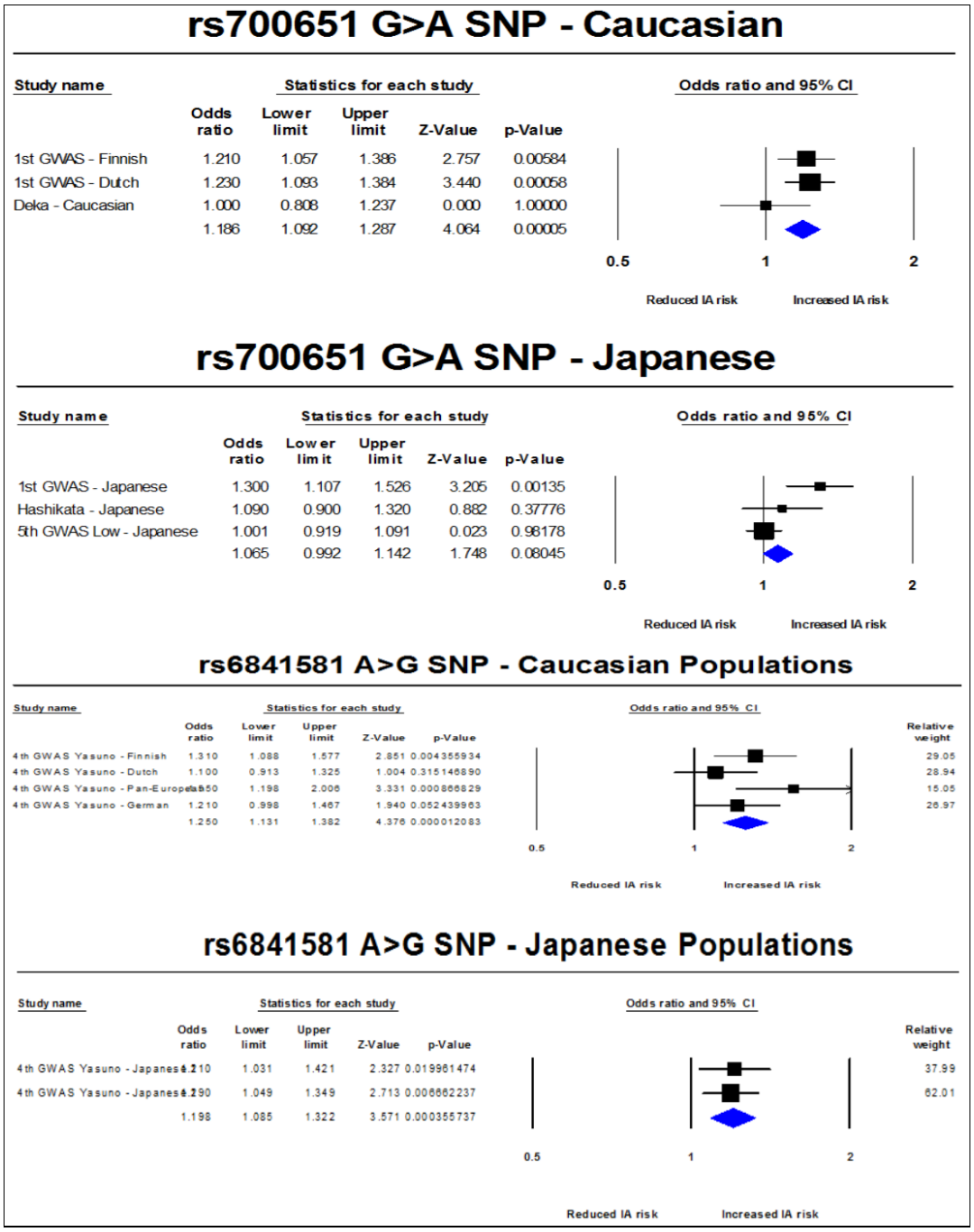
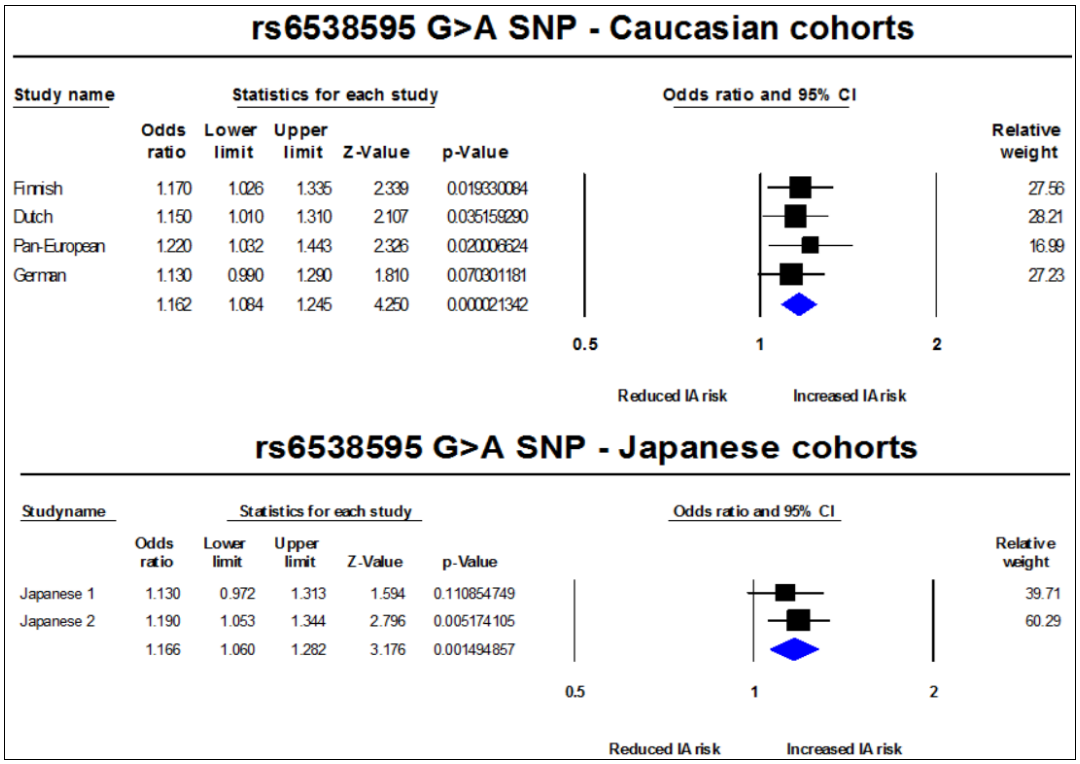

rs700651 G>A SNP - Caucasian
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
1st GWAS - Finnish 1.210 1.057 1.386 2.757 0.00584
1st GWAS - Dutch 1.230 1.093 1.384 3.440 0.00058
Deka - Caucasian 1.000 0.808 1.237 0.000 1.00000
1.186 1.092 1.287 4.064 0.00005
0.5
1
2
Reduced IA risk
Increased IA risk
rs700651 G>A SNP - Japanese
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
1st GWAS - Japanese 1.300 1.107 1.526 3.205 0.00135
Hashikata - Japanese 1.090 0.900 1.320 0.882 0.37776
5th GWAS Low - Japanese 1.001 0.919 1.091 0.023 0.98178
1.065 0.992 1.142 1.748 0.08045
0.5
1
2
Reduced IA risk
Increased IA risk
rs6841581 A>G SNP - Caucasian Populations
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
4th GWAS Yasuno - Finnish 1.310 1.088 1.577 2.851 0.004355934 29.05
4th GWAS Yasuno - Dutch 1.100 0.913 1.325 1.004 0.315146890 28.94
4th GWAS Yasuno - Pan-European 1.550 1.198 2.006 3.331 0.000866829 15.05
4th GWAS Yasuno - German 1.210 0.998 1.467 1.940 0.052439963 26.97
1.250 1.131 1.382 4.376 0.000012083
0.5
1
2
Reduced IA risk
Increased IA risk
rs6841581 A>G SNP - Japanese Populations
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
4th GWAS Yasuno - Japanese 1.210 1.031 1.421 2.327 0.019961474 37.99
4th GWAS Yasuno - Japanese 1.290 1.049 1.349 2.713 0.006662237 62.01
1.198 1.085 1.322 3.571 0.000355737
0.5
1
2
Reduced IA risk
Increased IA risk

