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"A Small Animal Model for the Creation of Pre-Aneurysmal Change in the Intracranial Vasculature"

Masters Philosophy (Health)

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Australia

February 2016

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Acknowledgements

This work was supported by an educational stipend from Medtronic Australia and grants from the Australian National Health and Medical Research Council (NHMRC 1079193, 1079369, 1021416, 1000967) and the Queensland Government.

Professor Jonathon Golledge holds a Practitioner Fellowship from the NHMRC, Australia and a Senior Clinical Research Fellowship from the Queensland Government.

I would like to thank Australian Institute of Tropical Health and Medicine for providing research infrastructure.

I would like to thank Associate Professor Laurence Marshman, Professor Jonathon Golledge, and Dr Corey S Moran for their assistance and guidance with this project.

Thanks also to Dr Yutang Wang and Dr Sai-Wang Seto for their assistance with the laboratory component of this study.

A special thanks to Professor Rhondda Jones and Dr Leslie Kuma for their invaluable assistance with the statistical and pathological analyses respectively.

Statement of Contribution of Others

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		Dr Corey S Moran
Financial:	Stipend	Medtronic Australia Ltd.
		Australian National Health and Medical Research Council
		(NHMRC 1079193, 1079369, 1021416, 1000967)
		Queensland Government
Data Collection:	Research Assistance:	Dr Yutang Wang
		Dr Sai-Wang Seto

Our work as detailed in this thesis has formed the basis of two novel publications[148, 149]:

1. **“A small animal model for early cerebral aneurysm pathology**”

   James A. Lee, Laurence A.G. Marshman, Corey S Moran, Leslie Kuma, Eric P. Guazzo, David S. Anderson, Jonathan Golledge

   Journal of Clinical Neuroscience 34 (2016) 259-263.[148] 10.1016/j.jocn.2016.05.039

2. **"Mouse models of intracranial aneurysm."**

   Wang, Y., Emeto, T. I., Lee, J., Marshman, L., Moran, C., Seto, S. W., Golledge, J.

   Brain Pathol **25**(3): 237-247. 10.1111/bpa.12175 (2015)

Our work as detailed in this thesis has formed the basis of an oral presentation at the Neurosurgical Society of Australasia Annual Scientific Meeting, Sydney, Australia, 2016.

Glossary

AAA	Abdominal Aortic Aneurysm
ACA	Anterior Cerebral Artery
ACom	Anterior Communicating Artery
ACROSS	Australasian Co-Operative Research On Subarachnoid Haemorrhage Study
A-II	Angiotensin-II
aSAH	aneurysmal Subarachnoid Haemorrhage
BA	Basilar Artery
BAPN	beta-amino propionitrile
BBB	Blood Brain Barrier
BP	Blood pressure
C-	Complement Factor
CA	Cerebral Aneurysm
CoW	Circle of Willis
CSF	Cerebrospinal fluid
CT	Computer Assisted Tomography
dBP	diastolic Blood Pressure
DOCA	Deoxycorticosterone acetate
ECM	Extracellular Matrix
EEL	External Elastic Lamina
eNOS	endothelial-derived Nitric Oxide Synthase
EPC	Endothelial Progenerator Cells
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
HO	Haem-Oxygenase
HTN	Hypertension
ICH	Intracranial Haematoma/Intracerebral Haemorrhage

IEL	Internal Elastic Lamina
IL	Interleukin
INF	Interferon
JCU	James Cook University
LO	Lipoxygenase
LT	Leukotriene
mBP	mean Blood Pressure
MCA	Middle Cerebral Artery
MCP	Monocyte Chemo-Attractant Protein
MH	Myointimal hyperplasia
MMP	Matrix Metalloproteinase
NF	Neutrophil Factor
NO	Nitric Oxide
PAF	Platelet Activating Factor
PAR	Population Attributable risk
PBS	Phosphate Buffered Saline
PCA	Posterior Cerebral Artery
PCom	Posterior Communicating Artery
ROS	Reactive Oxygen Species
SAH	Subarachnoid Haemorrhage
sBP	systolic Blood Pressure
TF	Tissue Factor
Th-	T- helper type cell
TIMP	Tissue Inhibitors of Metalloproteinases
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
VBU	Vascular Biology Unit

VSMC	Vascular Smooth Muscle Cells
vWF	von Willebrand Factor
WHO	Word Health Organisation

“A Small Animal Model for the Creation of Pre-Aneurysmal Change in the Intracranial Vasculature”

Masters Philosophy (Health)

Dr James Anthony Lee

Part 1

Introduction and Historical Perspective

There is considerable interest in further understanding the pathophysiology of human cerebral aneurysm formation. Many human studies involving histopathological analysis of surgically dissected aneurysms[1, 2], gene linkage analysis[3-10] and epidemiological studies[11-18] have been performed with varying degrees of success, revealing some specific elements which may be of importance in aneurysm pathogenesis. These studies, however, have a number of important limitations[19]. Histopathological studies rely upon samples obtained during neurosurgical intervention, which in of itself may increase the risk of aneurysm rupture[2, 20]. The tendency of aneurysms to cluster in families can complicate genetic studies[21-23]. Further, histological data from human studies is obtained at a single point in time; this does not allow for examination of sequential changes in the arterial wall during aneurysm pathogenesis.

Intracranial aneurysms have been a subject of investigation since the mid-19th century. During this period, attaining a diagnosis of intracranial aneurysm in a living patient was difficult, with most cases being identified at necropsy. Indeed in 1859, Gull[24] expressed pessimism that such a feat would ever be achieved, concluding, after a review of both his personal experience with 7 patients and a review of previously reported cases: *"Although we may from the circumstances sometimes suspect the presence of an aneurysm within the cranium, we have at best no symptoms on which to ground a more probable diagnosis".* This dictum remained widely accepted for almost half a century; Beadles[25], on analysing 555 aneurysm cases, reached the same conclusion, declaring: "*Only two or three have ever been diagnosed during life, and even in those cases it can scarcely be said to have been an absolutely certain diagnosis"*. A more proactive stance in the diagnosis of aneurysms was taken by Fearnsides[26], who in a review of the subject, and broadly agreeing with the earlier statements by Gull, suggested that the infrequency of clinical diagnoses in living patients was probably due to the diagnosis of aneurysm rupture not being considered by clinicians. This new

focus on diagnosis during life appeared to re-kindle interest in intracranial aneurysms and their pathogenesis and treatment [27].

The pioneering work of Turnbull[28] in 1914 promulgated the theory that aneurysms were congenital in origin. In investigating 30 aneurysms, he noted that arterial wall atheroma was absent or mild in 21, with little to no evidence of arterial wall hyperplasia in 17. This contrasted sharply with previous observations of intracranial arteries after haemorrhagic stroke, leading the author to conclude that "…*direct rupture is usually associated with evidence of much more excessive blood pressure and greater arterial degeneration… If, therefore, these cerebral aneurysms were entirely the result of excessive blood pressure and arterial degeneration, they would precede and be more common than direct rupture …direct rupture is much more common. There appears, therefore, to be an additional factor in the formation of these cerebral aneurysms… this factor is, probably, an inherent weakness due to a congenital abnormality in the structure of the arteries at their points of junction*"[28, 29].

Although widely accepted, this hypothesis failed to explain the relatively late onset of aneurysm rupture and the rarity of SAH in children.

Although proposed as a contributory rather than the principal aetiology in aneurysm pathogenesis by Turnbull[28], interest in the role of hypertension and haemodynamic stress on cerebral vessels increased after Forbus' experiments in 1930[30]. By use of glass tubing and manometer measurements, these studies determined that haemodynamic pressure exerted on an arterial wall was maximal at arterial branch points, which correlated with the origin site of many aneurysms. The author attributed this propensity to small, congenital defects in the media layer or "*loci minoris resistentiae"*, which appeared to justify Gull and Turnbull's earlier assertions. Forbus concluded that the strength of the vessel wall is a function of the muscular coat, with breaches of this layer responsible for aneurysm formation. Critically, however, Forbus also found many media defects without apparent aneurysm formation; a point further explored by both Schmidt[31] and Strauss[32]

who despite accepting of the proposal that congenital lesions predisposed to aneurysm formation, nevertheless stressed the importance of atheroma formation in aneurysm pathogenesis.

The aetiological relationship between both medial defects and presence of atheroma was questioned in 1940 by Glynn[29], who showed that even in the presence of medial defects, unfixed, unsupported elastic elements in the vessel wall could withstand very high pressures without rupture. This hypothesis was tested by insufflation of a sample vessel with air from a foot-pump; pressures of up to 400-600mHg were achieved "without any visible sign of localised bulging". Although the author conceded that this experiment was not an accurate physiological representation of the in-vivo situation (the tissue being dead and without muscle tone), it nevertheless proved the concept that the unsupported elastic elements of the vessel wall could withstand very high pressures without loss of structural integrity or vessel rupture. These results were consistent in both vessels with naturally occurring and artificial medial defects, again pointing toward the elastic lamina as the critical element in maintenance of vessel wall integrity, leading the author to conclude that lesions of the elastic lamina are "*of paramount importance in the genesis of these aneurysms"*.

Glynn disputed Forbus' contention that the observed medial defects were congenital, citing increased frequency of observed defects in adult when compared to paediatric samples in his observed slides, and asserting that due to the frequency with which medial defects occur in the cerebral vessels of normal subjects, they were unlikely to be a significant factor in aneurysm pathogenesis. Interestingly, he described these changes as "*physiological response(s) to the increase in blood pressure which normally occurs between infancy and the attainment of maturity"* [29]". He noted the presence of intimal hyperplasia independent of either atheroma formation or medial defects, which he proposed to be a result of *"a local condition of strain"*, which occurred to a greater extent in older subjects with an increased frequency at arterial bifurcation points. Two explanations were suggested to account for this observation, namely:

1. At bifurcation points, a greater pressure was exerted on the arterial wall at the point of bifurcation due to it being in direct opposition with the forces exerted by the moving column of blood, and that
2. Eddy currents arising from turbulent blood flow at bifurcation points resulted in a pathological process, the end result of which was intimal hyperplasia.

Carmichael[33], in 1950, proposed that aneurysm formation was dependent on the combination of both a medial defect and a superimposed lesion in the internal elastic lamina. Findings of his histological study of 13 small aneurysms suggested that their development was due to the combined effects of "*developmental deficiency*" and "*arterial degeneration*". The author observed that aneurysm development occurred exclusively at the site of "*substantial breaches in the muscular and elastic coats, and these coats are breached in different ways*". The muscular gaps were attributed to foci of medial aplasia which became enlarged by superimposed degenerative changes, or alternatively areas of primary hypoplasia where an underdeveloped media had been destroyed by primary degeneration and fibrosis. Gaps in the internal elastic lamina, however, were attributed to degenerative change alone, which the author ascribed chiefly to atheroma formation. Although it appeared that the relative degree of each of these two factors varied in the aneurysms studied, Carmichael concluded that "*both developmental and degenerative factors are concerned in the genesis of all of these aneurysms and no distinction can be made between the so-called congenital (i.e. developmental) and arteriosclerotic types*"[33].

Du Boulay[34] further explored the association between atheroma formation and aneurysm pathogenesis in a radiological, surgical and pathological analysis of the size and shape of 252 aneurysms in 197 patients. This work found arterial irregularities or atherosclerosis in 35% of the aneurysms studied. Whilst concluding that an association between atheroma and aneurysm formation may exist, he did not accept that atheroma was in of itself the principal factor in aneurysm pathogenesis, rather that it was a "*most important factor*", and that although early

atherosclerotic change was closely associated with early aneurysm pathogenesis, development of a macroscopic saccular aneurysm was dependent on a balance between healing processes and the progression of atheromatous change . Du Boulay also reported several important aspects regarding the natural history of intracranial aneurysms, noting simple, unilocular aneurysms to be more common in young patients, with an increased incidence of larger, multilocular lesions in older patients. In patients with multiple aneurysms, 18 of 32 exhibited both simple saccular aneurysms and irregular, multilobed ones, suggesting that progressive changes affected individual aneurysms in the same patient at different rates, and new aneurysms may form at different stages. He noted the significance of loculation of aneurysms, especially the development of de novo loculation, correctly determining that a change in morphology was often an event which preceded aneurysm rupture.

Epidemiology and Risk Factors for Aneurysmal Subarachnoid Haemorrhage

Aneurysmal subarachnoid haemorrhage, that is, subarachnoid haemorrhage resulting from rupture of a saccular intracranial aneurysm, is a highly lethal medical condition, with a mortality approaching 50% in some studies and significant morbidity amongst survivors [36-39]. Although aneurysms are present in approximately 2-5% of the population, the incidence of aneurysmal subarachnoid haemorrhage in Australia is only 8 in 100,000 people per year [17]. In the vast majority of cases, aneurysms remain occult prior to rupture. A feature of aneurysmal subarachnoid haemorrhage is the relatively young age at which it strikes, with peak incidence occurring between 40 and 60 years of age[40]. Although attributable to only 7% of all strokes, aneurysmal subarachnoid haemorrhage is responsible for 27% of all stroke related years of life lost before age 65[38]

Why human aneurysms form remains unclear. Most studies examining pathophysiology of aneurysm formation have focused on physiological parameters such as hypertension, intra-arterial wall shear stress[41-43], and immunological factors such as endothelial dysfunction, inflammation and remodelling of the vascular wall [44-51]. The majority of historical studies investigating human aneurysm pathogenesis have relied on specimens obtained during autopsy or surgery. These histopathological samples are now less commonly available due to the increasing use of endovascular treatment[52-54]. Many potential risk factors for aneurysm formation and rupture such as hypertension, cigarette smoking, alcohol consumption and female gender have been identified[21, 55-59]. Exposure to any or all of these factors does not necessarily result in the formation of an aneurysm[35, 37, 60-62], and aneurysms may also arise spontaneously in the absence of any obvious risk factors[60, 63, 64].

Although significant difference of opinion exists amongst clinicians, aneurysm size predicts risk of rupture [37]. Contradictory data, however, show that up to 60% of ruptured aneurysms would have been classified as “low-risk” prior to rupture if aneurysm size alone is used to stratify risk of rupture[15, 65] [66]. Management of unruptured aneurysms is controversial; aneurysms deemed at

low risk of rupture are typically kept under radiological surveillance, with surgical clipping or endovascular occlusion offered to patients deemed to be at “higher” risk of SAH[37]. Regardless of treatment modality, for many patients the peri- and intra-procedural morbidity may equal or exceed the risk of conservative management[67].

The International Study on Unruptured Intracranial Aneurysms (ISUIA)[18] and subsequent follow-up study (ISUIA-II)[37], sought to stratify risk of aneurysm rupture on the basis of size and location alone. Although criticisms relating to inherent methodological biases[68], and utility of these data in a clinical setting[69, 70] have been raised, the size and location risk-stratification paradigm has gained favour in contemporary neurosurgical practice. However, it is also accepted that up to 60% of ruptured aneurysms would have been classified as “low-risk” and potentially remained untreated with potentially catastrophic consequences were clinical risk stratification protocols to rely only on the ISUIA study[15, 65] [66]. Thus, the broad extrapolation of this data to general neurosurgical practice may well be inappropriate[71, 72]

Mechanical obliteration of aneurysms and limiting the effects of aneurysmal subarachnoid haemorrhage after its occurrence are the mainstay of modern management of ruptured aneurysms. Despite decades of research and innovation, aneurysm pathogenesis and the processes leading to rupture are currently poorly understood, and the consequences of aneurysmal subarachnoid haemorrhage remain a significant burden both on the community and the individual. Further defining, clarifying, and understanding aneurysm pathogenesis may have important clinical and therapeutic implications

Pathophysiology of Human Aneurysm Formation

Anatomy of cerebral vessels

The brain is an integral part of the body which demands and uses 20% of the blood ejected from the heart over each cardiac cycle[73]. The principal function of the cerebrovascular network is to provide a stable blood flow to the brain over a range of activities whilst simultaneously avoiding potentially detrimental effects of abnormal physiological stressors such as increased haemodynamic stress. Optimum design of biological structures is an established theoretical biological principle which states that the "optimum" biological structure is the one which involves the least metabolic cost, or "work" (i.e. that which requires the minimum energy expenditure to build and maintain its infrastructure along with performing physiological tasks[74]). The biological efficiency of the vascular system is determined for the most part by the arrangement of vessels of variable luminal diameter arranged in a branching hierarchy from large to small calibre, designed in such a manner as to maintain the continuity of blood flow at the lowest energy cost and blood volume required, whilst simultaneously avoiding increased haemodynamic stresses[75].