Figure 6


rs6538595 G>A SNP - Caucasian cohorts
Study name
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
Finnish 1.170 1.026 1.335 2.339 0.019330084 27.56
Dutch 1.150 1.010 1.310 2.107 0.035159290 28.21
Pan-European 1.220 1.032 1.443 2.326 0.020006624 16.99
German 1.130 0.990 1.290 1.810 0.070301181 27.23
1.162 1.084 1.245 4.250 0.000021342
0.5
1
2
Reduced IA risk
Increased IA risk
rs6538595 G>A SNP - Japanese cohorts
Studyname
Statistics for each study
Odds ratio and 95% CI
Odds ratio
Lower limit
Upper limit
Z-Value
p-Value
Relative weight
Japanese 1 1.130 0.972 1.313 1.594 0.110854749 39.71
Japanese 2 1.190 1.053 1.344 2.796 0.005174105 60.29
1.166 1.060 1.282 3.176 0.001494857
0.5
1
2
Reduced IA risk
Increased IA risk

Funnel Plots for positively associated SNPs

Figure 7

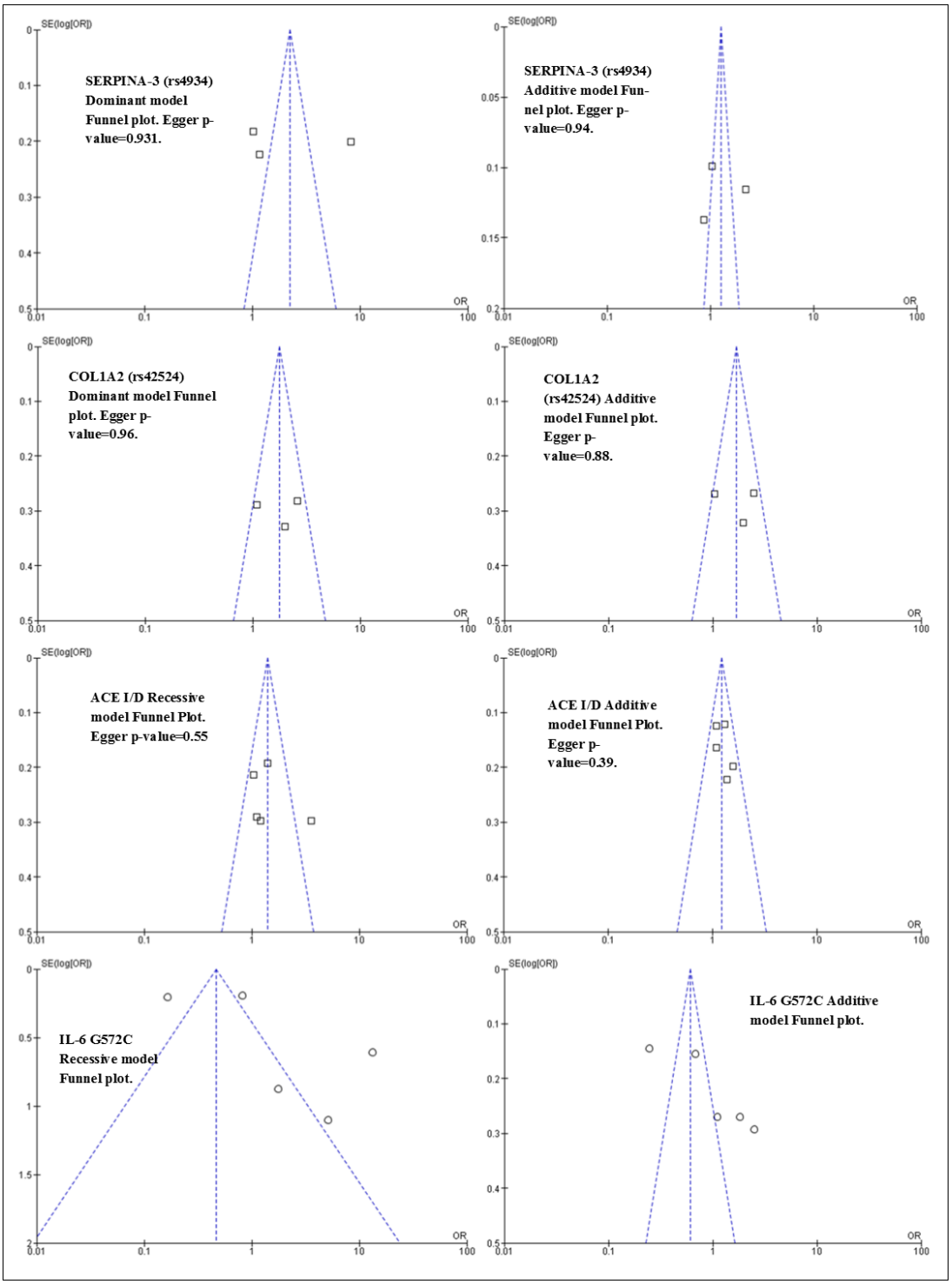


Figure 8

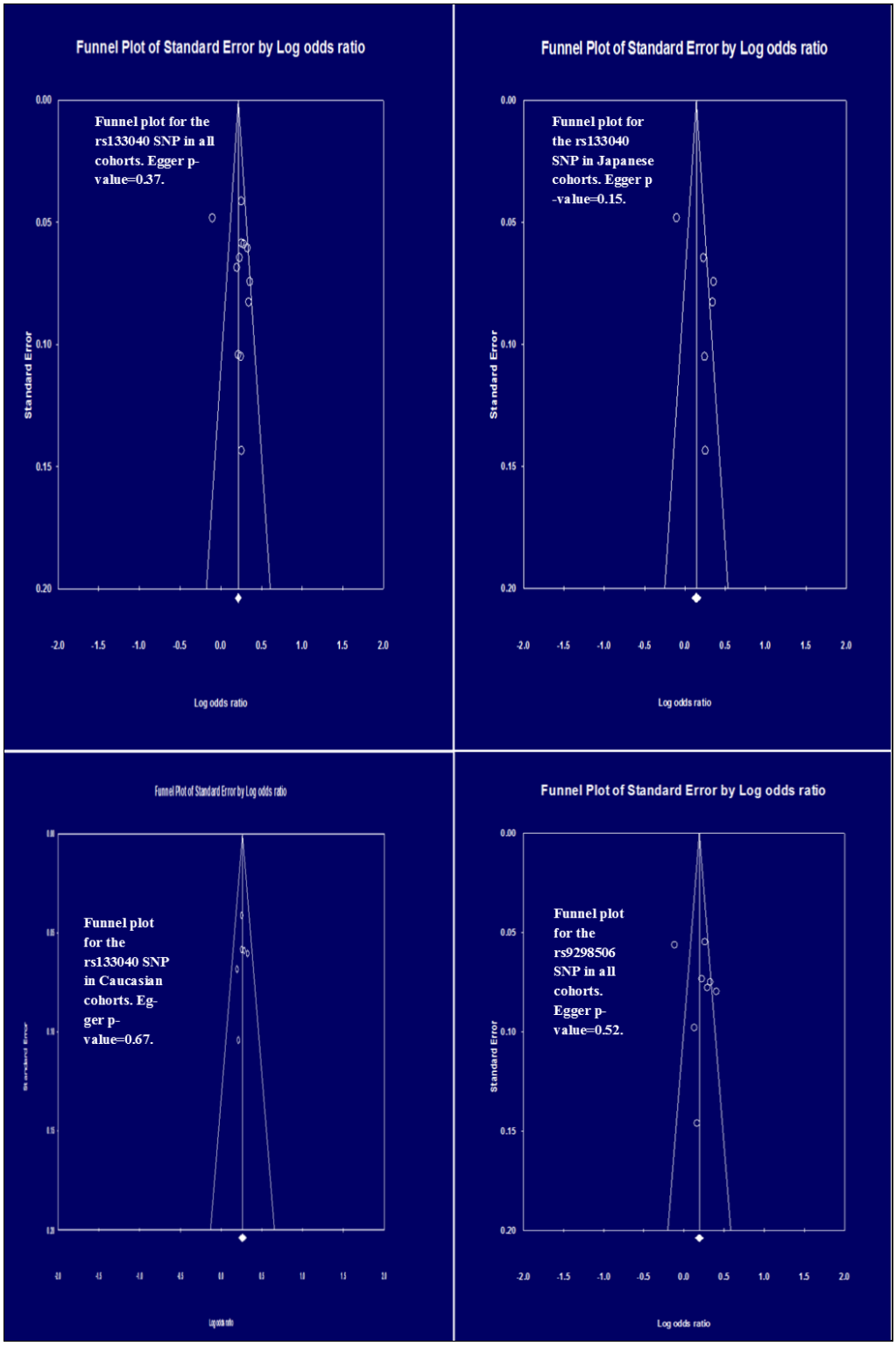

Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs133040 SNP in all cohorts. Egger p-value=0.37.
Standard Error
Log odds ratio
Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs133040 SNP in Japanese cohorts. Egger p-value=0.15.
Standard Error
Log odds ratio
Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs133040 SNP in Caucasian cohorts. Egger p-value=0.67.
Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs9298506 SNP in all cohorts. Egger p-value=0.52.
Standard Error
Log odds ratio

Figure 9

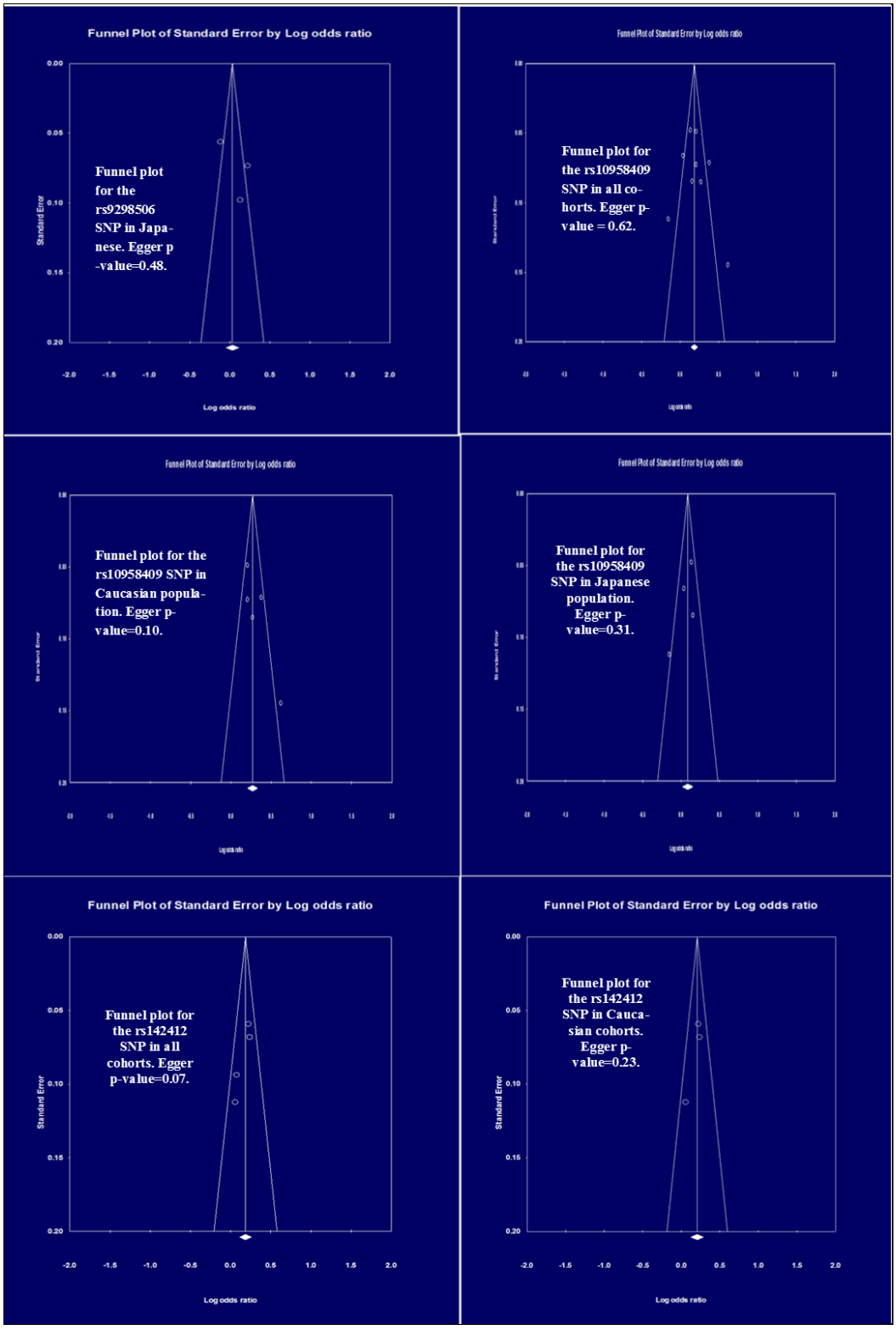

Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs9298506 SNP in Japanese. Egger p-value=0.48.
Standard Error
Log odds ratio
Funnel plot for the rs10958409 SNP in all cohorts. Egger p-value = 0.62.
Funnel plot for the rs10958409 SNP in Caucasian population. Egger p-value=0.10.
Funnel plot for the rs10958409 SNP in Japanese population. Egger p-value=0.31.
Funnel plot for the rs142412 SNP in all cohorts. Egger p-value=0.07.
Funnel plot for the rs142412 SNP in Caucasian cohorts. Egger p-value=0.23.

Figure 10

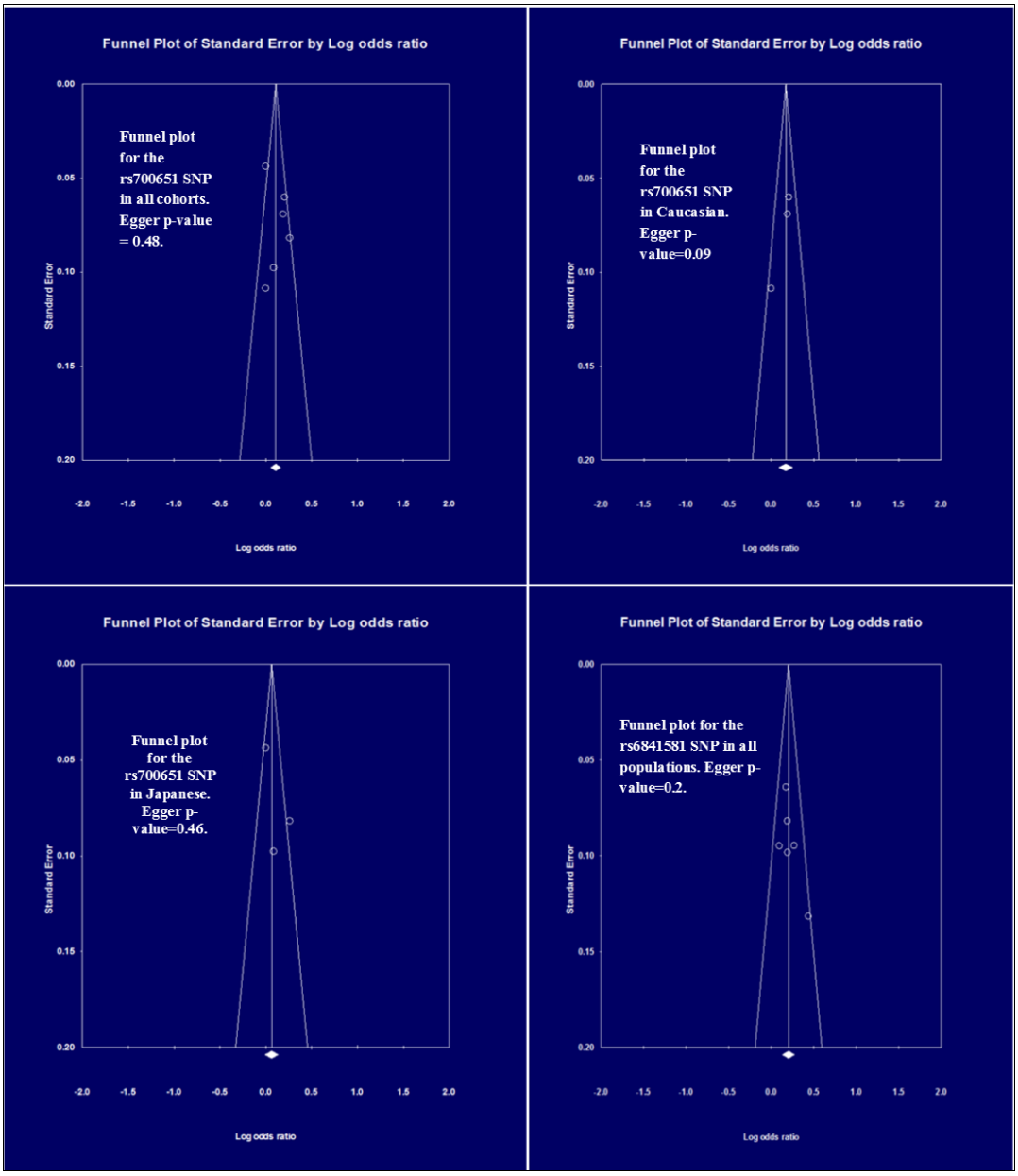

Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs700651 SNP in all cohorts. Egger p-value = 0.48.
Standard Error
Log odds ratio
Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs700651 SNP in Caucasian. Egger p-value=0.09
Standard Error
Log odds ratio
Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs700651 SNP in Japanese. Egger p-value=0.46.
Standard Error
Log odds ratio
Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs6841581 SNP in all populations. Egger p-value=0.2.
Standard Error
Log odds ratio