In mammals, cerebral blood supply is via three major intracranial arteries; two internal carotid arteries and the basilar artery. The internal carotid arteries supply primarily the cerebrum, with the basilar supplying the brainstem, cerebellum and the remainder of the cerebrum. In humans, the Circle of Willis is formed from the three main intracranial arteries and the anterior cerebral, anterior communicating, posterior communicating and posterior cerebral vessels. The vertebrobasilar junction represents an area of unique haemodynamic profile in human vascular anatomy. The basilar artery (BA) is formed by the confluence of the paired vertebral arteries, representing a convergence of arteries rather than the usual divergent bifurcation; blood flow is in the "opposite" direction to that experienced elsewhere in the body.

Macroscopic Anatomical variations

The Circle of Willis is a polygon or closed arterial circuit, through which (by means of component vessels) blood may circulate from and return to the point of entrance[76].

Suggested reasons for the development of this unique pattern range from preservation of cerebral blood flow via collateral channels in the event of occlusion of one of the main feeding arteries [77] to protection of the brain from ischaemia whatever the position of the head may be relative to the cervical spine[78, 79]. Although there is no unanimity regarding what constitutes a "normal" or typical anatomical pattern[76], generally speaking the paired anterior cerebral arteries must be present, and no vessel may be less than 1mm in external diameter [80-82].

The Circle of Willis exhibits high anatomical variability in humans, with only approximately 40% of the population thought to exhibit a "textbook" configuration; indeed some studies have described variant arterial patterns to be more common than the anatomical pattern [76, 81]. Common anomalies include absence, hypoplasia, or multiplicity of component vessels. Persistence of the embryonic configuration of arteries, i.e. hypoplasia of the proximal segment of the aneurysm due to failure of the medial branch of the posterior branch of the aneurysm to properly join the basilar artery during early embryogenesis is probably the most frequently encountered anomaly and is usually seen unilaterally [76, 82, 83]. Growth of new vessels secondary to haemodynamic and genetic factors can result in other deviations from the expected pattern [76]. Alternatively, vascular anomalies may manifest more discreetly as subtle structural anatomical variations, or differences in the bifurcation angles between parent and daughter arteries. In most cases, however, physiological continuity of the arterial collateral circulation is maintained[75, 84-87].

Effect of Anatomical Variations on Cerebral Blood Flow

It has long been postulated that anatomical variability in the Circle of Willis be a predisposing factor to aneurysm formation[80, 83, 88, 89]; aneurysms arising from the Circle of Willis show a propensity

for arterial bifurcation points, and develop more often at bifurcations where one artery is hypoplastic relative to the other than at those where the calibre of the parent vessels is more equal. For example, A1 hypoplasia begets a tendency to anterior communicating artery aneurysm formation[90]. Likewise, aneurysms are seen with increased frequency at acute rather than more obtuse bifurcations, where laminar blood flow deviates more markedly from that of the parent artery [91].

The work of Padget et al[83] was one of the first studies to suggest a statistically significant correlation between anatomical variability and aneurysm formation, however was criticised due to poor statistical methodology. Kayembel[89] described a definite correlation between anatomical variation and aneurysm formation, and emphasised the importance of ascribing macroscopic aneurysm formation not on the presence of an anatomical variant per se, but rather that the pathophysiological processes resulting in aneurysm formation arose from alterations in cerebral blood flow caused by these variations. Modern, quantitative, in vivo imaging studies have confirmed this hypothesis, with significant variations in flow rates in both the carotid and basilar arteries demonstrated on MRI in the presence of variations in the Circle of Willis [92, 93]. In their study of 117 healthy volunteers using two-dimensional cine-phase contrast enhanced MRI, Tanaka et al[93] measured volume flow rates of the bilateral internal carotid arteries and the basilar artery. Anatomical variations were classified as:

1. A “textbook” Circle of Willis, where the pre-communicating segment of the anterior cerebral artery (A1) and posterior cerebral artery (P1) were normal in size,

2. Right/left A1 hypoplasia,

3. Right/left P1 hypoplasia, and

4. “Other” which consisted of other unclassified variations.

Total volume flow rate and volume flow rates for, and the relative contributions of each artery of groups 1, 2 and 3 were measured. The relative contribution of each of the proximal arteries was found to correlate significantly with variations of blood flow in the Circle of Willis. The ratio of contribution of the bilateral internal carotid and basilar artery were estimated at 40:40:20 for the "textbook" type. Assuming that both anterior cerebral arteries were supplied by a single internal carotid in the A1 hypoplasia variant, and the posterior cerebral artery ipsilateral to the hypoplastic P1 is supplied only by the ipsilateral posterior communicating artery, ratios of contribution of the bilateral internal carotid arteries and basilar artery were estimated at 30:50:20 for the A1 hypoplasia variant and 50:40:10 for the P1 hypoplasia variant. These results confirmed the earlier findings of Hendrikse[94] et al, who measured volume flow in the internal carotid arteries and basilar artery in 208 patients with symptomatic atherosclerosis or risk factors for atherosclerosis using three-dimensional time-of-flight MR angiography. In this study, significantly increased volume flow was demonstrated in the contralateral internal carotid artery in patients with A1 hypoplasia compared to "textbook" circulation. In the presence of P1 hypoplasia, significantly increased volume flow was demonstrated in the ipsilateral internal carotid , with decreased basilar artery flow. This effect appeared most significant for anterior circulation anatomical variants (i.e. A1 hypoplasia or absence) indicating that asymmetries in volume flow between the internal carotid arteries may not necessarily be due to vascular disease but may instead be due to variations in the anatomy of the Circle of Willis.

Ultrastructural organisation of intracranial arteries

Human intracranial arteries are muscular vessels comprising of three main layers:

1. An outer tunica adventitia which consists mostly of type-1 collagen,
2. A tunica media comprised primarily of vascular smooth muscle cells (VSMCs) with an extracellular matrix consisting of type I and III collagen fibres, and
3. An inner layer, the tunica intima, comprising of a single layer of endothelial cells adjacent to the lumen, separated by a distinct internal elastic lamina (IEL) of elastin[84, 95, 96].

Structural integrity of these vessels is maintained by the media layer via the highly organised, circumferential orientation of the VSMCs and IEL [96, 97]. Malleability of the vessel wall is imparted by the VSMCs. Thus, the VSMCs, in concert with the IEL and collagen fibres, allow the vessel wall to withstand significant the haemodynamic stressors imparted upon it over the course of the cardiac cycle without sustaining any significant injury. The tensile strength of the artery wall is derived from the thin layers of type-I and –III collagen fibres, [98], with types I, III, IV and V embedded in endothelial and VSMC basement membranes[99].

Despite these characteristics, intracranial arteries are structurally weaker than their systemic counterparts. Intracranial arteries are generally thinner walled, do not exhibit an external elastic lamina (EEL), nor vasa vasora, and have a thin tunica media. They are also relatively “exposed”; they do not benefit from an organ capsule or wrapping hilar ligaments (i.e. structures such as the hepatic ligament), with the surrounding CSF usually exerting a pressure of approximately 12cm H_2O.

Remodelling of the extracellular matrix has been associated with many vascular diseases, including abdominal aortic aneurysm formation and atherosclerosis[100, 101]. Degenerative changes in the arterial wall manifest as a decrease in the number of mural cells and excess deposition of myointimal fibrous tissue[102, 103], with constant remodelling of wall structures. This results in intimal hyperplasia, loss of clear demarcation between the various layers, and disorganised muscle layer[103, 104]. The composition of the affected vessel wall compared to normal arteries is also

changed. Diseased arteries exhibit type 1 collagen and fibronectin dispersed throughout the tissues; in normal arteries the distribution of type 1 collagen is confined to the adventitia, and fibronectin to the media. Thinning of the media layer due to VSMC loss is seen in aneurysm propagation and progression [46, 105].

The unique anatomy of intracranial arteries may make them more predisposed to haemodynamic stress, and thus, aneurysm formation. As discussed, a unique microscopic feature of intracranial arteries is small foci of discontinuity of the tunica media, variously named the medial gap, medial defect, or medial raphe[29, 30, 96]. Medial gaps are almost invariably present at bifurcation points, however why this is so remains unexplained, however their morphology and width appears to be dependent on the angle subtended at the bifurcation, with wider gaps seen at narrower branch angles[84]. Their frequency also appears to increase with age [29, 106]. The organisation of collagen and smooth muscle cells along a straight length of artery is mainly circumferential, with a lesser presence of longitudinal fibres in the outer adventitia[97, 98, 107] At arterial bifurcations, however, the collagen fibres in medial gaps exhibit a highly aligned organisation with fibres and fibrils running parallel to each other; at the apex of the bifurcation these track perpendicular to the long axis of the parent artery [97]. Finlay et al[97] likened this structure to a “tendon under load”; which imparted increased strength at the bifurcation apex. This increased strength, however, was noted by the authors to increase the vulnerability of the vessel walls immediately adjacent to haemodynamic stress, with distension of the vessel wall more likely at the discontinuity at the edge of the band.

Role of Genetic Factors

Genetic factors are thought to substantially contribute to intracranial aneurysm formation[4, 108]. Multiple identical twin[12, 109], family[4, 63, 110-112] and population-based epidemiology studies have suggested genetic predisposition plays a significant role in aneurysm formation, however no study has yet identified a definite association to any particular gene[4]. It would appear that a combination of genetic and environmental factors working in concert lead to aneurysm pathogenesis; no one factor is sufficient to cause an aneurysm in isolation. Thus, the identification of a single gene exerting a dominant effect in aneurysm pathogenesis seems unlikely, however this remains the focus of considerable interest.

Unruptured aneurysms are identified in approximately 10-15% of first-degree relatives of patients presenting with SAH [113, 114]. These aneurysms are more usually multiple [115], and appear to confer an increased risk of rupture at a smaller size and at a younger age; 70% of familial aneurysms rupture by the age of 50 versus 43% of non-familial aneurysms [111, 116] [117]. Interestingly, aneurysms in siblings tend to rupture in the same decade, with female family members exhibiting a higher incidence of rupture[114, 118]. This may reflect an interaction between genetic predisposition and a contributory effect of oestrogens.

Although the familial nature of aneurysms is well recognised, this determination has largely arisen based on studies of first-degree relatives of selected cases. More detailed, population-based investigation is limited by the lack of large, comprehensive population-based genealogical databases. In their population-based analysis of the familial nature of aneurysms (intracranial, abdominal aortic and other types), Cannon-Albright[119] et al identified a high incidence of intracranial, abdominal aortic and other aneurysm types in certain families, suggesting a genetic component in aneurysm pathogenesis. Data obtained from 279,002 death certificates and weighting for mean degree of

relatedness ascribed a relative risk for aneurysm formation in first-degree family members of 3.67, 2.07 and 13.0 for intracranial, abdominal aortic and other aneurysms respectively. Each aneurysm type showed significant evidence of increased familiality. Although the authors conceded insufficient evidence to prove the hypothesis of a genetic susceptibility towards aneurysm formation, their data was suggestive of such a trait. Although some pedigree clusters of abdominal aortic aneurysm revealed concomitant intracranial and other aneurysms amongst their descendants, presence of an intracranial aneurysm did not appear to confer an increased risk of abdominal aortic or other aneurysms.

Over the past decade, a number of studies have described an anticipation effect in familial aneurysms, i.e. that in family pedigrees where aneurysms are seen in consecutive generations, the mean age of aneurysm rupture in patients is significantly higher than that of their affected children[120, 121], with mean differences in age of onset of aneurysmal subarachnoid haemorrhage reported at 19.8 years (range -3 to 38)[120] and 21.2 years[121]. Ruigrok et al, referencing other hereditary, autosomal dominant conditions, hypothesised that this phenomenon may be due to the transmission of an unstable trinucleotide repeat sequence, increasing in size through subsequent generations. Huntington's disease, myotonic dystrophy, and spinocerebellar ataxia type 1, 2, 3 and 7 also exhibit an anticipatory effect[120, 122, 123]. As anticipation is a likely feature of familial aneurysm transmission, an unaffected parent may subsequently develop an aneurysm after diagnosis in their offspring[120].

Connective tissue disorders

Approximately 5% of intracranial aneurysms are thought to be associated with a recognised heritable connective tissue disorder[109, 114, 124]. Connective tissue and extracellular matrix disorders have also been associated to varying degrees with pre-aneurysmal change, with Autosomal Dominant Polycystic Kidney disease (ADPKD, polycystin)[125], Ehlers-Danlos Syndrome (EDS IV, COL3A1 collagen)[126], pseudoxanthoma elasticum (PXE)[127], supravalvular aortic stenosis (SVAS, elastin)[128] and Fibromuscular Dysplasia being long recognised [15, 114, 129].

The most well-known association of a heritable condition with intracranial aneurysms is Autosomal Dominant Polycystic Kidney Disease (ADPKD), intracranial aneurysms being identified in up to 10% of patients with the condition. Although SAH is a major cause of morbidity and mortality in these patients, ADPKD patients make up a tiny minority (estimated to be approximately 0.3%) of all SAH patients[57, 130].

In a similar manner, although studies have suggested an association between Ehlers-Danlos IV and aneurysm formation[131, 132], the association is weaker again than that between ADPKD and aneurysm formation, with these patients comprising an even smaller number of total patients presenting with SAH. Furthermore, aneurysms in patients with ED IV tend to be of the fusiform type in contrast to the saccular type more usually seen in patients with idiopathic aneurysms[133]. Other heritable disorders associated with an increased incidence of aneurysm pathogenesis include Osteogenesis Imperfecta, Pseudoxanthoma elasticum, Supravalvular Artic Stenosis, Neurofibromatosis type-1 and Marfan's syndrome.

The overall incidence of heritable connective tissue disorders is probably underestimated due to variability in phenotypic expression and although many are inherited in an autosomal dominant fashion, family history is frequently negative as the disease arises as a new mutation [109]. The

causal relationship, however, between many of these disorders and aneurysm pathogeneis remains to be conclusively proven and uncertainty exists as to the significance of the contribution of any of these conditions to risk of aneurysmal subarachnoid haemorrhage. Prevalence of aneurysms is higher in some of these disorders (especially APKD and ED IV) than in the general population, but this increase is only modest, with systemic studies of larger groups of affected patients demonstrating that aneurysms are an infrequent clinical manifestation in the disorder[131, 134] Polymorphisms in genes encoding for elastin (ELN), A-1 antitrypsin (AAT), Collagen-III (COL3A1), endothelial nitric oxide synthase (eNOS), endoglin (ENG), Polycystin (PKD-1), (FBN1) and transforming growth factor –beta receptors have all been implicated in aneurysm pathogenesis[15]. Large, genome-wide studies have identified potential loci for aneurysms on 2p13[7] on 7q11, 14q22, 5q22-31[135], and on 19q13.3[8]. An allelic association for aneurysm formation has been shown for genes encoding for elastin (ELN, 7q11.2) and Collagen type 3A2 (COL1A2, 2q31)[14, 135]. Such associations, however, may give rise to the identification of false positives, and there are indications for locus and allelic heterogeneity between different ethnic groups[136]

Collagen, Elastin and Connective Tissue.

Abnormalities in collagen and elastin formation and turnover are thought to play a key role in aneurysm formation.

Elastin is synthesised in fibroblasts, endothelial cells, smooth muscle cells and chondroblasts. It is secreted as a soluble, 72kDA monomer (tropoelastin) which alternates hydrophobic (responsible for elastic properties) and lysine rich (required for lysl oxidase-mediated covalent cross-linkage between monomers) sequences. The net effect of these alternating sequences is formation of a highly insoluble network of elastic fibres in the extracellular space[137, 138]. Elastic fibres are formed from an insoluble core of elastin surrounded by a lattice of myofibrils. Elastin is secreted as a precursor

protein (tropoelastin) which is cross-linked into a pre-existing microfibrillar matrix [128, 139]. The major microfibrillary components are Fibrillin 1 and 2 (FBN-1, -2) [140].

Types I and III collagen are the major extracellular matrix components of vessels, representing approximately 80-90% of the total arterial collagen content, and are responsible for most of the tensile strength of the vessel wall[136]. It appears only Type-III collagen is associated with vascular disease[140]. Type-III collagen is composed of three identical a- subunits which are secreted as procollagen precursors[141]. After secretion, these procollagens are processed to form mature, insoluble collagens, which aggregate to form banded fibrils[128]. Type-III, or reticular fibrils are mostly found in tissues exposed to periodic stresses and assist in the organisation of Type-I collagen networks. These fibrils are the primary source of tensile strength of the vessel wall[128].

Collagen Disorders

Ehlers-Danlos Syndrome Type IV

Ehlers-Danlos syndrome is a heterogenous group of connective tissue disorders characterised by hypermobility of joints, fragile or hyperelastic skin, easy bruising and abnormal scarring[109]. Patients exhibit characteristic facies of expressive eyes, thin nasal bridge, thin lips, lobeless ears and a “prematurely aged” appearance[109, 115]. Of the ten currently classified subtypes, Type IV (ED IV) is the least common but most lethal, with a prevalence of approximately 1 in 50,000 to 500,000 persons[109, 128, 142].

Type III collagen is the major constituent of distensible tissues, including arteries and veins. The gene responsible for ED IV encodes for the pro-a1 chains of type III pro-collagen (COL3A1) and is located on chromosome 2. Multiple mutations of this gene have been identified, and are acquired in either an autosomal dominant manner (~50%) or as a de-novo mutation (~50%)[143]. There does not appear to be a correlation between the nature or location of the mutation and the expression or severity of the phenotype, however some studies suggest null mutations may result in more severe disease [131]. Although multiple studies have revealed evidence of abnormal type III collagen production in the absence of ED IV, COL3A1 mutations are uncommon amongst patients with spontaneous intracranial aneurysms[109, 144, 145]

Osteogenesis Imperfecta

Osteogenesis Imperfecta (OI) is a heterogenous group of connective tissue disorders characterised by bone fragility and decreased bone mass [146, 147]. It is predominantly caused by dominant mutations affecting synthesis of type I collagen, however recessive and X-linked inheritance have also been described[146, 147].The prevalence of OI in all its forms has been estimated at between 6-

7 per 100,000 people [148]. Typical extra-skeletal manifestations of the condition include hyperlaxity of ligaments and skin, dentinogenesis imperfecta, blue sclerae and hearing loss. The original Sillence Classification of OI described four subtypes by primary clinical characteristics and pattern of inheritance, namely (i) mostly dominant inheritance with blue sclera, (ii) lethal perinatal subtype, (iii) progressively deforming subtype and (iv) mostly dominant inheritance with normal sclera. These subtypes were classified on the basis of clinical findings to reflect the spectrum of severity of the condition from mild (OI type I) to lethal (OI type II) and severely deforming (OI type III) to minimally deforming (type III). The observation that each of the four subgroups displayed slightly different modes of inheritance (predominantly autosomal dominant) indicated genetic heterogeneity in OI[146]. An internal deletion in COL1A1 was first described in OI type II[149]; subsequent gene studies revealed mutations in COL1A1 (encoding for a-1 collagen chains) and COL1A2 (encoding for a-2 collagen chains) in all OI types. A total of 17 genetic causes of OI have since been described, however COL1A1/2 mutations resulting in qualitative deficiencies of Type I collagen still account for approximately 90% of OI in patients of European origin where a defect has been detected[132, 150]. The number of subtypes has since been expanded up to OI XIV with the discovery of these multiple genetic defects, however these phenotypes may not be mutually exclusive and retain comparable clinical and radiological characteristics of the original clinical description of subtypes I-IV. Thus, the preferred nosology remains phenotypic, adding PI type V (presumed autosomal dominant).

Neurological complications of OI such as basilar invagination and fragility of blood vessels are well recognised[148, 151] and may be more prevalent in OI type IV[146]. Neurovascular manifestations of OI such as spontaneous carotid-cavernous fistula[152], vertebral artery dissection[132] and aneurysm formation[153-155] have been reported. As type III rather than type I collagen provides most of the tensile strength in vessel walls; this may account for the relative paucity of vascular complications in OI relative to other disorders of collagen biosynthesis such as ED IV.

Autosomal Dominant Polycystic Kidney Disease

Autosomal Polycystic Kidney Disease (ADPKD) is a systemic hereditary disorder accounting for 8-10% of cases of end-stage renal disease. It is characterised by cyst formation in ductal organs, principally the kidneys and liver, and is associated with various cardiovascular, musculocutaneous and gastrointestinal abnormalities [156, 157]. It occurs in 1 in 400-1000 persons. The gene responsible has been located on the short arm of chromosome 16 (16p13.3)[158]. PKD1 encodes for polycystin-1, a, 11-pass membrane protein mediating cell-cell and cell matrix interactions and is responsible for 85% of ADPKD cases. PKD2, mutations of which gene are responsible for the majority of the remainder, codes for polycystin-2; a 6-pass membrane protein homologous to voltage-gated Ca++ channels. PKD1 is believed to interact with PKD2 in signal transduction from extracellular ligands [159-161]. Germline mutations in both genes comprise missense, splice site and both small deletions and insertions[162] Both polycystin-1 and -2 are strongly expressed in the vascular smooth muscle and endothelium of patients with ADPKD, suggesting that the development of vascular pathology is directly linked to the PKD mutation and polycystin dysfunction in the arterial wall rather than secondary to the arterial hypertension arising from chronic renal dysfunction[163-166].

The prevalence of intracranial aneurysms in the ADPKD population has been estimated at approximately 8%, approximately five times higher than that of the general population [166]. Aneurysms are identified in up to 25% of patients at autopsy[132, 158]. Persistent foetal carotid-basilar anastomoses are a relatively frequent angiographic variant in ADPKD; it has previously been suggested that this aberrant anatomy may be a predisposing factor in aneurysm formation[167, 168]. In contrast to the general population, aneurysms in these patients arise most commonly from the middle cerebral artery appear to show a slight propensity for males, and rupture at an earlier age[132].

The incidence of SAH in ADPKD has been estimated at approximately 1 in 2000 person years[158, 169], which although five times the rate in the general population, is roughly proportionate to the increased prevalence of aneurysms in this group, thus the overall risk of SAH is probably similar to that of the general population [169]. The earlier mean age of SAH and the potentially devastating sequelae of this, however, has prompted some authors to advocate screening for asymptomatic aneurysms in either all patients with ADPKD[162] or in certain subgroups (e.g. younger patients, family history of aneurysm or SAH, uncontrolled hypertension etc.)[158]. This targeted screening, along with general risk factor reduction (cessation of smoking, control of hypertension, etc.) may represent a sensible stop gap approach pending further studies [166]. Interestingly, in one study by Chapman[167]et al, patients with ADPKD experienced a significantly increased incidence of transient neurological deficits after undergoing Digital Subtraction Angiography, which appeared to subsequently resolve without neurological deficit. This may have implications for the undertaking of catheter angiography in ADPKD in future clinical or research settings.

Elastin Disorders

Supravalvular Aortic Stenosis

Supravalvular Aortic Stenosis (SVAS) is an autosomal dominant systemic elastin arteriopathy which usually manifests as thickening of the media and intima of the great arteries. Its prevalence is approximately 1 in 13,000-20,000 live births [128, 137].

SVAS is classically associated with Williams-Beuren Syndrome (WBS); a complex developmental syndrome manifesting with neurobehavioural, craniofacial, cardiovascular and metabolic abnormalities[137]. SVAS may arise in conjunction with WBS or as a non-syndromic variant. The gene causing the syndromic variant has been isolated to a microdeletion at 7q11.23, which encompasses 27 genes, including the elastin gene (ELN) [170]. The non-syndromic version is

recognised as separate entity as the patients do not present with the other features of WBS syndrome, however is also caused by a mutation of ELN[171, 172]. When WBS SVAS patients and spontaneous SVAS patients are compared, it appears that hemizygosity for elastin causes SVAS, but not the other characteristic features of WBS[128, 173]. Point mutations, deletions and translocations involving the ELN gene have been described in the non-syndromic version, and the genetic defect has been implicated in hypertension and intracranial aneurysms[174].

Pseudoxanthoma Elasticum

Pseudoxanthoma elasticum (PXE) is a rare inherited disorder of the elastic fibres of the skin, ocular and cardiovascular systems. Its' prevalence has been estimated at approximately 1 in 100,000 per population [175]. Both autosomal dominant and recessive inheritance has been described[175-179]. The disease gene has been localised to the short arm of chromosome 16[175].

Both extracranial and intracranial vessels are affected. Cerebrovascular complications typically present as cerebral infarction (presumably via carotid stenosis or occlusive disease of the carotid or vertebral arteries) which are often multiple, typically present in the fifth or sixth decade (sometimes as early as the third decade)[180]. Infarction of the cervical spinal cord has also been described[132]. Several cases of aneurysmal subarachnoid haemorrhage associated with PXE have been reported [127, 181, 182]. Aneurysms associated with PXE are frequently located in the cavernous sinus, and may present with acute oculomotor palsy[127, 183, 184]. Several cases of spontaneous intracranial haemorrhage without reference to an underlying aetiology have also been reported[185, 186].

Marfan's Syndrome

Marfan's syndrome is an autosomal dominant connective tissue disorder arising from mutations in the gene encoding for fibrillin-1 (FBN-1), giving rise to specific skeletal, ocular, cardiovascular,

pulmonary, connective tissue and CNS findings. The gene encoding for Fibrillin has been localised to chromosome 15. Although multiple mutations have been identified in patients with Marfan's syndrome [187, 188], a specific mutation causing Marfan's syndrome in the fibrillin gene is yet to be identified. The syndrome affects approximately 1 in 10,000-20,000, and is usually easily recognised by the characteristic phenotype of tall stature, dolichostenomelia, arachnodactyly and sternal deformities, however the variable expression of the phenotype may make diagnosis difficult [189].The most common cause of death from the syndrome in children is severe aortic or mitral valve regurgitation, whereas in adults it is acute aortic dissection [132]. The most common vascular complication is probably aortic dissection extending to the innominate and internal carotid arteries, causing sudden death, cerebral ischemia, or, in the case of lesions involving the spinal arteries, paralysis [190]. Spontaneous dissection of the common carotid arteries and extracranial internal carotid and vertebral arteries has also been reported[132, 191].

Intracranial aneurysms in patients with Marfan's syndrome may be saccular, fusiform or dissecting [109, 115, 192]. These aneurysms tend to be large, exert mass effect on surrounding structures[132] and appear to show a propensity for the cavernous segment of the proximal internal carotid artery[132, 193]. Vessels may be excessively fragile and tortuous.

Despite multiple reports proposing an association between Marfan's and a propensity to intracranial aneurysm formation, this link now appears doubtful[194]. This may in part be due to the natural history of the disease. Acute aortic dissection causing death peaks in Marfan's patients in the fourth decade[195], preceding the peak age of incidence of subarachnoid haemorrhage in the sixth decade[18, 37, 196, 197], thus fewer patients with Marfan's syndrome may have the opportunity to develop a mature aneurysm. Stehbens [198] et al described atrophic changes in the middle cerebral artery and with aneurysm formation in a patient with Marfan's syndrome, suggesting the pathogenesis of intracranial aneurysm formation in patients with Marfan's is no different to the mechanism seen in those without the syndrome.

Neurofibromatosis Type-1

Neurofibromatosis Type-1 (NF-1) is a progressive systemic disease caused by mutations in the gene encoding for neurofibromin on chromosome 17q11.2. The neurofibrin protein is similar to other tumour suppressor gene products, with a centrally located domain homologous to GTPase-activating protein (GAP). Neurofibromin is thought to have a regulatory role in the development of connective tissue, including vascular connective tissue, via an effect on microtubular function[109, 199], however as the gap domain encompasses only 10% of the protein, neurofibromin may have multiple other as-yet-to-be-determined functions[200].

The most common vascular pathology in patients with NF-1 is hypertension secondary to renal artery stenosis [201]. Many small series studies and case reports [202, 203] associate NF-1 with intracranial aneurysm formation, most large series of NF-1 patients do not [194, 204]. Where they occur, they may be saccular, fusiform or dissecting, and often coexist with intracranial arterial occlusive disease, which may complicate endovascular management [109, 205]. Surgical intervention for these aneurysms is often complicated by excessive vascular fragility and distortion of the normal anatomical landmarks caused by sphenoid wing dysplasia [109]. The clinically important question as to whether a definite association between NF-1 and intracranial aneurysm formation can be made remains unclear, however there are an increasing number of reported cases [206-208], leading some authors[207] to advise screening for aneurysms in these patients. Other large studies, however, have failed to demonstrate an increased incidence of intracerebral aneurysms[209].

Angiogenic factors

Renin Angiotensin System

The renin-angiotensin system (RAS) has been long recognised as playing an important role in pathological vascular remodelling seen in several cardiovascular diseases. Angiotensin-converting enzyme (ACE) is an ectoenzyme expressed in two forms in humans; a somatic form and a smaller isoenzyme exclusive to the testis. Somatic ACE circulates in the plasma and is particularly abundant on the endothelial surface[210]. The system plays a critical role in the regulation of systemic blood pressure. Briefly, ACE catalyses the conversion of inactive angiotensin-I (A-I) to physiologically active angiotensin-II (A-II). A-II a potent vasoconstrictor and the major effector of the system which exerts its effect via an AT-1 receptor. ACE may also influence blood pressure indirectly by inhibition of other vasoactive compounds such as the hypotension-inducing peptide bradykinin [211].

Several studies support the role for ACE in intracellular signalling in endothelial cells[210]. In addition to its effects on blood pressure, A-II has positive inotropic and chronotropic effects on the myocardium, and is a regulator of vascular growth[212, 213]. Considerable interest has been focused on the association between deletion and insertion polymorphisms in the ACE gene and vascular disease [210, 211]. At the genomic level, the insertion ([I] allele) or deletion ([D] allele) in intron 16 of the ACE gene correlate with decreased and increased plasma levels of ACE respectively. The D allele has been associated with a number of vascular pathologies including hypertension[211], left ventricular hypertrophy[214] and myocardial infarction[215] [216], however these associations remain the subject of debate and have not been consistently replicated[217]. Up-regulation of the RAS plays a major role in the pathogenesis of abdominal aortic aneurysm formation via facilitation of SMC migration, proliferation and hypertrophy, resulting in a thickened arterial wall. However, in cerebral aneurysm formation a thinning of the arterial wall is seen due to a decreased number of SMCs[218, 219]. The medial thinning seen in cerebral aneurysmal change has been associated with

significantly decreased local expression of ACE, AT-1 receptor and A-II, however exactly how reduced levels of ACE might contribute to aneurysm formation remains unclear. One possible explanation is that a decreased level of ACE might result in a blunted response of the vessel wall to haemodynamic stress, in turn resulting in decreased medial remodelling [220]. The [I] allele and genotype of ACE has been reported to be significantly associated with cerebral aneurysm formation and risk of SAH[212, 221]. These findings have been contradicted by others[6, 216]. A comprehensive meta-analysis undertaken by McColgan et al did not find a statistically significant association[222]between ACE polymorphism and increased risk of cerebral aneurysm formation.

Nitric Oxide Synthase

The role of Nitric Oxide Synthase (NOS) in cerebral aneurysm pathogenesis is explored further under "Role of Endothelium". The gene encoding for endothelial Nitric Oxide Synthase (eNOS) is located on chromosome 7q35-36 which expresses a number of variants in humans [223]. Functional polymorphisms of the gene may predispose to vascular pathology such as atherosclerosis, hypertension, myocardial infarction, coronary vasospasm and abdominal aortic aneurysm formation [224].

Similarly, eNOS gene polymorphisms have been associated with cerebral aneurysm formation and rupture. Khurana[225, 226] et al, in a series of works analysing genetic data from patients with radiologically proven aneurysmal subarachnoid haemorrhage, identified multiple genetic loci of interest for aneurysm pathogenesis and rupture. One such study reported a statistically significant difference in the distribution of genotypes for eNOS 27 VNTR (Variable Number Tandem Repeats) polymorphism in patients with SAH compared with controls, with heterozygosity for this polymorphism almost three times as prevalent amongst the SAH group [225]. In this study, a minor

allele (4a) containing four repeats of the 27 VNTR gene was found to be over represented in patients with aneurysmal subarachnoid haemorrhage, and appeared to confer an increased odds ratio of 3.95 (p0.007) for aneurysm rupture after controlling for other known risk factors (age, sex, smoking history). This replicated a previous finding by the same authors that polymorphic variants in eNOS alleles conferred an increased risk of aneurysm rupture, the magnitude of which increased in the presence of two or more variant alleles [224]. The same authors have also suggested[226] that a single nucleotide polymorphism (T-786C) may be a factor influencing the size at which an aneurysm ruptures. In this study, heterozygosity for the C-allele was significantly more prevalent in patients with aneurysms greater than 10mm in size at time of rupture. All homozygotes, whether wild-type (i.e. T/T) or abnormal (i.e. C/C) had ruptured aneurysms less than 10mm diameter. The authors postulate homozygosity versus heterozygosity for the gene, rather than the allele itself may be a differentiating factor between small and large diameter ruptured cerebral aneurysms, i.e. differential expression of local eNOS amongst heterozygotes compared with homozygotes may affect the capacity of the vessel wall to withstand other factors leading to aneurysm formation and ultimately rupture [226]. Other similarly structured studies focusing on these polymorphisms, however, have failed to replicate these results [227-229].