Figure 11

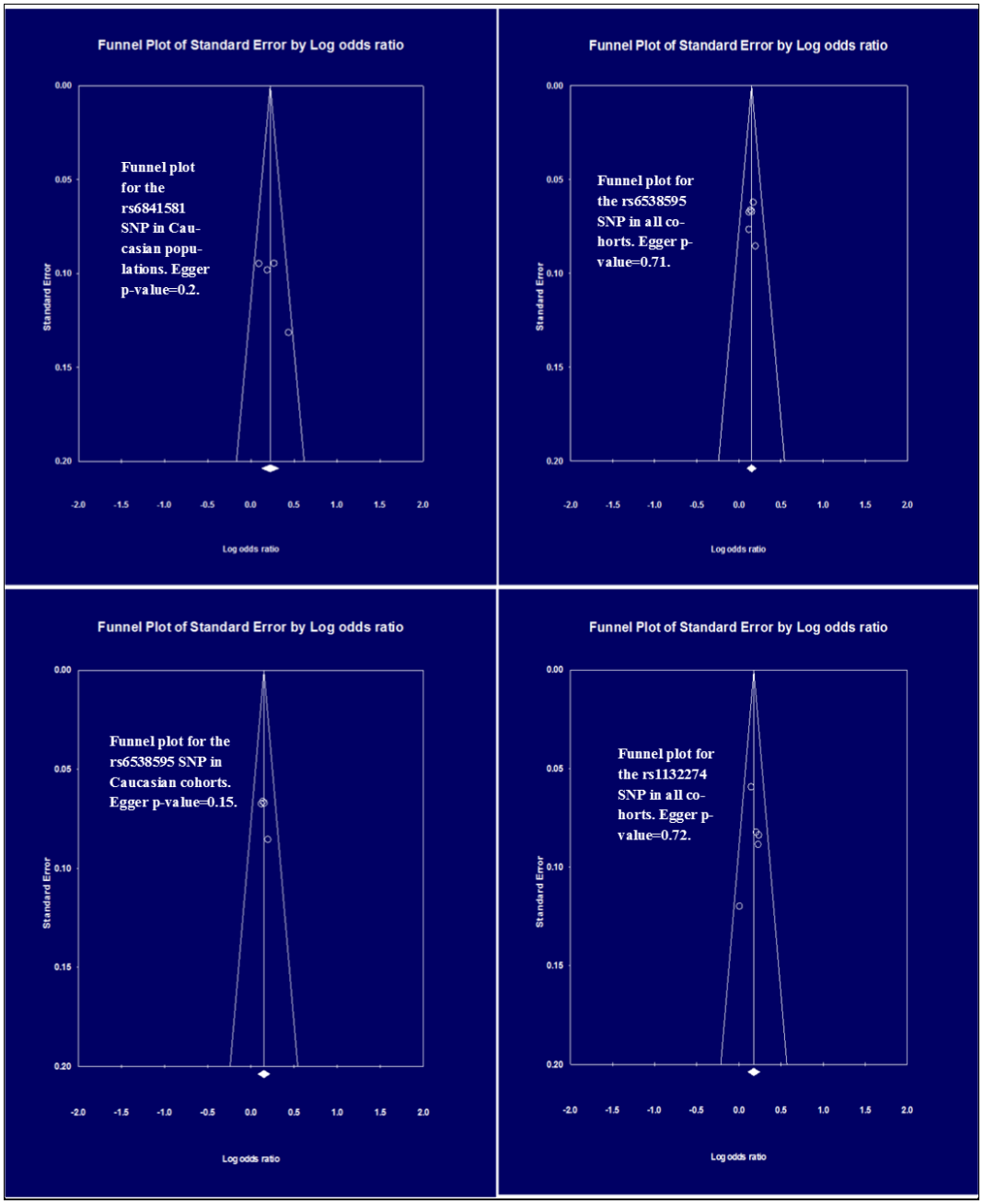

Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs6841581 SNP in Caucasian populations. Egger p-value=0.2.
Standard Error
Log odds ratio
Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs6538595 SNP in all cohorts. Egger p-value=0.71.
Standard Error
Log odds ratio
Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs6538595 SNP in Caucasian cohorts. Egger p-value=0.15.
Standard Error
Log odds ratio
Funnel Plot of Standard Error by Log odds ratio
Funnel plot for the rs1132274 SNP in all cohorts. Egger p-value=0.72.
Standard Error
Log odds ratio

Table 2 – Candidate gene SNP not associated with IA in any genetic model.