Platelet Adhesive Glycoproteins

Platelets play a key role in haemostasis at sites of vascular injury. Endothelial damage exposes type-I collagen fibrils, which absorb von Willebrand factor. Platelets adhere to this exposed subendothelial tissue via binding of membrane glycoproteins (GP Iba to immobilised von Willebrand factor), tethering the platelets to the reactive surface. This interaction also requires synergistic binding of integrins a2b1 and aIIbb3 (GP IIb-IIIa complex) to their substrates. On the subendothelial surface endogenous von Willebrand factor and adsorbed plasma von Willebrand factor initiate platelet

recruitment, however a2b1 and aiibb3 are key factors for thrombus development[230, 231]. Stable platelet attachment is the first, essential step in this process.

Gene mutations causing modification of platelet function may predispose to haemorrhagic or thrombotic disorders.[232, 233]. Some studies suggest a protective role of prothrombotic clotting factor polymorphisms in primary intracranial haemorrhage [234]. Identification of functional polymorphisms of platelet function has attracted considerable interest [235, 236], with a particular focus on those polymorphisms affecting the structure or levels of expression of adhesive receptors[237-240]. GPIba is the functionally dominant subunit of the platelet GPIb-IX-V receptor complex, with the n-terminal domain of the GPIba chain containing the binding sites for a-thrombin and von Willebrand factor[241]. A number of polymorphisms causing significant structural change on the GPIba subunit affecting binding of von Willebrand factor have been identified [236]. GPIIIa HPA-1 and GPIb HPA-2 are responsible for structural change [237], and GPIa C807T and HPA-5 polymorphisms are associated with levels of expression of GPIb. Iniesta et al[236] prospectively evaluated the role of these common functional polymorphisms in the development and severity of SAH, finding no significant associations between GPIba -HPA-2, -VNTR, and GPIa -C807, -HPA-5 polymorphisms in the incidence or severity of SAH. A significant correlation was, however, identified between patients with the GPIIIa HPA-1b genotype (both a/b and b/b) and aneurysm size and incidence of rupture, with patients carrying this allele significantly under-represented in the SAH cohort compared with controls. These patients also presented with aneurysms of significantly larger size than those with HPA-1 a/a genotype. The protective effect appeared most pronounced in patients exhibiting aneurysms <9mm in diameter, however was less apparent in patients with aneurysms >9mm. Clinical and radiographic severity of SAH was also significantly lower in this cohort.

No difference in mortality was identified, however a trend towards increased 30-day survival was seen, suggesting the thrombophilic phenotype arising from this polymorphism conferred a

decreased incidence of aneurysm rupture in patients with small aneurysms, and a reduction in severity of haemorrhage in the event of aneurysm rupture. These patients did, however, have an increased incidence of ischaemic events than those with the HPA-1 a/a genotype, supporting a previous hypothesis that polymorphisms in genes involved in the clotting cascade may exert a mild, but opposite effect in the pathogenesis of haemorrhagic and thrombotic disorders[234, 242]. A small number of control patients in this study were found to carry both the GPIIIa –HPA-1 and GPIba – HPA-2 polymorphism; this appeared to confer a strong protective influence against aneurysm rupture, again strengthening previous suggestions that a combination of polymorphisms in may amplify their overall protective effects, which may explain their relatively high frequency in the general population [234, 236]

Lipoprotein Metabolism

Role of Apolipoprotein A

The association between apolipoprotein metabolism and cerebral aneurysm pathogenesis remains the subject of debate. Elevated serum levels of apoA-1 are an independent risk factor for atherosclerosis[243]. Elevated levels of apoA-1 have been associated with aneurysm pathogenesis in some studies[244-246], however no such association was found in others[247]. Given the correlation between high levels of apoA-1, atherosclerosis, and the presence of asymptomatic intracranial aneurysms, Caird et al[248] investigated the role of apoA-1 in early aneurysmal change and subsequent pathogenesis. Using immunohistochemical analysis with anti-apoA-1 monoclonal antibodies on the walls of 25 human aneurysms and 23 feeding vessels, apoA-1 immunopositivity was demonstrated in aneurysm walls both in the presence and absence of atherosclerosis, and in 86% of feeding vessels. This suggests a possible role for apoA-1 in early aneurysmal change. Of note, deposition of apoA-1 in the aneurysm wall was observed to occur in a multilayered fashion, suggesting intermittent deposition of apoA-1 over multiple growth and repair cycles. Similar

patterns of mural deposition were observed in cerebral arteriovenous malformations and atherosclerotic plaques in Circle of Willis vessels however no deposits were seen in cerebral cavernous malformations. This observation strongly suggests that apoA-1 deposition occurs via a pressure mediated effect. Whilst no direct correlation has yet been proven between apoA-1 polymorphisms and early aneurysmal change, the possibility remains that elevated levels of apoA-1 may be involved in the induction of early aneurysmal change in the vessel wall [246, 248].

Zhao et al[249] observed an association between a pentanucleotide repeat polymorphism of the apoA-1 gene and early aneurysmal pathogenesis using PCR and non-denatured polyacrylamide gens electrophoresis in a small study of 58 patients with angiographically diagnosed aneurysms. The data revealed two sites of sequence variance in the 5' control region of the apoA-1 gene which differed significantly between the aneurysm and control cohorts.

Role of Apolipoprotein E

Apolipoprotein E (apoE) is a plasma glycoprotein involved in lipid metabolism, specifically the highly atherogenic apo B containing lipoproteins, and mediates the cellular uptake of lipid complexes through interaction with specific apoE and low-density lipoprotein receptors [250, 251]. ApoE is highly polymorphic, and is the primary mediator of cholesterol and lipid transport in the brain [252]. ApoE is produced by astrocytes in response to neuronal injury[253, 254] and exhibits complex neuroprotective functions [255]. The gene is located on the long arm of chromosome 19. It has three common alleles: e2, e3, and e4 each of which encode for three major isoforms of the protein: apo –E2, -E3 and –E4, thus six different genotypes may be expressed. ApoE is an effective free radical scavenger and ApoE polymorphisms have been shown to modify the acute response to brain trauma, which may exert a detrimental effect in patients with brain injury and subarachnoid haemorrhage [252, 255, 256]. Other polymorphisms have been associated with ischaemic cerebrovascular disease[257], lobar intracerebral haemorrhage associated with cerebral amyloid

angiopathy [258-260], and neurodegenerative disorders such as Alzheimer's disease[261]. Although some studies have identified an association between ApoE e4 and subarachnoid haemorrhage in certain populations [257], similar findings have not been replicated in others[251].

Growth Factors and Cytokines

Remodelling of the vessel wall both in the course of normal cell turnover is mediated by growth factors such as vascular endothelial growth factor (VEGF), transforming growth factor b (TGFαb), basic fibroblast growth factor and its receptors (bFGF) and (bFGFRs)[262]. Altered expression of these factors may predispose to aneurysm formation (previous studies have suggested differences in allelic frequency at the FGF1 locus on 5q31 may be associated with findings in the vessel wall consistent with those seen in early aneurysmal change). Although a haplotype association was observed with the combination of 10 SNPs of bFGFR1, significant haplotype associations were not observed with combinations of two, three or four SNPs[9]. Increased expression of various growth factors such as TGFαa [263], VEGF[264], local RAS and platelet derived growth factor (PDGF)[265] have been observed in models of aneurysm pathogenesis, however identification of a genetic polymorphism leading to increased expression of any one of these factors remains elusive.

Role of Haemodynamic Stress

The concept of haemodynamic stress causing pathological vascular remodelling and early aneurysmal change has been extensively investigated. Environmental risk factors such as hypertension and cigarette smoking exert a generalised pathological effect on the vasculature, One would expect that this would lead to the formation of pathological lesions a random distribution[43]. However the opposite is the case; in the case of aneurysms these occur with a high degree of predictability in specific locations[100, 266]. Most aneurysms are located along the Circle of Willis, showing a propensity for arterial bifurcations at the branch points of parent and daughter arteries, or, less commonly, along the convexity of curved arteries[43, 266-268]

Haemodynamic forces

Cyclic stretch refers to the elongation of cells due to periodic vascular distension, pulsatile pressure (force applied across the luminal surface as a pressure wave) and Wall Shear Stress (WSS); the tangential or "frictional" force applied to the vessel wall by normal blood flow[269-272, 273.]. In the microcirculation, WSS is regulated to a set point that is a function of local transmural pressure[274]. The cerebral arteries exhibit an optimum blood flow/vessel radius relationship in so far as their luminal diameter is determined locally in order to maintain a constant WSS, both under physiological and pathological conditions[275].

WSS acts as a biological stimulator governing a wide variety of biological processes in the vasculature, including maintenance of optimum blood flow/vessel luminal diameter relations by regulation of vasoactive substances derived from the endothelium (mainly NO and prostacyclins) [42, 274, 276-278]. Under normal conditions, sustained physiological levels of WSS lead to the endothelial cells adapting a structural organisation and alignment, which firmly adheres them to the underlying substratum thus minimising friction between the endothelium and blood mass flow[43,

279]. In addition, the endothelial cells exhibit changes in gene expression, with up-regulation of factors conferring an atheroprotective phenotype and down-regulation of transcription of potentially harmful genes[280-283]

Haemodynamic micro-environment at bifurcations

Computational Fluid Dynamics (CFD) refers to a computer simulation method which can be used to map in-vivo the flow field associated with intracerebral aneurysms to construct a time dependent, spatially resolved three-dimensional velocity flow field. This method allows accurate calculation and visualisation of the fluid dynamics and blood particle paths, giving a better understanding of the relationships between local haemodynamics associated with aneurysms. Modern CFD studies have enabled three dimensional characterisation of flow fields in human and large animal studies, allowing spatial and temporal correlation between measured haemodynamics and specific tissue responses[284, 285]. Findings of these studies have largely confirmed the earlier assertions of mathematical modelling studies in human and animal models, specifically:

1. Intracranial aneurysms occur more often at cerebral bifurcations which experience higher WSS and stronger flow acceleration[286]
2. A combination of high WSS and WSS gradient predisposes to aneurysm formation in the apical vessel wall[284], and that high WSS is an important initiating factor in early aneurysmal change[276].

It would appear, therefore, that the true measure of departure from optionality of work is not the difference between the measured and predicted optimal arterial branch angles, but rather the difference in energy expenditure of the system. Although the overall increase in energy cost involved as a result of deviations from optionality in branching angles may be relatively low (approximating 2-

5%), this minor increase in WSS appears to be sufficient to initiate early aneurysmal changes in vessel walls[85, 287].

The temporal shear stress (TSS) gradient refers to the increase or decrease of shear stress over a small period of time at the same location. This occurs throughout the circulatory system due to the pulsatile nature of blood flow; the magnitude becomes significantly increased at the interface between the main flow in a vessel and the recirculation zone.

The spatial shear-stress (SSS) gradient is the difference in shear stress between two close points of a cell at the same point in time. SSS gradients are primarily seen at recirculation zones and bifurcations [277]. In vitro models have shown both TSS and SSS to be dependent on the initial onset of flow and the geometry of the flow chamber [288]. In addition, in-vitro models have indicated that TSS gradients stimulate endothelial cell proliferation, but SSS gradients affect endothelial proliferation no differently than steady uniform shear stress[289].

In vivo, the pulsatile nature of arterial blood flow results in a variation in the magnitude of absolute shear stress on the endothelium over the cardiac cycle. Where the blood flow is unidirectional with no recirculation, these time-averaged fluctuations are positive (i.e. blood flow is in a forward direction), and temporally and spatially uniform, resulting in mean positive shear stress[277, 289]. Arterial bifurcations result in departures from unidirectional flow; in in-vitro models, this results in relatively predictable separation, re-attachment and recirculation patterns. These are recognisable in in-vivo models, and have long been linked with the localisation of atherosclerotic lesions [277].

In order to understand the specific haemodynamic insults leading to maladaptive vascular wall remodelling, Meng[284] et al surgically created new branch points in the common carotid artery in a canine model. After an observational period of either two weeks or two months, the artificial bifurcation was evaluated using in-vivo angiography, the results of which were then spatially

correlated with the histological features of the artery to show the tissue response. The study identified three angiographically distinct flow patterns:

1. Region 1 (Region of Impingement): characterised by a stagnation point, with low to normal WSS relative to the baseline level. These are typically seen in straight vessels
2. Region II (Region of Accelerating Flow): characterised by high positive WSS and WSS gradient.
3. Region III (Recovery region): characterised by negative to zero WSS gradient and high WSS.

The impingement region occurs where blood flow from the parent vessel impinges on the bifurcation apex, resulting in the creation of a stagnation point which raises local pressure by approximately 1mmHg, increasing WSS at the boundaries of the region. This rapid increase in WSS produces a large spatial gradient.

The acceleration region is characterised by high wall stress due to the increase in flow velocity from the impingement region until maximum WSS is reached, marking the distal boundary of the region. The magnitude of the WSS gradient becomes elevated, which persists into the recovery region.

The recovery region is reached when the WSS returns to baseline levels and the WSS gradient returns to zero.

The stagnation point (i.e. where the WSS is zero) arises at a point on the vascular wall of flow re-attachment (usually along the outer wall of the bifurcation), resulting in an area of low mean WSS which is more pronounced in systole [290]. During the downstroke of the systolic phase a reversal of flow occurs, which alters the size and spatial migration of the secondary flow patterns [277, 290]. The net effect is a migration of the stagnation point along the outer wall, resulting in a focal area of low temporal and spatial WSS.

Using three-dimensional rotational angiography and computational fluid dynamic modelling, Meng mapped these three loci to photomicrographs, enabling the biologic properties of the endothelium and vessel wall cells to be evaluated with reference to the local haemodynamic microenvironment. Although initially histologically indistinct, two distinct patterns of wall remodelling were identified:

1. Hyperplasia forming an intimal pad at the bifurcation apex

   This was predominantly a feature of the impingement region. Two morphologies were identified; early intimal hyperplasia, and development of a more mature intimal pad. The early intimal hyperplastic state was characterised by an increased number of cells on the luminal side of the internal elastic lamina, beneath which was deposited a thick layer of largely acellular collagen. The mature intimal pad state demonstrated fewer intimal cells in the hyperplastic region, but a thicker layer of subendothelial collagen with additional layers of elastin, resembling the intimal pad at the apex of a natural bifurcation.

2. Destructive remodelling in the acceleration zone.

   The acceleration zone was noted to contain a “shallow groove” in the vessel wall secondary to an overall decrease in the thickness of the media and intima. Microscopic analysis revealed loss of the internal elastic lamina and overlying endothelium, and a prominent collagen matrix containing fewer medial smooth muscle cells.

Given both began as biologically and histologically identical segments, the observed remodelling suggested a distinct response to the specific haemodynamic microenvironment. Constructive hyperplastic remodelling events were localised to the flow-impingement area, suggesting that this may be a physiological repair response similar to wound repair and scar formation. This hypothesis was further strengthened by the author’s observation of fibronectin (an important matrix component during tissue repair) was increased in the intimal pad region.

Conversely, the destructive events localised to the acceleration region (i.e. destruction of the internal elastic lamina and loss of the endothelium and smooth muscle cells) resembled those previously observed in both animal and human histological studies of aneurysm pathogenesis[1, 45, 291-294]. These histological findings were spatially correlated with areas of high WSS and WSS gradient, however the other expected histopathological changes of early aneurysm formation were absent. Rather than the expected endothelial cell proliferation in the acceleration region, authors noted instead loss of endothelium. This may have occurred due to the high WSS gradient in the acceleration region, which when combined with the high WSS resulted in endothelial cell dysfunction and denudation. The authors noted early aneurysmal change localised to the acceleration zone, suggesting that high WSS contributes to aneurysm pathogenesis.

Meng's model unsuccessfully attempted to induce hypertension in the dogs using both a high-salt diet and renal ligation prior to the carotid artery procedure. Previous animal models of intracranial aneurysm formation have shown that neither systemic hypertension nor degeneration of the elastic lamina alone is sufficient to cause formation of an aneurysm[295, 296]. It would appear, therefore, that Meng had inadvertently demonstrated that the combination of high WSS with a high WSS gradient is sufficient to induce early aneurysmal change; a potentially crucial insight into the role of haemodynamic stress in macroscopic aneurysm formation.

It is likely that both high and low WSS, working in tandem, have a synergistic effect on early an aneurysmal change; i.e. that pre-aneurysm change in the arterial is caused by high WSS, but further propagation and aneurysm growth are a function of endothelial dysfunction and wall degeneration caused by low WSS. Confirmation of this hypothesis using in-vivo measurements of WSS is obviously impractical, however modern CFD studies using various mesh algorithms have been shown to recreate accurately the cerebral haemodynamic pathophysiology surrounding aneurysms[276, 297-301]. Shojima[276] et al determined that the magnitude of WSS in well-developed aneurysms is insufficient to mechanically tear the aneurysm wall. Rather, excessively low WSS results in further

wall degeneration by inducing apoptosis in endothelial cells[302], wall remodelling and further aneurysm growth[301]. Modern CFDs have revealed considerable variability in aneurysm haemodynamic profiles [276, 297, 303]. Although determining haemodynamic pathophysiology leading to aneurysm growth remains a challenge, there is mounting evidence that morphology, and intra-aneurysm flow dynamics may be more predictive of rupture risk[58, 60, 197, 285, 300, 304-306]}.

Role of Endothelium

The endothelium is a continuous layer of cells connected to each other and the substratum by various adhesive molecules. Previously viewed as an inert membrane lining the circulatory system with the primary function of maintaining vessel wall permeability[307], the vascular endothelium is now known to serve as an important paracrine and autocrine organ in the regulation of vascular wall function [293, 294]. The earliest description of the endothelium as a secretory cell system was made by Heidenhahn in 1891, however it was not until the advent of electron microscopy and the studies of Palade and Gowan [308] in the 1950s that interaction between the endothelium of post capillary venules and lymphocytes was described.

The endothelium is a heterogeneous organ critical in the performance of vital secretory, synthetic, metabolic and immunological functions[309]. The endothelial cell surface area in adults is approximately 1-7m^2, consisting of approximately 1-6x10^{13} cells and lines vessels in virtually every organ system[310]. The endothelium regulates the flow of nutrient substances, blood cells, and other molecules via membrane bound receptors for proteins (e.g. pro- and anti- coagulant factors, growth factors), lipids (e.g. low-density lipoproteins), metabolites (e.g. nitric oxide) and hormones (e.g. endothelins), along with specific junctional proteins and receptors governing cell-cell and cell-matrix interactions[307].

The endothelium also provides a structural barrier between the circulating blood and surrounding tissue, and secretes vascular mediators of critical importance in the maintenance of physiological haemodynamics. Quiescent endothelial cells maintain an antithrombotic luminal surface which facilitates laminar blood flow. Pro-thrombotic, antifibrinolytic microenvironments may be induced by endothelial cells where disturbances to laminar flow are created, such as sites of high WSS or inflammation. Blood flow and pressure are also regulated in part by endothelial release of vasoconstrictors (endothelin, platelet–activating factor) and vasodilators (nitric oxide (NO),

prostacyclins). These are not stored as intracellular granules; their major effects are regulated via localisation of specific cell-surface receptors, through rapid metabolism, or through rapid gene transcription. Some mediators such as prostacyclins, endothelin, and platelet activating factor are expressed primarily in response to external stimuli. The endothelium may also act in a paracrine manner to external stimuli, resulting in vasoconstriction or vasodilation in specific vascular beds[307].

Putative endothelial stem cells migrate, proliferate and differentiate during vasculogenic and angiogenic processes. Mature endothelial cells are highly heterogeneous for different vascular sites and form distinct, system-specific morphotypes[310]. By way of example, in humans, vessels of the liver and spleen are lined with discontinuous endothelial cells to allow trafficking of substances between intercellular gaps. Intestinal villi, endocrine glands and the kidneys are lined with fenestrated endothelial cells to facilitate the selective permeability required for absorption, secretion and filtration respectively[307, 311]. This heterogeneity extends to different vessel calibres within the same organ or system[312].

Endothelial cells are also heterogeneous with respect to their surface phenotype and protein expression; e.g. the expression of von Willebrand factor is not uniform across all endothelial cells, and the expression of tissue-plasminogen activator is limited in-vivo to approximately 3% of the endothelium[307, 311, 313, 314]. This structural heterogeneity highlights the adaptative capacity of the endothelium and allows the endothelial cell to best adapt to the local environment to perform its structural and metabolic functions. In the brain, the endothelium is continuous and connected by tight junctions, which maintain the blood-brain barrier (BBB).

Nitric Oxide

Nitric oxide (NO) is a heterodiatomic free radical product generated from the oxidation of L-arginine to L-citrulline by nitric oxide synthase (NOS). NOS is expressed in microorganisms, plants and mammals and participates in a diverse range of physiological and pathological functions including neurotransmission, regulation of vascular tone, cellular communication, inflammation and immune responses[315]. In the circulatory system it is constitutively expressed by endothelial cells and its production is modulated by a number of exogenous chemical and physical stimuli and receptor-dependent agonists. There are three different isoforms of NO synthase; endothelial-derived NOS (eNOS), neuronal-derived NOS (nNOS) and inducible NOS (iNOS). The formerly accepted distribution of these isoforms in the vascular wall had been that endothelium expressed eNOS, that perivascular nerves in some (but not all) vessels secrete nNOS, and VSMCs do not express NOS at all[316]. More recent studies have challenged this hypothesis with the discovery that all three isoforms are expressed by VSMCs, with muscular arteries demonstrating a greater magnitude of expression than elastic arteries[317-320]

eNOS derived NO is produced by endothelial cells in response to physiological WSS, and is a crucial factor in autoregulation of the vessel diameter in response to exercise[321]. eNOS mRNA levels increase in direct proportion to the magnitude and duration of the WSS exerted on the endothelium which elicits a biphasic response [322]. The early (i.e. <15min) phase is dependent on the rate of change of shear and is mediated by calcium, calmodulin, and pertussis-toxin insensitive G-Proteins. The later phase is dependent on the magnitude of shear stress and is independent of calcium and calmodulin [323]. eNOS may also be expressed in response to bradykinin, acetylcholine, and aggregating platelets[285]. Endothelial-derived NO has several important effects:

1. It maintains basal tone by causing relaxation in vascular smooth muscle cells via its action on guanyl cyclase[324].

2. It inhibits platelet aggregation, adhesion, and promotes disaggregation via a cyclic guanosine monophosphate (cGMP) dependent mechanism, and acts synergistically with PGI_2 to inhibit other steps in the platelet coagulation cascade[325, 326].
3. It also inhibits leukocyte adherence to the to the endothelium[327], smooth muscle cell migration[328] and proliferation[329]

All of these effects serve to limit neointimal proliferation, suggesting a role for NO in vascular reparative mechanisms[307].

nNOS derived NO has been implicated in a wide variety of physiological roles in the cerebral circulation including maintenance of cerebral perfusion, CO_2 reactivity, neurogenic and excitatory amino-acid induced vasodilation and flow-metabolism coupling [426]. nNOS expression is upregulated by various tissues such as endothelial cells, vascular smooth muscle cells, macrophages and nerves[316, 330]. In addition, nitrergic (non-adrenergenic, non-cholinergic) perivascular nerves of the cerebral vasculature contain nNOS. As a neurotransmitter, nNOS allows for relaxation of vascular smooth muscle cells, counterbalancing sympathetic vasoconstriction[316].

Thus, both eNOS and nNOS have a coordinate role in relieving haemodynamic stress. Although originally identified as a constitutively expressed enzyme, nNOS exerts important vasoprotective effects, suppressing both neointimal formation and constrictive vascular remodelling in response to vascular injury in animal models[331]. Studies have suggested that up-regulation of nNOS can compensate for decreased function of eNOS in eNOS -/- mice[332]. nNOS may therefore compensate for the downregulation of eNOS in cerebral arteries[331, 333]. In aneurysm walls, eNOS function is also down-regulated, probably due to endothelial damage. nNOS upregulation by VSMCs may functionally compensate for this[333]; this compensatory effect appears especially relevant in the modulation of leukocyte-endothelial cell interactions [332].

Functional expression of nNOS has been suggested in human, porcine and rodent arteries [334-336]. But due to its upregulation in response to eNOS dysfunction or vascular injury, nNOS cannot be considered a wholly "constitutive" enzyme. Its role in early aneurysmal change in the vessel wall is probably protective as eNOS expression is downregulated due to damage to and hypofunction of endothelial cells in the vessel wall, and nNOS expression is upregulated by VSMCs as a compensatory measure. As damage to the vessel wall persists and the media of the wall becomes more degenerate, nNOS expression may be further reduced, resulting in progression of this early change and potentially macroscopic aneurysm formation.

This proposed compensatory effect has only been observed in small-to-medium sized arteries, and not in the aorta[333]. Sex hormones, particularly oestrogen, have been shown to exert a vasoprotective and anti-inflammatory effect[337], and both up-regulate and down-regulate nNOS production[338, 339]. Oestrogen receptors have been identified on endothelial cells and VSMCs; the interaction of oestrogen with these receptors stimulates the function and proliferation of endothelial cells, reducing vascular tone and oxidative stress[340, 341].

Further evidence suggestive of the compensatory effect of nNOS for eNOS may lie in the observation of the significantly decreased incidence of cerebral aneurysm formation in female eNOS-deficient mice; in these models it would appear that oestrogen exerts a significant protective effect against early aneurysm pathogenesis and development of macroscopic aneurysms[342]. Human epidemiological studies suggest that reduced oestrogen levels in post-menopausal women may predispose to a higher incidence of aneurysms due to a diminution of the collagen content of cerebral arteries[342-344]

The small amount of NO produced by eNOS and nNOS under physiological conditions is thought to be important in cellular signalling events such as blood pressure regulation and neurotransmission[315]. Inducible NO synthase (iNOS), on the other hand, is transcriptionally regulated, and is not normally produced by most cells[345, 346], but can be induced in virtually

every cell if appropriately stimulated[315]. iNOS functions as both a regulator and effector during inflammation and infection; one effect includes direct cytotoxicity towards microorganisms, tumour cells and host cells, in many circumstances as a result of interaction of NO with superoxide to form peroxynitrite, a potent oxidant[315]. An association between tissue damage and NO produced by iNOS has been made in animal models[347]. Shear stress is a major stimulant of iNOS expression in the vascular wall, and its increased expression in aneurysmal microenvironments exposed to variations in WSS has been demonstrated in animal models[347]. When chronically elevated, WSS causes an over-expression of iNOS, generating 100-1000 fold more NO than eNOS or nNOS, which results in endothelial and VSMC damage and degenerative change[347, 348].

iNOS activation is also dependent on cellular exposure to inflammatory stimuli such as bacterial endotoxins, TNF or interlukin-1. iNOS expression is therefore thought to play a central role in chronic inflammation and connective tissue degeneration via endothelial cell proliferation and decrease in cell-cell adhesion within the vessel wall [349]. It is a key upstream regulator of the inflammatory response[350]. In animal models of aneurysm pathogenesis, expression of iNOS appears to be particularly increased at the juxta-apical groove and the intimal pad near the bifurcation apex, which coincides with the area of maximum tissue damage [347]

Increased expression of iNOS and elevated plasma nitrite/nitrate levels have been demonstrated in animal models of AAA formation, where iNOS inhibition leads to the development of smaller AAAs[351]. These studies also suggest ablation of the iNOS gene may delay the progression of AAA formation[348, 350]. Although other studies have demonstrated increased iNOS expression during AAA development, it is not thought to be a crucial factor in induction of experimental intracerebral or aortic aneurysms[348, 349]. iNOS may, however, play a role in macrophage induced VSMC apoptosis. The capacity of NO to induce apoptosis has been well documented in numerous cell types including macrophages and neurons[352, 353], and is thought to occur via accumulation of the tumour suppressor protein p53, inducing cell cycle arrest[354]. In-vitro, this apoptotic effect appears

to arise from natural cell-protective mechanism becoming overwhelmed by high levels of exogenous NO, although whether this is also the case in-vivo remains unclear[315]. Alternatively, this effect may result from the interaction of NO with a superoxide anion to produce peroxynitrite, inducing apoptotic DNA fragmentation and subsequent p53 dependent apoptosis[315, 354, 355].

The peroxynitrite hypothesis is complicated in that NO also exhibits an antiapoptotic effect via induction of cytoprotective stress proteins, cGMP-dependent inhibition of apoptotic signal transduction, or suppression of caspase activity[315]. Therefore, the role of NO-induced apoptosis in cerebral aneurysm pathogenesis is probably dependent on the relative magnitude of expression of NO in the vessel wall. At physiological levels, NO suppresses the apoptotic pathway at multiple points[315]. Reduction and suppression of the caspase protease caspase-3 by NO has been demonstrated using purified human caspases[356], and has been shown to be at least partially responsible for the suppression of NO-induced apoptosis in endothelial cells[357]. Once NO levels increase to supraphysiological levels, however, cellular protective mechanisms become overwhelmed and shift the balance towards apoptotic death[315].

Role of Inflammation

Introduction

The inflammatory process is crucial in maintaining homeostasis of the circulatory system[358]. It is initiated and maintained by a complex system of interactions between multiple factors and receptors, leading to changes in the integrity of the vessel wall. In the same manner, inflammation plays a central role in the pathophysiology of a number of vascular diseases including atherosclerotic plaque formation, arteritis, and development of abdominal aortic and intracerebral aneurysms[73, 358-360].

Chyatte et al[45] demonstrated a link between inflammation and aneurysm growth and rupture by describing extensive inflammatory and immunological reactions in unruptured aneurysms. These findings were replicated by further studies demonstrating the presence of inflammatory cells (macrophages, T-lymphocytes, B-lymphocytes) in the walls of human aneurysms[45, 103], and increased levels of proteolytic enzymes and MMPs in the serum of some patients harbouring unruptured aneurysms[361]. Complement factor (C-), an important part of the innate and adaptive immune response, was found in almost all subjects with aneurysms[73]. C3, the most abundant complement protein in serum, and C9, the terminal protein of the complement cascade, are used as markers of activation of the complement cascade; both were found in human aneurysms[45]. The findings of Kataoka et al[103], demonstrating inflammatory infiltrates in half (n=10/20) of unruptured, and all (n=40/40) ruptured aneurysms suggests strongly that inflammation plays a key role in early aneurysmal change, progression to macroscopic aneurysm formation, and, ultimately rupture. Whether or not different subsets of inflammatory leukocytes influence the natural history of aneurysms requires further study.

Endothelial Dysfunction

The endothelium of cerebral arteries maintains blood flow by inhibiting coagulation and adhesion of leukocytes and serves as a selectively semi-permeable barrier between the central nervous system and blood cells. It plays a pivotal role in the inflammatory response to pathogens, regulating both innate and adaptive immune responses[362, 363]. Under physiological conditions, the endothelium has a very low level of cell turnover[364].

Activation of endothelial cells initiates an inflammatory cascade resulting in recruitment of leukocytes to the damaged site, which has been proposed as an initiating factor in early aneurysmal change. Inflamed tissue is entered by leukocytes through non-specialised postcapillary venules which have been transiently activated by an inflammatory stimulus to capture leukocytes from the circulation and attract them into tissue[363].

Leukocyte/endothelial cell interactions are facilitated by a number of adhesion molecules and chemo-attractants which function in a multistep cascade initiated by a small group of cell-adhesion molecules (selectins) which capture leukocytes from the bloodstream via transient ligand interactions. These interactions slow down the flow of leukocytes, resulting in them “rolling” along the endothelium (the “rolling phase”). Multiple selectins with subtly differing properties facilitate this process; P-selectin and its ligand P-selectin glycoprotein-1 initiate capturing, E-selectin stabilises and slows down the rolling process, and integrin leukocyte function-associated antigen-1 (LFA-1) further supports rolling and transforms it into the arrested state[363, 365-368].