Gene	SNP	Genetic Model	No. of studies	Cases	Controls	FE - OR (95%)	p-value (corrected)	p_{HET} (I^2)
eNOS	VNTR 4a/4b[a]	Dominant	4[e334-338]	818	710	0.97 [0.71-1.34]	0.82	0.91 (0%)
		Recessive				1.33 [0.54-3.31]	0.42	0.28 (21%)
		Additive				1.01 [0.76-1.33]	0.95	0.80 (0%)
eNOS	T786C[b]	Dominant	6[e334, 336-341]	1002	1137	1.24 [0.95-1.61]	0.04	0.83 (0%)
		Recessive				1.18 [0.66-2.10]	0.47	0.30 (18%)
		Additive				1.18 [0.95-1.47]	0.05	0.86 (0%)
Endoglin Intron 7	Wt/INS	Dominant	6[e232-237]	934	992	1.00 [0.78-1.28]	0.98	0.38 (6%)
		Recessive				1.23 [0.78-1.94]	0.25	0.08 (49%)
		Additive				1.04 [0.86-1.27]	0.60	0.14 (39%)
eNOS	G894T[c]	Dominant	3[e334-338, 342]	413	535	1.08 [0.74-1.57]	0.60	0.56 (0%)
		Recessive				0.82 [0.32-2.10]	0.59	0.89 (0%)
		Additive				1.03 [0.75-1.42]	0.80	0.74 (0%)
IL-6	G174C	Dominant	3[e154, 196, 343]	541	3457	0.89 [0.67-1.19]	0.32	0.03 (73%)
		Recessive				0.76 [0.52-1.11]	0.06	0.06 (65%)
		Additive				0.88 [0.72-1.07]	0.09	0.01 (78%)
IL-1β	C5111T	Dominant	2[e312, 313]	446	386	1.07 [0.74-1.53]	0.64	0.30 (5%)
		Recessive				1.55 [0.88-2.71]	0.05	0.05 (74%)
		Additive				1.15 [0.88-1.50]	0.18	0.12 (60%)
MMP-3	5A/6A	Dominant	2[e344, 345]	146	323	0.81 [0.42-1.57]	0.42	0.40 (0%)
		Recessive				1.14 [0.65-2.03]	0.55	0.99 (0%)
		Additive				0.99 [0.68-1.44]	0.95	0.74 (0%)
Elastin	Intron 20 T>C (rs2856728)	Dominant	2[e129, 346]	354	384	0.93 [0.62-1.40]	0.65	0.12 (58%)
		Recessive				1.72 [0.56-5.27]	0.21	0.76 (0%)
		Additive				1.00 [0.70-1.43]	0.99	0.21 (38%)
Elastin	Intron 23 C>T	Dominant	2[e129, 346]	343	373	0.78 [0.51-1.18]	0.12	0.21 (35%)
		Recessive				1.05 [0.65-1.69]	0.81	0.55 (0%)
		Additive				0.91 [0.69-1.20]	0.37	0.25 (24%)
PAI-1	4G/5G[d]	Dominant	2[e344, 347]	240	539	1.15 [0.72-1.85]	0.44	0.20 (38%)
		Recessive				1.11 [0.71-1.72]	0.54	0.11 (61%)
		Additive				1.10 [0.83-1.46]	0.39	0.09 (65%)
GP IIIa A2	A1/A2	Dominant	2[e348, 349]	391	560	0.89 [0.61-1.30]	0.43	0.04 (77%)
		Recessive				1.19 [0.31-4.65]	0.74	0.75 (0%)
		Additive				0.92 [0.66-1.29]	0.53	0.08 (68%)
Factor XIII	Val/Leucine	Dominant	3[e347, 350, 351]	675	790	1.12 [0.84-1.50]	0.30	0.14 (50%)
		Recessive				1.31 [0.75-2.27]	0.22	0.09 (58%)
		Additive				1.13 [0.90-1.42]	0.18	0.14 (48%)
APOE	ε2	Dominant	5[e139, 250-252, 352]	477	2102	1.05 [0.77-1.44]	0.77	0.41 (21%)
		Recessive				1.41 [0.48-4.12]	0.53	0.26 (5%)
		Additive				1.15 [0.91-1.27]	0.10	0.13 (0%)
APOE	ε4	Dominant	5[e139, 250-252, 352]	477	2102	1.13 [0.78-1.63]	0.52	0.11 (0%)
		Recessive				1.57 [0.71-3.45]	0.26	0.37 (15%)
		Additive				1.22 [0.77 – 1.3]	0.25	0.45 (43%)
COL3A1	C>T (rs2138533)	Dominant	2[e190, 353]	788	2666	0.89 [0.71-1.10]	0.15	<0.00001 (96%)
		Recessive				0.73 [0.51-1.05]	0.03	0.007 (86%)
		Additive				0.87 [0.74-1.03]	0.03	<0.00001 (96%)
COL3A1	A>G (rs11887092)	Dominant	2[e190, 353]	788	2666	1.07 [0.83-1.38]	0.49	0.44 (0%)
		Recessive				1.16 [0.49-2.71]	0.66	0.98 (0%)
		Additive				1.07 [0.85-1.34]	0.46	0.49 (0%)
COL41A	C>T (rs3783107)	Additive	2[e151, 177]	1316	1742	1.07 [0.96-1.2]	0.20	0.016 (83%)
FBN2	C>G (rs331079)	Additive	2[e151, 177]	1316	1742	1.15 [0.96-1.38]	0.14	0.035 (78%)
MMP-2	C>T (rs243865)	Additive	2[e148, 206]	2175	2069	1.01 [0.86-1.19]	0.88	0.7 (0%)
MMP-9	C1562T (rs3918242)	Additive	3[e148, 345, 354]	427	562	0.95 [0.73-1.24]	0.7	0.196 (39%)
9p21.3	C>T (rs10757272)	Additive	2[e163, 355]	2478	6770	1.01 [0.94-1.09]	0.76	$6.5x10^{-09}$ (97%)
18q11.2	A>C (rs11661542)	Additive	3[e46, 163]	7272	19660	1.04 [0.97-1.11]	0.26	$3.83x10^{-06}$ (95%)

[a] Ref e-1 (Khurana et al.) excluded due to control genotypes not meeting HWE.
[b] Ref e-3 (Krischek et al.) excluded due to control genotypes not meeting HWE.
[c]3 of the 6 studies for the ENOS G894T were excluded as genotype counts not consistent with HWE ($p<0.05$).
[d] Ref e-25 (Ladenvall et al.) excluded due to control genotypes not meeting HWE.

Appendix II for Chapter 4

Figure 1 -GOSH CRF proforma for ruptured intracranial aneurysms

GENETICS OF GOSH SUBARACHNOID HAEMORRHAGE

DATE CRF COMPLETED: ___/___/_____

GOSH SAH CRF ENTRY PROFORMA

PATIENT NAME: SEX:
HOSPITAL NUMBER:
DOB: ___/___/_____ **CENTRE CODE: 17**
RECRUITED INTO STASH **(YES/NO)**
CENTRE: MOUNT GOULD
STUDY ID NUMBER: 17-

Page 1 of 4

ANGIOGRAM-CONFIRMED ANEURYSMAL SAH?

YES☐ NO☐ → **END**

EXCLUSION CRITERIA?

Known ADPKD (Autosomal Dominant Polycystic Kidney Disease)? No☐ Yes☐
Non-aneurysmal cause? No☐ Trauma ☐
AVM (Arteriovenous Malformation) ☐
SBE (Subacute Bacterial Endocarditis) ☐
DEAD ☐

☐YES *Exclusion Present*

NO☐ *No Exclusions*

Please complete the information boxes below

1. CLINICAL DETAILS *(please tick all boxes that apply)*

Diagnostic test: CTA (CT Angiogram) ☐

MRA (Magnetic Resonance angiogram) ☐

DSA (Digital Subtraction Angiogram) ☐
Date of acute admission: ____/____/_____

Date/time of event (ictus):____/____/_____ ___h:___min

Sudden severe headache <2 mths prior to ictus? No☐

Yes☐ (*if yes, please state date:* ____/____/_____)

Activity at time of ictus: Asleep☐ Sedentary☐

Mild-mod activity☐ Strenuous activity☐ Unknown☐
Specific activity……

Symptoms at ictus: Headache No☐ Yes☐

Vomiting No☐ Yes☐ Seizures No☐ Yes ☐

LOC (Loss of Consciousness)/Collapse No☐ Yes ☐

Focal neurologic deficit No☐ Yes☐ (State).....
Aneurysm location(s): (*please tick all that apply*)

AComm ☐☐ L R	PComm ☐☐ L R	ICA ☐☐ L R
MCA ☐☐ L R	ACA ☐☐ L R	Basilar ☐☐ L R
AICA ☐☐ L R	PICA ☐☐ L R	Superior ☐☐ Cerebellar L R

OTHER LOCATION (state).....
Location of index (ruptured) aneurysm if >1 aneurysm present.....
Size of index aneurysm: _____mm/cm (*please circle*)

Size of index aneurysm not known ☐

2. PAST MEDICAL HISTORY

Hypertension (HTN): N☐Y☐
Last known BP pre-ictus: _____/_____ mmHg
Date: _____/____/_____

HTN Drug(s) N☐ Y☐ Duration.....mths/yrs

Angina N☐Y☐ **MI** N☐Y☐ **CABG** N☐Y☐

Periph Vasc Dis N☐Y☐ **D.Mellitus** N☐Y☐

Ischaemic stroke N☐Y☐ **↑Cholesterol** N☐Y☐

Statin: N☐ Y☐ (drug).....
Admission Total Cholesterol level (if available)……..

Intracerebral haemorrhage N☐Y☐ **SAH** N☐Y☐

On antiplatelet/anticoagulants N☐ Y☐.....yrs

Aspirin☐ Clopidogrel ☐ Dipyridamole☐

Warfarin☐ If on Warfarin, admission INR:.....

Pregnant N☐Y☐ **Oral contraceptive pill** N☐Y☐

Hormone Replacement Therapy N☐Y☐

Post-menopause N☐Y☐

Connective tissue disorder? N☐ Unknown☐ Y☐

Marfan☐ Ehlers-Danlos☐ Pseudoxanthoma elasticum☐

3. SOCIAL HISTORY

PREMORBID DISABILITY - Modified Rankin Score (MRS): ☐ (See Page 4 for how to score the MRS)

Current Smoker? No☐ Yes☐

Ex-Smoker? No☐ Yes☐
How many years of smoking? .....years
No. of cigarettes smoked per day……..
Age started..... Months/years of abstinence.....

Alcohol: Current drinker? No☐ Yes☐

Ex-Alcohol drinker? No☐ Yes☐
Units/wk.....

Type of Alcohol you drink: Beer☐ Spirits☐ Wine☐
Age started..... Months/ years of abstinence.....