Chemotactic factors present on the endothelial surface bind to G-protein coupled receptors on the leukocyte surface leading to the activation of leukocyte integrins which further improve the affinity for ligands such as intercellular adhesion molecule-1 (IaneurysmM-1) and vascular cell-adhesion molecule-1 (VaneurysmM-1) on the endothelium (the “firm-adhesion phase”)[363] [369]. These two families of aneurysm are also responsible for the subsequent migration of leukocytes through the

endothelium and into the vessel wall ("emigration phase") where they promote further inflammation.

Endothelial erosion promotes leukocyte adherence to the vessel wall, allowing invasion of the IEL by macrophages and leukocytes [103]. The degree of endothelial damage seen in the vessel lumen is dependent on the magnitude of injury to the endothelial cells and capacity for endothelial repair. Although this repair may be facilitated by migration and differentiation of the surrounding endothelium, mature endothelial cells are terminally differentiated and thus have a limited capacity for further proliferation and repair of damaged areas [364].

Endothelial progenitor cells (EPCs) are circulating, bone marrow derived cells which share properties with embryonic angioblasts[370]. They are produced by bone marrow in an immature form and change their progenitor properties in the circulation[364]. Endothelial damage and dysfunction appears to be the trigger for release of these cells from the bone marrow, and is regulated by a variety of enzymes, growth factors, ligands and cell surface receptors[364]. Acute myocardial infarction, burn injuries, or vascular trauma such or acute limb ischaemia or coronary artery bypass grafting have been shown to elicit rapid mobilisation of EPCs into the circulation[364, 371]. The process by which these cells subsequently home in upon the damaged vessel, differentiate into mature endothelial cells and effect repair is unclear. Lower levels of circulating EPCs have been correlated with an increased overall cardiovascular risk profile[364, 372-374]; these patients exhibit a decreased number of circulating EPCs which become senescent more rapidly than those of controls. Risk factors specifically associated with intracerebral aneurysm pathogenesis such as hypertension and cigarette smoking have likewise been reported to correlate with decreased levels of circulating EPCs; whether these factors impact directly upon the mobilisation and half-life of EPCs, or, owing to continuous cycles of damage and repair, simply deplete the circulating pool of EPCs requires further study[364, 372, 374].

Wall remodelling and Inflammation

The central nervous system is immunologically active, with various immune and inflammatory mediators interacting in a highly orchestrated environment[375]. An inflammatory component to aneurysm pathogenesis was first suggested by Virchow in 1847[376], with other historic studies describing inflammatory cell infiltration at the aneurysm neck[377]. The importance of these early findings were mostly ignored for decades, however various studies intermittently hypothesised inflammation to be a critical factor in aneurysm formation[45, 291]. Interest in this area was rekindled after a number of studies reported inflammatory infiltrates, macrophages, T- and B-lymphocytes in the walls of aneurysms [45, 103, 361] and increased levels of proteolytic enzymes in the serum of patients harbouring aneurysms[361]. An increased level of inflammatory infiltrate and degradation of matrix proteins in the walls of ruptured relative to unruptured aneurysms has been demonstrated[103].

Recent studies have demonstrated inflammatory vessel wall remodelling to be key factors in aneurysm pathogenesis, from early aneurysmal change to rupture[44, 47, 49, 104, 113, 293, 378]. The net effect of the inflammatory response is the balance between pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines such as monocyte chemo-attractant protein-1 (MCP-1), IL-1, 8, 18, INF-g and, TNF-a are predominantly secreted by macrophages and lymphocytes. Several studies have demonstrated the presence of these cytokines in aneurysm walls, however it is unclear whether their relative over-activity induces aneurysm pathogenesis, or occurs in response to other pathological insults inducing early aneurysmal change[73, 350, 379]. The relative paucity of anti-inflammatory mediators such as IL-10 in the wall suggests either absence or suppression of T helper type-2 (Th-2) cells, however Th-2 cells have previously been demonstrated in the aneurysm wall[73, unpublished data].

Other pro-inflammatory mediators such as leukotrienes (LTs) and various lipid mediators may also contribute to this pro-inflammatory state. LTs are a group of pro- inflammatory lipid mediators

derived from arachidonic acid. 5-lipoxygenase (5-LO) is involved in LT production and has been implicated in various pathophysiological inflammatory disorders including asthma, rheumatoid arthritis and atherosclerosis[73]. They may play an indirect role in the induction of pro-inflammatory cytokines such as TNFα via LT[380]. 5-LO inhibitors significantly decrease TNFα production; it's exact role in aneurysm pathogenesis remains to be determined, however it may represent a promising target for future therapies.

Inflammatory cell infiltration of vessel walls

Several studies report inflammatory cell infiltration in the aneurysm wall [45{Frosen, 2004 #88, 113, 291, 378]. Factors triggering inflammatory cell adhesion to and infiltration of the damaged arterial wall are incompletely understood, however loss of normal endothelial cell function is thought to be an important initiating factor[381, 382]. Therapies aimed at attenuating the inflammatory response including NF-KB inhibitors[383], free-radical scavengers, statins[384] and inhibitors of mast cell degeneration [385] have been investigated in animal models. Although these appear to slow aneurysm formation and progression, their role in prevention of aneurysm rupture remains less clear.

As discussed, Chyatte[45] et al demonstrated inflammation and immunological reaction due to focal pockets of matrix metalloproteinases and other proteolytic enzymes in the aneurysm walls of patients with both ruptured and unruptured aneurysms. These infiltrates were dispersed throughout the wall, indicating a generalised inflammatory response. Leukocyte infiltration and VSMC damage in both unruptured and ruptured aneurysms was also observed by Kataoka[103].

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) include collagenases, gelatinases, and membrane-type MMPs. These are a family of structurally related, zinc containing proteolytic enzymes which play a key role in embryonic development, wound healing, vascular remodelling, cellular migration, extracellular matrix haemostasis and neointimal formation [386]. They are produced by many tissues and are either secreted from the cell or remain anchored to the plasma membrane.

Their site of action is primarily the cell surface or extracellular space, and activity is tightly regulated on many levels including inhibition of endogenous inhibitors such as a-2 macroglobin and tissue

inhibitors of metalloproteinases (TIMPs)[387]. Like MMPs, the expression of TIMPs is controlled under physiological conditions to maintain a balance of production and turnover of the extracellular matrix[388]. Disruption of this balance may result in pathological turnover of matrix. Once activated, MMPs participate in a broad range of physiological and potentially pathological processes. Other molecules whose biological activity is regulated by MMPs include pro-inflammatory cytokines such as TNFα[389], fibroblast growth factors and receptors (FGF, FGFR) [390], plasminogen and activators of plasminogen[391], and endothelin [392].

Nuclear factor

Nuclear factor-kappa B (NF-κB) is a transcription factor implicated in the inflammatory response[393]. It is present in the cytoplasm in a non-active form and is activated by cleavage of an inhibitor subunit (IκB) in response to inflammatory stimuli. The result is mRNA expression of multiple cytokines, adhesion molecules and growth factors[393]. Transcription and activation of NF-κB from the endothelium upregulates adhesion molecules, triggers macrophage infiltration and creates a pro-inflammatory environment in the adventitia and media[394], and is a major contributing factor in the pathogenesis of atherosclerosis[395]. Activated NF-κB has also been demonstrated in aneurysm walls and particularly so in the endothelium. Haemodynamic stress and endothelial injury may be precipitating factors in its transcription[278]. Inhibition of NF-κB has been shown to significantly reduce the incidence of early aneurysmal change when administered within one week of initiation of aneurysm pathogenesis in animal models, suggesting a role for NF-κB in very early aneurysmal change[383] Genes upregulated by NF-κB activation include MMPs, iNOS, IL-1b and MCP-1[396], which are functionally important in aneurysm pathogenesis.

Tumour Necrosis Factor

Tumour Necrosis Factor–alpha (TNFα) is a potent pro-inflammatory cytokine which triggers endothelial activation with increased monocyte adhesion; the hallmark of the inflammatory response[73]. It alters the function of the blood brain barrier (BBB) allowing inflammatory cells to cross the endothelium and enter the vascular lumen matrix. In addition to induction of specific cytokines, TNFα may further contribute to matrix degeneration indirectly by activating other pro-inflammatory signalling molecules such as Tissue Factor (TF), Nitric Oxide (NO), von Willebrand factor and platelet activating factor(PAF)[397]. TNFα may also exert an indirect effect on the aneurysm wall via activation of other signalling molecules such as TF, adhesion molecules for leukocytes, release of IL-1, von Willebrand factor, PAF and endothelin[73, 397].

TNFα amplifies several pro-inflammatory gene pathways and exerts a pro-apoptotic effect on VSMCs. The principal cells producing TNFα are macrophages and monocytes, however it is also produced by B cells, T cells, NK cells, Kupffer cells, glial cells and adipocytes[397]. It acts via distinct receptors, TNFR-1 and -2, the principal difference between which is the presence of a death domain (DD) on TNFR-1 which is absent in TNFR-2[73, 398]. TNFR-1 is constitutively expressed in most tissues and is the key mediator of TNFα signalling for most cell types[398]. It is an important activator of both cell death and survival signals[398-400]. TNFR-2 is expressed in endothelial and immune related cells and has an affinity for TNFα five times that of TNFR-1[400]. Interaction of TNFα with TNFR-1 or -2 induces either an inflammatory response or apoptosis/survival of the cell via recruitment of different adaptor proteins.

Jayaraman et al[73] demonstrated significant expression of TNFα in the walls of ruptured aneurysms. TNFα upregulation appears to correlate with a number of known risk factors for aneurysm formation including haemodynamic stress, hypertension, and increasing age. Its production is also upregulated in response to environmental insults such as tobacco smoke and alcohol, which are recognised independent risk factors for aneurysm formation and rupture[73]. TNFα is selectively expressed at regions of increased haemodynamic stress such as arterial branch points and along the curvature of major vessels, and appears to be more significantly expressed in the walls of ruptured aneurysms[73].

The effects of TNFα in a murine animal model of intracranial haemorrhage were explored by Siren et al[401]. Histological examination of the brains of the rats most impaired by intracerebral haemorrhage demonstrated increased infiltration of leukocytes expressing immunoreactive IL-1b, IL-6 and TNFα around blood vessels, cerebral ventricles and meninges. Treatment with a recombinant type-1 soluble human (rh-) TNF receptor completely prevented intracerebral haemorrhage in this model, suggesting TNF-a to be a necessary causal factor for intracranial blood vessel activation and rupture.

Conversely, in small animal models of atherosclerosis, accelerated plaque development was observed in mice lacking the TNFR-1 receptor, suggesting that at least some level of TNFR-1 mediated signalling has a protective effect on the vessel wall.

These seemingly contradictory findings underline the importance of the inflammatory system in cerebrovascular haemostasis. They also suggest that a threshold of TNFα activity is important in intracerebral aneurysm pathogenesis. Jayaraman[73] hypothesised that TNF–a is expressed during early aneurysmal change and is mostly membrane-bound on luminal endothelial cells, leading to endothelial cells activation and further TNFα expression. This in turn leads to endothelial cells

dysfunction and apoptosis, providing an opening for aneurysm formation and infiltration of inflammatory cells into the inner layers of the arterial wall. Subsequent TNFα secretion by these inflammatory cell infiltrates initially causes increased VSMC growth[402], which is swiftly followed by apoptosis due to increased macrophage infiltration, leading to MMP generation and structural breakdown of mural proteins. However, when these inflammatory infiltrates are inactivated or reduced beyond a certain threshold by either anti-inflammatory cytokines or other modulators of the inflammatory response, the increased VSMC growth caused by the now attenuated inflammatory response may lead in increased stability in the aneurysm wall, lessening the risk of rupture.

Although there is little definitive evidence implicating TNFα in aneurysm formation, its strong association with known risk of aneurysm formation and its significant expression in aneurysm walls and role in inflammatory cell infiltration provide a provocative correlation with aneurysm pathogenesis. Characterisation of TNFR- mediated signalling events during aneurysm growth may lead to a better understanding of aneurysm pathogenesis.

Significance of Fibrosis in aneurysm walls

In contrast with the rapidly evolving vascular changes, oedema and neutrophilic infiltration characteristic of acute inflammatory reactions, fibrosis is an end–phase process typically resulting from chronic inflammation. An inflammatory process, having continued for a protracted period of time (typically several months) leads to tissue remodelling and repair[403]. Most chronic fibrotic disorders have in common a persistent irritant which sustains the ongoing production of growth factors, proteolytic enzymes, angiographic factors and fibrogenic cytokines, which stimulate the deposition of connective tissue elements which progressively remodel and destroy normal tissue architecture[403-406].

Aneurysm walls may remain intact for a very prolonged period[2, 407], suggesting strong maintenance and repair mechanisms. They do, however, tend to increase in size[197, 407], with the aneurysm wall undergoing morphological changes which are likely different in those which remain intact versus those which ultimately rupture[45, 103]. These morphological changes appear to be independent of aneurysm size, location or bleb formation, however some may be associated with younger patient age at presentation [2]

Frosen et al[2], in their histological study of unruptured and ruptured human aneurysms, observed morphological changes associated with remodelling of the aneurysm wall in prior to rupture, allowed the description of four distinct wall-types:

1. Endothelialised wall with linearly organised VSMC
2. Thickened wall with disorganised VSMC
3. Hypocellular wall with myointimal hyperplasia or organised thrombus
4. Extremely thin, thrombus lined hypocellular wall (the most prevalent type in ruptured aneurysms),

In keeping with earlier studies[103], they found thick, intima like walls, and very thin, degenerate walls with hyaline deposits to be more prevalent in unruptured and ruptured aneurysms respectively. “Stable”, unruptured aneurysms, on the other hand, demonstrated more proliferation and migration of VSMCs, resulting in the formation of a thick, fibroid layer on the luminal surface of the vessel.

The observation of this fibrotic layer in the aneurysm wall led Frosen[408] to consider whether “macrophages may stimulate (V)SMCs to change phenotype and proliferate, thus promoting fibrosis”. Interestingly, the degree of VSMC proliferation and T-Cell and macrophage infiltration was increased in aneurysm walls resected less than 12 hours post rupture, suggesting the presence of at least some inflammatory cell infiltrates in the aneurysm wall preceded the acute inflammatory cell

infiltration associated with rupture; in healthy arterial walls, such infiltrates generally occur in response to injury during the first 24 hours or later[409]. It would appear that in response to an undefined event prior to rupture, the aneurysm wall becomes unstable and undergoes morphological change reflecting an imbalance between the effect of factors predisposing to rupture and those maintenance and repair mechanisms attempting to prevent it. Although adaptive responses of arteries to luminal stressors attempt to negate these stressors, it would somehow appear that in aneurysmal subarachnoid haemorrhage, the responses themselves may predispose to rupture due to increased matrix proteolysis[2, 50, 408, 410]

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“A Small Animal Model for the Creation of Pre-Aneurysmal Change in the Intracranial Vasculature”

Masters Philosophy (Health)

Dr James Anthony Lee

Part 2

List of Figures

Part 2

Abstract

As naturally occurring aneurysms in laboratory animals are very rare, various animal models of intracerebral aneurysm pathogenesis have been designed in multiple species including rodents, rabbits and also higher mammals such as dogs, sheep, pigs and primates[1]. There are two major classes of animal models of aneurysm development; those used to evaluate underlying mechanisms of aneurysm formation, which are discussed in detail below, and those principally used in the trialling novel endovascular techniques and devices. [2] (these are generally canine, ovine or porcine models using surgically induced aneurysms, usually in the carotid artery)[3-6].

Relatively few animal models of cerebral aneurysms are available, and most incorporate a high incidence of rupture [2, 7-11]. Some have incorporated induction of systemic hypertension and stereotactic intracranial elastase infusion[2]. The aneurysms produced by such studies have been rapidly formed (2-6 weeks), and have been relatively large (even giant) [7, 8, 10-14]. This questions their representativeness to human pathogenesis as most human aneurysms are small to medium in size relative to their parent vessels and appear to develop over a protracted period[15-23].

Appreciation of the limitations of these models is of paramount importance; information gained from animal studies should be carefully evaluated for relevance[24]. The aim of this study was to develop an animal model of the changes associated with the early aneurysm pathogenesis.

Animal Models of Intracranial Aneurysm Formation

Introduction

Surgically Induced Aneurysms

Various techniques for inducing aneurysm formation in animal models have been described in the early literature[25-30]. Broadly speaking, these can be subdivided into two categories; those involving aneurysms resulting from direct arterial injury or surgery, and those induced by stimulating known risk factors for aneurysm formation in humans[31].

Vein pouch models involve microsurgical grafting of a patch of autologous vein to a surgically created arteriotomy. German et al[32] first reported this technique in 1954. In brief, a small wedge is cut from the medial corner of the left common carotid artery, a partial end-to-side anastomosis of the left common carotid to the right common carotid is fashioned, and a previously harvested vein segment is sutured to the notch formed by the anastomosis. A number of variations on this technique have been reported including end-to-side anastomosis of an external jugular vein graft to the common carotid artery [33], suturing of an autologous vein graft to an end-to-side anastomosis of the left common carotid artery to right common carotid artery[34], via an arteriovenous fistula method[35] or via microsurgical construction on the wall of the basilar artery [27]. Although these models may accurately model the geometry and haemodynamic microenvironment of human aneurysms, all spontaneously manifest histologic changes not seen in true saccular arterial aneurysms[36, 37]. In addition, the manipulation of the arterial and venous pouch endothelium during construction of the aneurysm releases various factors which lead to scarring and obliteration of the aneurysm over time, confounding any long-term observations or results of therapeutic intervention[37].

Aneurysms induced by haemodynamic manipulation alone

The early work of Hassler[25] described induction of pre-aneurysmal change and macroscopic aneurysm formation in a rabbit model via ligation of the common carotid artery. Subsequent analysis of the intracerebral arteries revealed structural changes in the arterial walls with an increase in the size of medial defects. Macroscopic aneurysms were induced in six of 42 rabbits. The calibre of the posterior communicating, ophthalmic and anterior and middle meningeal arteries was noted to be increased to a greater degree in younger animals and tended to become more pronounced over time (i.e. the observed increase was greater in animals euthanized five months after ligation compared with those euthanized after one month.). These findings confirmed the authors' prior expectation that the increase in haemodynamic stresses secondary to carotid ligation would lead to histological changes in the arterial wall.

The alterations in vessel calibre on the side of the ligation led the authors to hypothesise that pathology of the internal carotid artery (e.g. stenosis) may cause variations in the calibre of the intracranial arteries. The paucity of these findings in younger animals was felt to be due to an increased ability of younger animals to develop anastomoses and collateral circulatory pathways between the internal and external carotid systems. The observed macroscopic and histological changes in the intracranial arteries demonstrated the susceptibility of the arteries to "hydraulic imbalance" or increased haemodynamic stress.

Aneurysms induced by haemodynamic manipulation and surgical induction of hypertension

Observations from these early models suggested that a critical factor in aneurysm induction and growth was altered circulation and haemodynamic forces in the Circle of Willis. This hypothesis was backed by later studies[38-41] inducing aneurysm formation via haemodynamic manipulation alone, however the long incubation time (approaching 12 months in some models) limited their utility.

Similarly, hypertension was determined to be an important, but non-essential factor in aneurysm pathogenesis in these models; although the combination of hypertension and carotid ligation was shown to aggravate degenerative changes in the vessel walls, non-hypertensive rats treated with carotid ligation alone still developed aneurysms[41]

The idea that increased haemodynamic stress, when coupled with other it factors causing fragility of the arterial wall may accelerate the pathophysiological process leading to aneurysm formation, Handa et al[31] developed a model of aneurysm induction in adult Sprague-Dawley rats via a combination of unilateral ligation of the common carotid artery, surgical induction of hypertension via right nephrectomy, and Beta-aminopropionitrile (BAPN) feeding.

BAPN is a lathrogen. its mechanism of action is inhibition of lysyl oxidase, which initiates cross-linkage formation between elastin and collagen. When fed to young or rapidly growing animals, connective tissue becomes abnormally fragile and a variety of connective tissue pathologies arise[42, 43]. This commonly manifests as loss of tensile strength and elasticity of great vessels such as the aorta[44]The action of BAPN is exclusive to collagen and elastin during the synthesis phase only; under physiological circumstances mature animals are not susceptible to its effects. Hypertensive stress, however, induces increased collagen and elastin synthesis in the arterial wall[45-47].

Renal hypertension was induced via a number of methods. Surgical hypertension was achieved via right nephrectomy at the time of carotid artery ligation followed by administration of Deoxycorticosterone acetate (DOCA) via a twice weekly subcutaneous injection commencing one week after surgery. DOCA is a mineralocorticoid used to augment surgically induced hypertension. It increases the permeability of cell membranes and capillaries, and accelerates the inflammatory process[48]. DOCA has a propensity to cause significant alterations in ion transport by VSMCs in the vessel wall, reducing the ability of calcium to stabilise the membrane. This may be a contributory factor in vascular reactivity and development of vascular hypertrophy in vessels under chronic

hypertensive stress[48]. DOCA is not essential in the development of aneurysms[31, 49], however its use increases the yield of aneurysms.

1% NaCl solution was also given as drinking water. Alternatively, renal infarction hypertension was induced in place of DOCA hypertension.

In the second week following the initial surgery, BAPN was added to the diet for all rats. As with the earlier study of Hashimoto[50], surgical hypertension was initially induced via unilateral nephrectomy and augmented by DOCA administration; this was replaced by induction of hypertension via bilateral ligation of the posterior branches of the renal artery bilaterally.

Regardless of methodology, all rats were made hypertensive and fed BAPN. They were then divided into three cohorts; those with no carotid ligation, those with unilateral carotid ligation, and those with bilateral carotid ligation. No aneurysms were found in rats without carotid ligation. Aneurysms developed at the anterior communicating artery/anterior cerebral artery junction and ipsilateral proximal PCA in those rats who underwent unilateral ligation, and aneurysms were found exclusively in the posterior circulation of those rats which underwent bilateral carotid ligation. These findings appeared to confirm earlier hypotheses on aneurysm development; namely that abnormal haemodynamic stress in the Circle of Willis is of primary importance in aneurysm induction and formation, and that in surgically manipulated models, aneurysms preferentially occurred at the sites where haemodynamic stresses were expected to increase.

The observed locations of aneurysm formation represented the expected areas of maximal haemodynamic stress. Similarly, rats having undergone bilateral ligation demonstrated preponderance to aneurysmal development in the posterior circulation, again in keeping with the expected increase in haemodynamic stress. Furthermore, aneurysms were invariably induced on the side contralateral to carotid ligation, in keeping with the intended haemodynamic manipulation.

These findings appeared to support previous hypotheses regarding aneurysm pathogenesis in man, where an increased incidence of aneurysm formation on the anterior communicating artery was observed in patients with inequalities in the proximal segment of the anterior cerebral artery. This anomaly resulted in formation of a considerable shunt of blood across the anterior communicating artery to the contralateral anterior cerebral artery, thus subjecting the small anterior communicating artery to increased haemodynamic stress. Similarly, human aneurysms had been observed in other vessels under increased haemodynamic stress, such as those associated with arteriovenous malformations[51] and anatomical anomalies such as agenesis of the internal carotid artery[52].

This study was important for a number of reasons. Firstly, it demonstrated that aneurysms were inducible in experimental animals. Its primary advantages over previously described models, as reported by the authors, were the apparent ease with which aneurysms were cultivated in a large number of rats, and the apparent similarities in anatomy and natural history with human aneurysms. Most of the induced aneurysms arose at the apex of arterial bifurcations. Gap formation and leukocyte adhesion was observed at the endothelial cell junction, similar to previously reported findings of human studies[53]. Some observed morphological characteristics, such as aneurysmal bleb formation which were associated with an increased risk of aneurysm rupture have subsequently been identified as independent risk factors for aneurysmal subarachnoid haemorrhage in humans[54-57].

The procedure for aneurysm induction described in this model have been largely superseded by more modern methods. However, the study was notable for the apparent ease in which aneurysms were induced and the relatively high number of aneurysms produced. By pharmacologically augmenting surgically induced hypertension, aneurysms were induced in 30-70% of animals within three or four months. This was a significant improvement on previous models using carotid ligation alone, which often required an incubation period of 12 months or more[41]. In addition, the aneurysms induced were similar in histological analysis and natural history to human cases, allowing an early perspective on the dynamic aspects of aneurysm pathophysiology “as a living and metabolising structure”.

Pharmacological facilitation of aneurysm induction

Induction of aneurysms using toxic materials to degrade the arterial wall in both intracranial and extracranial arteries has been described. Mc Cune et al[29] designed a model of abdominal aortic dissection in dogs involving injection of nitrogen mustard beneath the aortic adventitia. White et al[30] adapted this method to the intracranial arteries using hypertonic saline, hyaluronidase and nitrogen mustard directly into the walls of the intracranial arteries of dogs with varying results. Troupp et al[58] described a similar technique focused on the external carotid artery in a rabbit model. Although reasonably successful, the lesions produced were not histologically comparable to human aneurysms.

On the hypothesis that increased haemodynamic stress on weakened cerebral arteries might induce structural fragility in the arterial wall, Hashimoto et al described cerebral aneurysm formation in a rat model[1]. This model relied on multiple factors for aneurysm induction.

BAPN was used to induce structural fragility in the arterial wall. Whilst the authors accepted its administration was not essential for aneurysm induction, it significantly increased the number of aneurysms cultivated. Handa[31] declared that administration of BAPN alone without carotid ligation was insufficient for aneurysm induction, confirming the earlier work of Hashimoto[40]. BAPN administration was again demonstrated to significantly increase the yield of aneurysms formed.

The yield of well-formed, saccular aneurysms induced in animal models which omit BAPN is less than 30%[31, 59, 60]. Hashimoto and others cautioned that the tendency of BAPN to cause pathological changes in the arterial wall may confound any histological analysis of CA development, leading the authors to recommend its use only in models where a high incidence of CA formation was required, such as haemodynamic studies.

Hypertensive Models

Methods of induction of hypertension

Unilateral common carotid artery ligation, administration of BAPN, subcutaneous DOCA injection, and salt loading in drinking water are common methods of inducing hypertension in animal models. This strategy was first reported by Hashimoto et al[39, 50, 61, 62]. This model was hampered by its relatively low incidence of CA formation (n=11/30) and long incubation period (averaging 11-21 weeks). Subsequent variations added ligation of the posterior branches of the renal arteries bilaterally to induce hypertension[39] improving the yield of CAs induced (n=18/13) and shortening the incubation time to 16 weeks.

Jamous et al[63, 64] augmented the model further by using oophorectomy in an attempt to increase the aneurysm yield. Ligation of the right CCA and posterior branches of the bilateral renal arteries of 7-week old Sprague-Dawley rats was performed, with initiation of hypertension via salt loading in the drinking water one week later. Oophorectomy was performed at a second operation one month after the initial procedure. The incidence of aneurysm formation was 60% over an incubation period of three months in rats who underwent oophorectomy compared with 20% of rats who underwent an identical initial procedure without oophorectomy. In addition, the size of aneurysms produced in the oophorectomy cohort was significantly larger.

This concept of exposure to and/or modification of environmental risk factors to produce aneurysms in animal models was further developed by Abruzzo[36] et al in endothelial nitric oxide synthase-3 (eNOS-3 knockout) mice. This study reported induction of aneurysms in two of six eNOS-3 knockout mice after an average survival period of 20.4 months (+/- 1.5 months) post-surgical ligation of the left common carotid artery. The same study failed to induce any aneurysms in thirty wild-type, 7 NOS-2 knockout, or 8 plasminogen activator inhibitor (PAI)-1 female mice using the same technique after a similar survival period.

The Blotchy mouse phenotype exhibits one of a series of mutations at the X-linked "mottled" locus. In male hemizygotes, the different allelic variants result in various phenotypes with connective tissue disorders of varying severity[65]. Male Blotchy mice have a propensity to develop extracranial aneurysms (especially of the ascending aorta)[66]. Using common carotid ligation, a low yield of aneurysm development in normotensive Blotchy mice was reported by Coutard et al[67]. The aneurysms produced were described as "a close succession of small dilatations in arteries of the complex of the anterior artery and anterior communicating artery", with no macroscopic aneurysms seen. Ligation of the left common carotid artery alone similarly failed to induce macroscopic aneurysm formation in a normotensive wild-type control group. The authors compared this with both wild-type and blotchy mice which had undergone renal artery ligation to induce hypertension. A significantly increased incidence of CA formation in the blotchy mice was seen.

The Jamous model substituted administration of BAPN for oestrogen deficiency to increase the incidence of aneurysm formation. This model demonstrated that oophorectomy and oestrogen deficiency led to significantly increased yields of aneurysms in rats, consistent with previous epidemiological human studies identifying female sex, especially in post-menopausal years, as a significant independent risk factor for de novo aneurysm development [47, 68, 69] and supporting the hypothesis of a protective role of oestrogen against the development and progression of CAs.

Stereotactic Elastase Models

Degeneration or disruption of the internal elastic lamina of intracranial arteries is a recognised component of the pathophysiological process of aneurysm development. Histopathological analysis of aneurysm walls often exhibits a continuum of degeneration ranging from almost intact to severely degenerate in the neck to fundus direction[70]. These degenerate changes exhibit a decrease in the number of mural cells, specifically VSMCs, and gaps in the medial raphe filled with tendon-like fibres. The aneurysm wall itself typically lacks an internal elastic lamina. Human studies suggest that serum elastase and collagenase levels are elevated in patients with aneurysms[71-74]. Histological studies of

collagenase and elastase activity in aneurysm walls demonstrate high levels of MMP-2 and -9 activity[74] in both human and animal models[75].

Elastase and collagenase are the major proteinases present in the aneurysm wall, and are known to be produced by macrophages in other vascular pathologies such as Abdominal Aortic Aneurysm (AAA) [76-78]. Degradation of the Extracellular Matrix (ECM) within the vessel wall leads to further damage via further upregulation of proteinases. MMP-9, in addition to its proteinase activity, is also a regulator of macrophage migration and infiltration across the ECM[11, 79], Some studies suggest that inhibition of proteinase activity may slow or suppress the development of aneurysms[75].

Building on these findings of degeneration and disruption of the elastic lamina being key characteristics of cerebral aneurysm pathogenesis, Nuki et al[14] developed a small animal model of aneurysm induction via a single stereotactic injection of elastase into the basal cisterns of hypertensive mice. The aneurysms produced “recapitulated the key features of human intracranial aneurysms”.

Elastase was used to induce disruption of the internal elastic lamina, and hypertension induced via a continuous infusion of Angiotensin-II via an implanted subcutaneous pump. Using a dose of 35 milliunits of elastase with continuous infusion of Angiotensin-II at a rate of 1000ng/kg/min, aneurysms were induced in 77% (n=34 of 44) of mice along the Circle of Willis or one if its major branches. The authors demonstrated a dose-dependent relationship between aneurysm induction and elastase concentration, demonstrating yields of 0%, 10%, 30%, and 77% of aneurysms using concentrations of 0.0 (i.e. placebo), 3.5, 17.0, and 35.0 milliunits of elastase respectively. Histological assessment of the CAs produced revealed varying degrees of structural pathology similar to that reported in histological studies of human aneurysms; degenerate vascular walls with thick segments and loss of the internal elastic lamina.

This method of aneurysm induction demonstrated a dose-dependent relationship between the incidence of aneurysm formation and the concentration of the Angiotensin-II infusion. Higher yields of aneurysms were seen in mice receiving a more concentrated volume (0%, 20%, and 77% at 0 (PBS infusion), 500 or 1000 ng/kg/min Angiotensin-II respectively). The magnitude of hypertension induced appeared to be dependent Angiotensin-II concentration (111.0 +/- 6.7, 127.0 +/-18.8, and 142.0 +/- 37.0 mmHg at 0 (PBS infusion), 500 or 1000 ng/kg/min Angiotensin-II respectively), supporting the hypothesis underpinning this model; that exaggeration of known risk factors (in this case hypertension) may lead to CA formation.