Recreational drug use in the last 6 months? No☐

Cocaine☐ Opiates☐ Cannabis☐

Amphetamine ☐ Other (please state):……

GOSH CRF v1.3 OCTOBER 2012 © V S ALG & D WERRING **ION**

GENETICS OF GOSH SUBARACHNOID HAEMORRHAGE

GOSH CRF ENTRY PROFORMA

STUDY ID NUMBER: 17- _____

4. LIFE EVENTS

Significant life events in last 6 months pre-ictus:

None ☐

Death of close friend / relative ☐

Divorce or separation ☐

Major illness in self or relative ☐

Financial Problems ☐

Accidents, criminal attack ☐

Other ☐ (please state below):

.....
.....
.....
.....

* Intracranial haemorrhage
**Autosomal Dominant Polycystic Kidney Disease

5. GENETIC AND FAMILY HISTORY

Consanguinity (Marriage in 1st degree relatives)? No □ Yes □
Ethnicity: White - British□ White - Irish□ White - other□
Black – Afro-Caribbean□ Black - African□ Japanese□ Pakistani □ European□ Bangladeshi□ Chinese□ Indian□
Country of birth.....
Residency in UK.....MTHS/YRS
Mother's Ethnicity.....
Father's Ethnicity.....

Relative	Alive(A) Dead(D) Unknown(U)	Sex	Age at Death	Cause of death (U if unknown)	History of: hypertension / intracranial aneurysms / SAH / ICH* / Connective tissue disorder / ADPKD** / Other / None (please state)
Sibling 1					
Sibling 2					
Sibling 3					
Sibling 4					
Mother					
Father					
Paternal Grandmother					
Paternal Grandfather					
Maternal Grandmother					
Maternal Grandfather					

6. CLINICAL STATUS UPON ACUTE ADMISSION WITH SAH

Glasgow Coma Score (GCS)						WFNS Grade		Fisher Grade	
Motor		Verbal		Eye					
6 – Obeys commands	□	5 – Orientated	□	4 - Spontaneous	□	I – GCS 15	□	1 – No blood.	□
5 – Localises to pain	□	4 - Confused	□	3 – To Speech	□	II – GCS 13-14, no focal deficit.	□	2 – Diffuse, thin layer of blood <1mm.	□
4 – Normal Flexion	□	3 – Inappropriate	□	2 – To Pain	□	III – GCS 13-14, with focal deficit.	□	3 – Thick layer of blood >1mm, or localised clot.	□
3 – Abnormal Flexion	□	2 -Incomprehensible	□	1 – None	□	IV – GCS 7-12, with or w/o focal deficit.	□	4 – Intracerebral or intraventricular haemorrhage.	□
2 - Extension	□	1 - None	□			V – GCS 3-6	□		
1 – None	□								

Meningism	Yes □	Papilloedema	Yes □	Intraocular Haemorrhage	Yes □	Focal Neurological Deficit	Yes □ Please state.....
	No □		No □		No □		No □
	Unknown □		Unknown □		Unknown □		Unknown □
Admission BP	____/___ mmHg	Heart Rate	/min	O2 Saturations	%	Temp	___.__°C

SAH Complications	Yes □	Re-bleed □	Hydrocephalus □	Vasospasm □
	No □			
ITU/HDU Admission	Yes □	State Reason.....		
	No □			

ECG	Yes □	Please State findings (e.g. sinus rhythm, TW changes)
	No □	.....

STUDY ID NUMBER: 17- _____

7. DEFINITIVE ANEURYSM TREATMENT / SAH COMPLICATIONS (PRIOR TO TREATMENT)

Treatment: Endovascular Coiling☐ Surgical Clipping☐ Stenting☐ None☐ Other☐ (please state)……
Date(s) of treatment……

PO/NG Nimodipine 2-4 hourly☐ Simvastatin☐ Hypertensive-Hypervolaemic-Haemodilution R_x☐

☐Hydrocephalus warranting EVD (External Ventricular Drain) ☐ Haematoma Evacuation ☐ Decompressive Craniectomy

Post-intervention complications NO☐ YES☐ (*If yes please tick boxes below*)

Re-bleeding: Yes☐ No☐ Hydrocephalus: Yes☐ No☐ Seizures: No☐ Yes☐ Sepsis: No☐ Yes☐

Delayed Ischaemic Neurological Deficit (DIND) NO☐ YES☐ **(GOTO BOX 8)**

Nature of focal deficit: Hemiparesis? NO ☐ YES☐

Dysphasia? NO ☐ YES☐ Other focal deficit? NO ☐ YES☐ (please state):……

8. DELAYED ISCHAEMIC NEUROLOGICAL DEFICIT (DIND) – CEREBRAL VASOSPASM

Vasospasm present on treatment (1st) Angiogram NO☐ YES☐ (Please state territory +/- Intervention)……

DOES PATIENT FULFILL >1 OF CRITERIA 1-3 BELOW? YES☐ NO☐ (*If No please go to Box 10*)

DIND criteria

1. Clear new focal deficit developed 72 hours post-ictus? NO☐ YES☐

2. GCS drop by ≥ 2 or more points NO ☐ YES ☐

3. Severe worsening of headache 72 hours post-ictus with evidence of vasospasm? NO ☐ YES ☐

Date of onset post-ictus: ___/___/_____

Time of onset post-ictus: ___:___
Nature of focal deficit:

Hemiparesis? NO ☐ YES☐

Dysphasia? NO ☐ YES☐

Other focal deficit? NO ☐ YES☐ (please state):……

Glasgow Coma Score (GCS) prior to DIND:

E 1☐ 2☐ 3☐ 4☐ **V** 1☐ 2☐ 3☐ 4☐ 5☐

M 1☐ 2☐ 3 ☐ 4☐ 5☐ 6☐

GCS score when DIND developed:

E 1☐ 2☐ 3☐ 4☐ **V** 1☐ 2☐ 3☐ 4☐ 5☐

M 1☐ 2☐ 3☐ 4☐ 5☐ 6☐

9. CEREBRAL VASOSPASM INVESTIGATIONS AND INTERVENTIONS

Cerebral Vasospasm confirmed: No☐ Yes☐
If Yes please state vascular territory:

Middle Cerebral Artery ☐ ☐**L** ☐**R**

Anterior Cerebral Artery ☐ ☐**L** ☐**R**

Posterior Cerebral Artery ☐ ☐**L** ☐**R**

Other ☐ (please state details):
……

Method of confirming Cerebral Vasospasm:

CTA (Computed Tomography Angiogram) ☐

Transcranial Doppler (TCD) ☐

Suspected infarct: Yes☐ No☐

Confirmed by imaging? Yes☐ No☐

If Yes, CT☐ Diffusion-weighted MRI (DWI) ☐

Cerebral Angiography☐:

Endovascular intervention for DIND / cerebral vasospasm: Angioplasty☐ Stenting☐ Intra-arterial nimodipine☐ Intra-arterial papaverine☐

None☐

Admission Plasma Na^+ (Sodium): _____mmol/l

Plasma Na^+(Sodium) at DIND onset:_____mmol/l

GENETICS OF GOSH SUBARACHNOID HAEMORRHAGE

GOSH CRF ENTRY PROFORMA

STUDY ID NUMBER: 17- _____

Date of Discharge from Hospital: _____/____/_____ Discharged to: Home☐ Rehab Unit☐ Other☐

Is there any disability other than SAH that could affect the outcome scores?

NO☐ YES☐ If yes, please state.....

10. MODIFIED RANKIN SCORE (MRS) AT DISCHARGE

0 ☐ No Symptoms at all

1 ☐ No significant disability despite symptoms; able to carry out Activities of Daily Living (ADL)

2 ☐ Slight Disability. Unable to carry out normal activities, but can look after own affairs w/o assistance

3 ☐ Moderate disability requiring help but able to walk without assistance

4 ☐ Moderately-severe disability. Unable to walk or to attend to bodily needs w/o assistance

5 ☐ Severe disability. Bedridden, incontinent and requiring constant nursing care and attention

11. GLASGOW OUTCOME SCORE AT DISCHARGE

1 ☐ Good Recovery

2 ☐ Moderate Recovery

3 ☐ Severe Disability

4 ☐ Persistent Vegetative state

5 ☐ Dead

IS THIS CRF BEING COMPLETED MORE THAN 1 MONTH AFTER DISCHARGE FROM ACUTE ADMISSION TO HOSPITALWITH SAH?

☐NO → **END**

☐YES

Please complete the follow-up discharge outcomes below

12. MODIFIED RANKIN SCORE ON DATE OF COMPLETION OF THIS CRF

0 ☐ No symptoms

1 ☐ No significant disability

2 ☐ Slight disability

3 ☐ Moderate disability requiring help

4 ☐ Moderate-severe disability

5 ☐ Severe disability

(*MRS scoring details above*)

13. GLASGOW OUTCOME SCORE ON DATE OF COMPLETION OF THIS CRF

1 ☐ Good Recovery

2 ☐ Moderate Recovery

3 ☐ Severe Disability

4 ☐ Persistent Vegetative state

5 ☐ Dead

Name of person completing form:.....

Job Title: .....

Signature: .....

Date: ____/___/_____

THANK YOU FOR COMPLETING THIS CRF

PLEASE SCAN* (david.werring@nhs.net), *FAX* (020-7833-8613) *OR POST A COPY OF THIS CRF TO THE CHIEF SITE

Figure 2 – GOSH proforma for Unruptured Intracranial Aneurysms (UIA)

DATE CRF COMPLETED: ___/___/_____

GOSH UIA CRF ENTRY PROFORMA

PATIENT NAME:
HOSPITAL NUMBER:
DOB: ___/___/_____ SEX:
AGE:
CENTRE: **MOUNT GOULD** CENTRE CODE: **17**
STUDY ID NUMBER: 17-

ANGIOGRAM-CONFIRMED INTRACRANIAL ANEURYSM OR ANGIOGRAM NEGATIVE SAH?

YES☐ NO☐ → **END**

EXCLUSION CRITERIA?

Known ADPKD (Autosomal Dominant Polycystic Kidney Disease)? No☐ Yes☐
Non-aneurysmal cause? No☐ Trauma ☐
AVM (Arteriovenous Malformation) ☐
SBE (Subacute Bacterial Endocarditis) ☐
DEAD ☐

☐YES → *Exclusion Present*

NO☐ *No Exclusions*

Please complete the information boxes below

1. CLINICAL DETAILS *(please tick all boxes that apply)*

Diagnostic test: CTA (CT Angiogram) ☐

MRA (Magnetic Resonance angiogram) ☐

DSA (Digital Subtraction Angiogram) ☐

Aneurysm location(s): (*please tick all that apply*)

AComm ☐☐ **L R**	PComm ☐☐ **L R**	ICA ☐☐ **L R**
MCA ☐☐ **L R**	ACA ☐☐ **L R**	Basilar ☐☐ **L R**
AICA ☐☐ **L R**	PICA ☐☐ **L R**	Superior ☐☐ Cerebellar **L R**

OTHER LOCATION (state).....

Size of aneurysm: _____mm/cm (*please circle*)

Size of other aneurysms (if multiple present.....

Size of index aneurysm not known ☐

Angiogram negative SAH ☐

* Intracranial haemorrhage
**Autosomal Dominant Polycystic Kidney Disease

2. PAST MEDICAL HISTORY

Hypertension (HTN): N☐Y☐
Last known BP pre-ictus: _____/_____ mmHg
Date: _____/____/_____

HTN Drug(s) N☐ Y☐ Duration.....mths/yrs

Angina N☐Y☐ **MI** N☐Y☐ **CABG** N☐Y☐

Periph Vasc Dis N☐Y☐ **D.Mellitus** N☐Y☐

Ischaemic stroke N☐Y☐ **↑Cholesterol** N☐Y☐

Statin: N☐ Y☐ (drug).....
Admission Total Cholesterol level (if available).....

Intracerebral haemorrhage N☐Y☐ **SAH** N☐Y☐

On antiplatelet/anticoagulants N☐ Y☐.....yrs

Aspirin☐ Clopidogrel ☐ Dipyridamole☐

Warfarin☐ If on Warfarin, admission INR:.....

Oral contraceptive pill N☐Y☐

Hormone Replacement Therapy N☐Y☐

Post-menopause N☐Y☐

Connective tissue disorder? N☐ Unknown☐ Y☐

Marfan☐ Ehlers-Danlos☐ Pseudoxanthoma elasticum☐

3. SOCIAL HISTORY

PREMORBID DISABILITY - Modified Rankin Score (MRS): ☐ (See Page 4 for how to score the MRS)

Current Smoker? No☐ Yes☐

Ex-Smoker? No☐ Yes☐
How many years of smoking? .....years
No. of cigarettes smoked per day.....
Age started..... Months/years of abstinence.....

Alcohol: Current drinker? No☐ Yes☐

Ex-Alcohol drinker? No☐ Yes☐
Units/wk.....

Type of Alcohol you drink: Beer☐ Spirits☐ Wine☐
Age started..... Months/ years of abstinence.....

Recreational drug use in the last 6 months? No☐

Cocaine☐ Opiates☐ Cannabis☐

Amphetamine ☐ Other (please state):.....

4. GENETIC AND FAMILY HISTORY

Consanguinity (Marriage in 1st degree relatives)? No □ Yes □

Ethnicity: White - British□ White - Irish□ White - other□
Black – Afro-Caribbean□ Black - African□ Japanese□ Pakistani □ European□ Bangladeshi□ Chinese□ Indian□
Country of birth……
Residency in UK……MTHS/YRS
Mother's Ethnicity……
Father's Ethnicity……

Relative	Alive(A) Dead(D) Unknown(U)	Sex	Age at Death	Cause of death (U if unknown)	History of: hypertension / intracranial aneurysms / SAH / ICH* / Connective tissue disorder / ADPKD** / Other / None (please state)
Sibling 1					
Sibling 2					
Sibling 3					
Sibling 4					
Mother					
Father					
Paternal Grandmother					
Paternal Grandfather					
Maternal Grandmother					
Maternal Grandfather					

5. DEFINITIVE ANEURYSM TREATMENT / COMPLICATIONS (1st 24 hours post-procedure)

Treatment: Endovascular Coiling☐ Surgical Clipping☐ Stenting☐ None☐ Other☐ (please state)……
Date of Treatments(s)……

Post-intervention complications (within 1st 24 hours) NO☐ YES☐ (*If yes please tick boxes below*)

Re-bleeding: Yes☐ No☐ Hydrocephalus: Yes☐ No☐

Seizures: No☐ Yes☐ Sepsis: No☐ Yes☐

Delayed Ischaemic Neurological Deficit (DIND) NO☐ YES☐

Nature of focal deficit: Hemiparesis? NO ☐ YES☐

Dysphasia? NO ☐ YES☐ Other focal deficit? NO ☐ YES☐ (please state):……

Any Further treatment……

Date of Discharge from Hospital: _____/____/_____ Discharged to: Home☐ Rehab Unit☐ Other☐

Died ☐ Cause of death……

Is there any disability that could affect the outcome scores?

NO☐ YES☐ If yes, please state……

10. MODIFIED RANKIN SCORE (MRS) AT DISCHARGE

0 ☐ No Symptoms at all

1 ☐ No significant disability despite symptoms; able to carry out Activities of Daily Living (ADL)

2 ☐ Slight Disability. Unable to carry out normal activities, but can look after own affairs w/o assistance

3 ☐ Moderate disability requiring help but able to walk without assistance

4 ☐ Moderately-severe disability. Unable to walk or to attend to bodily needs w/o assistance

5 ☐ Severe disability. Bedridden, incontinent and requiring constant nursing care and attention

11. GLASGOW OUTCOME SCORE AT DISCHARGE

1 ☐ Good Recovery

2 ☐ Moderate Recovery

3 ☐ Severe Disability

4 ☐ Persistent Vegetative state

5 ☐ Dead

Name of person completing form:……

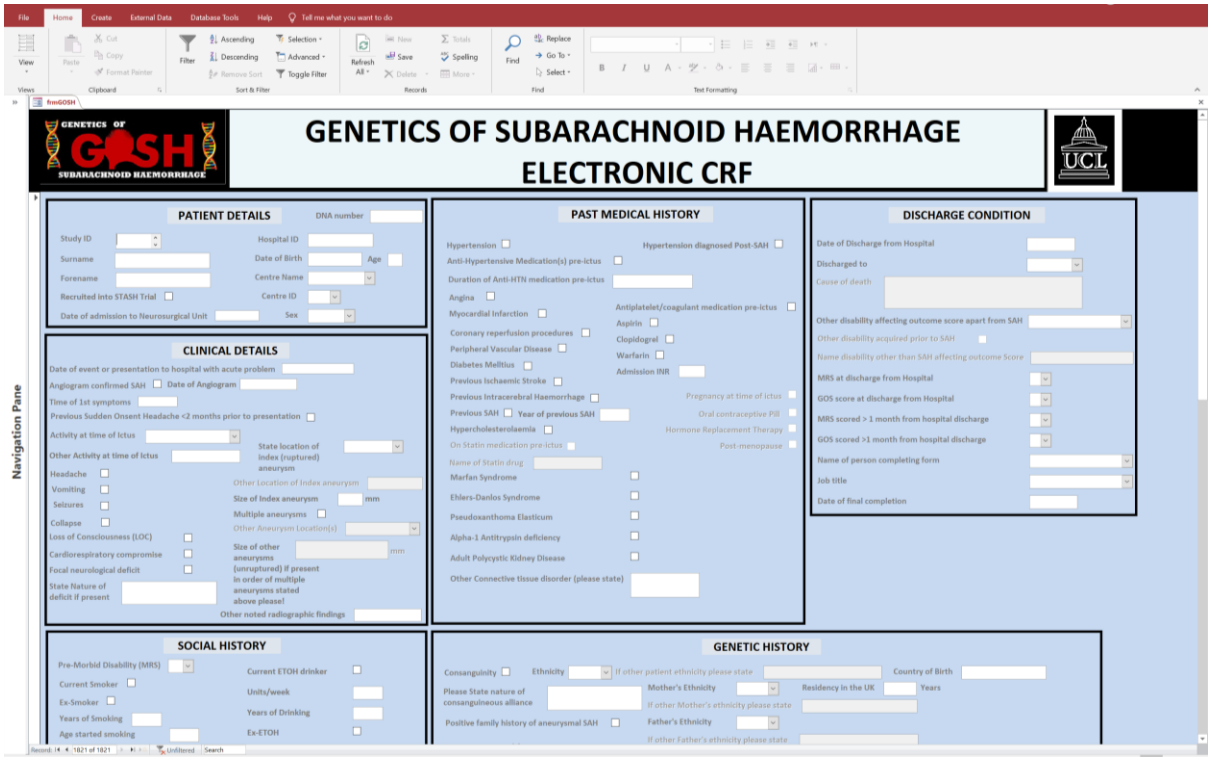
Job Title: ……

Signature: ……

Date: ____/___/_____

THANK YOU FOR COMPLETING THIS CRF

Figure 3 – GOSH e-CRF for data entry



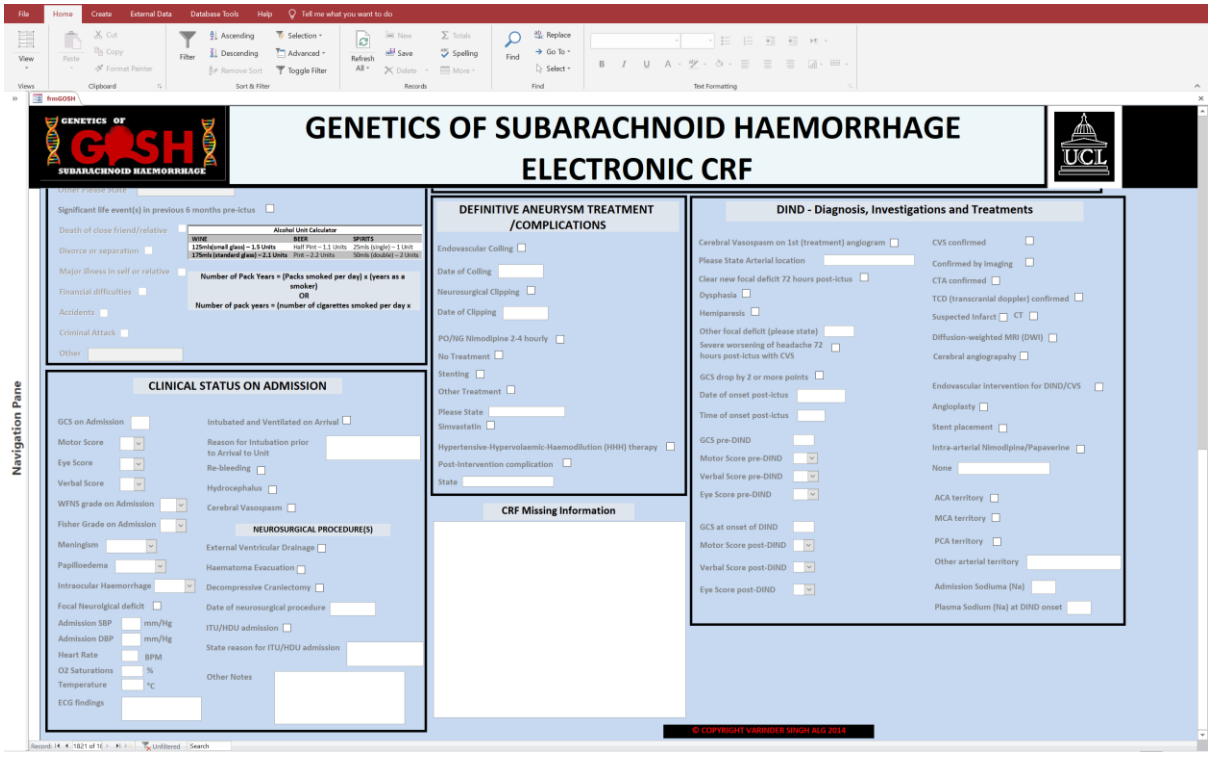


Figure 4 – Consent form patient with Capacity

INSERT HOSPITAL HEADERS AND LOGOS SPECIFIC TO EACH SITE

Centre Number: 07
Name of centre: Charing Cross Hospital — Project ID number: 7512
Patient Identification Number for this study: 07/___ — Form version: 1.3 28th April 2011

Genetics of Subarachnoid Haemorrhage (GOSH): CONSENT FORM

Principal investigator: Dr Pankaj Sharma — **Please initial box**

I confirm that I have read and understood the information sheet dated 28th April 2011 V1.3 for the above study and have had the opportunity to ask questions.	☐
I confirm that I have had sufficient time to consider whether or not I want to be included in the study.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
I understand that sections of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	☐
I agree to have a blood sample taken for a genetic test as part of the study protocol. I understand this is given as a gift.	☐
I agree to take part in the above study	☐

Name of patient	Date	Signature
Name of Person taking consent (If different from researcher)	Date	Signature
Researcher (to be contacted If there are any problems)	Date	Signature

Figure 5 – Consent forms signed by NOK on patient who lacked capacity

INSERT HOSPITAL LOGOS AND HEADERS SPECIFIC TO EACH SITE

INSERT HOSPITAL NAME AND CONTACT DETAILS FOR SPECIFIC SITE.

Centre Number: 07/_____
Centre Name: Charing Cross Hospital
Patient Identification Number for this study: 07/___

UCL Project ID number: 7512
Form version: 1.1 28th April 2011

Genetics of Subarachnoid Haemorrhage (GOSH): CONSENT FORM FOR CONSULTEE

Principal investigator: Dr Pankaj Sharma **Please initial box**

I confirm that I have read and understood the information sheet dated 28th April 2011 V1.3 the above study and have had the opportunity to ask questions.	☐
I confirm that I have had sufficient time to consider whether or not the patient would want to be included in the study.	☐
I understand that patient participation is voluntary and that they are free to withdraw at any time, without giving any reason, without their medical care or legal rights being affected.	☐
I understand that sections of patient medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to them taking part in research. I give permission for these individuals to have access to this patient's records.	☐
I agree for the patient to have a blood sample taken for a genetic test as part of the study protocol. I understand this is given as a gift.	☐
I agree for the patient to take part in the above study	☐

_____	_____	_____
Name of consultee providing consent on behalf of patient	Date	Signature
_____		
Relationship to patient		
_____	_____	_____
Name of Patient on whose behalf Consent is being taken	Date	Signature
_____	_____	_____
Name of Researcher taking consent	Date	Signature

1 form for Patient; 1 to be kept as part of the study documentation; 1 to be kept with hospital notes

Table 1 – Sites recruiting for the GOSH study

Name of site
NHNN
Kings College Hospital
Southampton General Hospital
Derriford Hospital, Plymouth
St George's Hospital
Imperial College Hospital
Mount Gould Hospital
Croydon University Hospital
Royal Preston Hospital
Leeds General Infirmary
Newcastle
Liverpool
Royal London
Oxford John Radcliffe
Cambridge Addenbrookes
Sheffield Royal Hallamshire
James Cook University Hospital, Middlesbrough
Salford Royal Manchester

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