The combination of stereotactic injection of elastase and Angiotensin-II induced hypertension represented an important new small animal model of CA induction. Nuki demonstrated the combination of hypertension and degeneration of the internal elastic lamina induced by a single stereotactic injection of elastase into the cerebrospinal fluid resulted in a high incidence of aneurysm formation in a comparatively brief period. By modifying the stereotactic co-ordinates (to 1.2mm rostral, 0.7mm lateral to bregma, and advancing the stereotactic injection needle to 0.3mm from the skull base), Hosaka et al[10] achieved even higher yields of aneurysms, inducing macroscopic aneurysm formation of 100% of mice receiving elastase doses of 5, 10, or 20ul of 10u/ml elastase solution, and in 90% of mice receiving 10uL of 1.0u/mL elastase solution (n=10 each cohort). The Hosaka model was the first murine model to consistently induce aneurysm rupture in a dose-dependent manner.

Rationale for this project and model chosen.

The aim of this study was to develop an animal model of the changes associated with the early aneurysm pathogenesis.

We sought to build on previous studies, using a contemporary model of murine aneurysm induction; the model described by Nuki[14] was chosen as our template. The methods used in our study differed slightly from those used in prior studies, for example, we used lower elastase doses to ameliorate the severity of vessel wall injury. Notwithstanding, the dose, rate and mode of human angiotensin II delivered in our study and magnitude of hypertension achieved, conformed to that of prior studies.

Methodology

Approval for animal studies was obtained from the local ethics committee (Approval no. A1926). Experimental work performed in accordance with the institutional and ethical guidelines of James Cook University, Australia, and conforming to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA).

20 male C57/BL6 mice aged 12-14 weeks, of mean weight 25.5±0.4g, were used. Under isoflurane general anaesthesia (2% isoflurane in 2L/minute oxygen), mice were secured in a stereotactic frame. A small right sided burr-hole was made 1.2 mm rostral and 0.7 mm lateral to the bregma. A 10µl Hamilton blunt tipped microliter syringe was adjoined to a semi-automated repeating dispenser and advanced until contact with the skull base was achieved, then withdrawn 0.3 mm and either methylene blue dye (control group) or elastase solution (experimental group) was infused into the basal cisterns. The incision was then closed with sutures.

To induce chronic hypertension, a constant infusion of Angiotensin-II (Angiotensin-II), dissolved in phosphate buffered saline, was continuously infused via an implanted micro-osmotic pump (placed

subcutaneously at a point 5 mm cephalad to the base of the tail immediately after closure of the cranial wound. The Angiotensin-II dose was determined by use of a weight-based algorithm delivering 1000 ng/kg/min [12, 14, 80, 81]

Two study groups were employed:

1. Sham operative controls

A dilute solution of methylene blue dye was infused into the right basal cistern of 5 mice at a rate of 2µl/min using the stereotactic method described above. Induction of hypertension via Angiotensin-II was not used via was not used in this group.

2. Experimental group

10µl of elastase solution (1.0u/ml) was injected manually into the right basal cistern of 15 mice at a rate of 2µl/min using the stereotactic method described above.

Following the procedure, animals were recovered under a heat lamp, housed individually, and observed for a period of 2-3 weeks using a previously validated rodent neurological scoring system[82, 83]. Blood pressure (BP) was measured by standard tail cuff manometry at 3 time points. At the completion of the observation period, each mouse was euthanized using CO2 and the entire head immersion-perfused in 10% formaldehyde for a period of 14 days.

Results

28 arterial bifurcations from 12 brains were examined. Massive sub-arachnoid haemorrhage was observed in 1 mouse. Formation of a macroscopic aneurysm without SAH was observed in 1 mouse. Pre-aneurysmal changes were observed in 8/12 (58%) brains: Endothelial change in 8/8 and Internal elastic lamina degeneration in 6/8. Hypertension was successfully induced in a time-dependent

manner. All 5 sham controls survived both the procedure and the observation period. Two of the 15 experimental mice died in the immediate post-procedural period. Four of the remaining 13 experimental died during the observation period (17-21 days).

Twelve brains from the 15 experimental mice were examined for histological changes associated with pre-aneurysm formation. In the experimental group, where n=28 bifurcations were harvested from n=12 brains, pre-aneurysm changes were observed in 7/12 (58%) brains, amongst 12/28 (43%) bifurcations. Internal elastic lamina degeneration was specifically observed in n=8. All bifurcations with internal elastic lamina degeneration also showed endothelial change. N=1 macroscopic aneurysm was identified at one bifurcation point (fig 3)

Of the sub-group of n=4/13 mice which died during the observation period (17-21 days), endothelial change was observed in n=3, whilst concomitant internal elastic lamina degeneration was observed in n=1 bifurcation.

Description of our model

Technique

Mouse model

Approval for animal studies was obtained from the local ethics committee (Approval no. A1926). and experimental work performed in accordance with the institutional and ethical guidelines of James Cook University, Australia, and conforming to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA). Mice were housed in an individually ventilated, temperature/humidity-controlled cage system (Aero IVC Green Line; Tecniplast) on a 12-hour light/dark cycle and maintained on normal laboratory chow and water *ad libitum*. 20 male C57/BL6 mice (Animal Resources Centre, Canning Vale, WA, Australia) aged 12-14 weeks, of mean weight

25.5±0.4g, were used. Under isoflurane general anaesthesia (GA) (2% isoflurane in 2L/minute oxygen), mice were secured in a stereotactic frame (Model 940 Linear Scale Digital Display, David Kopf instruments, Tujunga, CA, USA). A small right sided burr-hole was made 1.2mm rostral and 0.7mm lateral to the bregma (co-ordinates were obtained from the Mouse Brain Atlas[84] and had been validated by a previously described model[10]). A 10 µl Hamilton blunt tipped microliter syringe (Model 701N, Hamilton Instruments, Nevada, LV, USA) was adjoined to a semi-automated repeating dispenser (Model PB600-1, Hamilton Instruments, Nevada, LV, USA) and advanced until contact with the skull base was achieved. It was then withdrawn 0.3 mm and either methylene blue dye (control group) or elastase solution (experimental group) was infused into the basal cisterns (see below). The incision was then closed with sutures.

Two study groups were employed:

1. Sham operative controls

A dilute solution of methylene blue dye was infused into the right basal cistern of 5 mice at a rate of 2µl/min using the stereotactic method described above. Hypertension was not induced in this group

2. Experimental group

10µl of elastase solution (1.0u/ml) was injected manually into the right basal cistern of 15 mice at a rate of 2µl/min using the stereotactic method described above.

Following the procedure, animals were recovered under a heat lamp, housed individually, and observed for a period of 2-3 weeks using a previously validated rodent neurological scoring system[82, 83].

Mice were removed from the stereotactic apparatus and recovered under an infra-red heat lamp.

Once ambulant and feeding, and in the absence of any perceived neurological deficit, the mice were transferred to individual cages.

Operative time (i.e. from induction to completion of GA) for sham controls was 31 ± 4 mins. Total procedural time (i.e. from induction of GA to observable recovery of spontaneous movements) for sham controls was 45 ± 5 mins. Total procedural time for the experimental cohort was 60 ± 8mins: operative time was 34 ± 3.0 mins and recovery time 14 ± 2 mins.

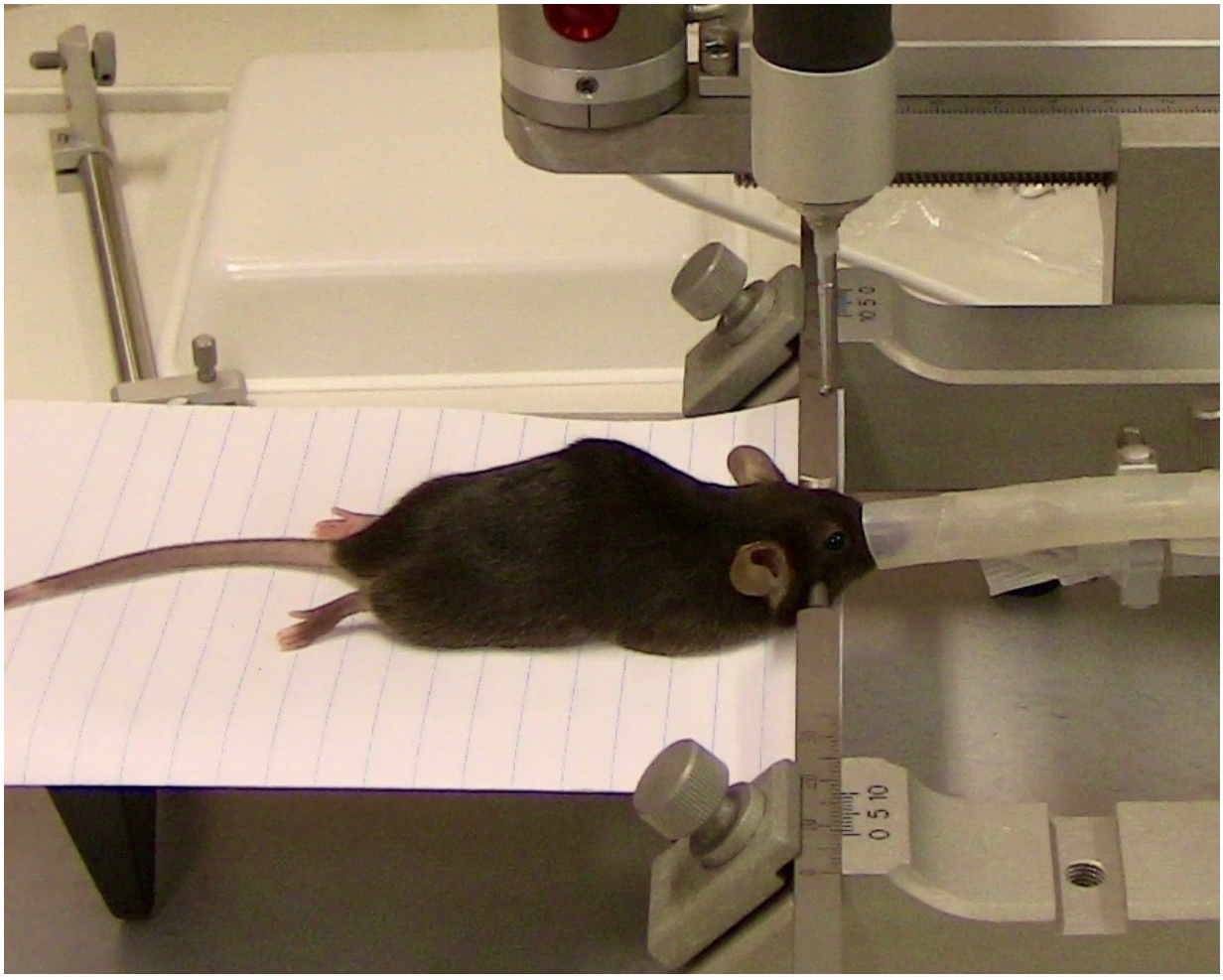

Figure 1. Placement of the mouse in the stereotactic apparatus. Prior to the surgical component of the procedure, the eyes were lubricated and taped shut and the surgical site prepared with an antiseptic solution. The mouse was covered with a warming blanket.

Induction of Hypertension

To induce chronic hypertension, a constant infusion of A-II, (Sigma Aldrich, Castle Hill, NSW, Australia), dissolved in phosphate buffered saline, was continuously infused. This was achieved via an implanted Alzmet micro-osmotic pump (model 1004, Durect Corporation, Cupertino, CA 95014) which was placed subcutaneously at a point 5mm cephalad to the base of the tail immediately after closure of the cranial wound. The A-II dose was determined by use of a weight-based algorithm delivering 1000ng/kg/min

Blood pressure (BP) was measured by standard tail cuff manometry at 3 time points: Baseline (24 hours pre-intervention); interim (8-10 days post-procedure); and final at 24 hours prior to euthanasia. Systolic (sBP), diastolic (dBP) and mean (mBP) were recorded.

Post-Procedural Observations

In the post-procedural period mice were housed individually and observed for a period of between two to three weeks, during which time they were checked twice daily. Each mouse was inspected visually for general condition and observations recorded using a previously described 5-point neurological scoring system (grade 0, normal function; grade 1, reduced eating or drinking activity demonstrated by weight loss >2g); grade 2, flexion of the torso and forelimbs upon lifting the whole animal by the tail; grade 3, circling to one side with normal position at rest; grade 4 leaning to one side at rest; grade 5, no spontaneous activity or death)[82, 83, 85-87].

Mice were observed for normal behaviours such as climbing, response of vibrissae to touch, and general activity such as spontaneous feeding and drinking. In addition to the above system, a neurological deficit was noted if an animal exhibited asymmetric limb movements, asymmetry of forepaw stretching when being held by the tail, or a slow/asymmetric reaction to vibrissae stimulation. None of the mice surviving the pre-defined incubation period exhibited a new-onset neurological deficit or signs of pain or distress during observation.

The surgical incisions were assessed twice daily. All incisions healed within the first three to four days, with no wound infection or dehiscence encountered. All the micro-osmotic pumps remained in situ for the duration of the observation period. The surgery appeared well tolerated by the mice during the healing period, with no signs of irritation such as excessive grooming, scratching or excessive loss of fur surrounding the incisions observed. The procedure was performed on a total of four cohorts of five mice each (n=20).

At the completion of the observation period, mice were placed under deep isoflurane GA (flow rate 4L/min) until agonal breathing was observed. Each mouse was then euthanized using CO2. The entire head was thereafter removed and immersion-perfused in 10% formaldehyde for a period of 14 days.

Necropsy

Removal of mouse brain from cranial vault.

This procedure was performed under x 3.5 loupe magnification using standard bench illumination. If during the dissection there was uncertainty regarding anatomical landmarks, or the plane of dissection became unclear, the remainder of the procedure was performed under x10 light microscopy. Dissection was performed using standard microsurgical instruments and technique.

The mouse head was removed from the 10% formaldehyde perfusion, and excess fluid removed with an absorbent tissue. A heavy arterial clamp was applied to the loose skin around the nose. Using a size 22 blade, a dorsal sagittal incision was made from the tips of the clamps to the back of the skull. The skin was reflected anteriorly, and soft tissue and musculature removed.

The skeletonised head was then placed supine. The calvarium was removed in a piecemeal fashion. Care was taken not to damage the underlying brain when crossing suture lines and on removal of the frontal and nasal bones due to increased thickness of the bone at these locations.

The base of the brain could now be visualised, and the skull base now removed using similar piecemeal technique. The basilar artery and posterior aspects of the temporal lobes served as useful landmarks when performing this stage of the procedure, and the utmost care was taken not to inadvertently damage the ventral surface of the brainstem.

Owing to the entry points of the internal carotid arteries and exiting points of the cranial nerves, removal of the remainder of the skull base proved to be the most technically challenging part of the procedure. In approximately half of the skulls this was best achieved by the use of gentle retraction of the brain from the skull base and careful division of the arteries and vessels close to the bone using a fine microsurgical scissors, working in a caudad-cranial direction.

For the remainder of the brains, application of this technique threatened to cause compressive deformity of the frontal lobes. Brains which exhibited this phenomenon were significantly less pliant than their counterparts. Why this occurred was unclear.

Average procedural time was 37 minutes respectively. Upon removal of each brain, arteries of the circle of Willis and their major bifurcation points were inspected under both x3.5 loupe and x10 light microscopy magnification, photographed, placed in fresh containers of 10% formaldehyde and stored at room temperature pending formal histological processing. All major bifurcation points visualised during dissection were processed, however owing to fragility of the specimens a variable number of bifurcations were harvested from each brain.

Statistical Analysis

Inter-group comparisons were compared using analysis of variance. Statistical significance was assessed at $P<0.05$.

Results

Of 20 mice, 5/5 sham controls survived both the procedure and the observation period. Two of the 15 experimental mice died in the immediate post-procedural period, presumably due to the stress of the procedure. Four of the remaining 13 experimental died during the observation period (17-21 days). The cause of death was not readily apparent in three of these mice. Three cerebral artery bifurcations were harvested from two of these mice. The remaining mouse was found to have suffered a massive SAH (fig. 3): owing to the severity of tissue disruption incurred, no viable bifurcations could be harvested; this specimen was excluded from analysis. Mortality was thus n=6.

Hypertension

Hypertension was successfully induced in a time-dependent manner. All mice underwent acclimatisation to tail-cuff plethysmography in the pre-operative period by performing a single daily reading over the course of three days. Ten pre-operative, interim and pre-euthanasia systolic blood pressure (sBP) measurements were taken per mouse, with the highest and lowest values excluded.

Pre-operative, interim and pre-euthanasia sBP was 91 ± 2.0 mmHg, 116 ± 2 mmHg and 157 ± 3 mmHg respectively ($p<0.001$).

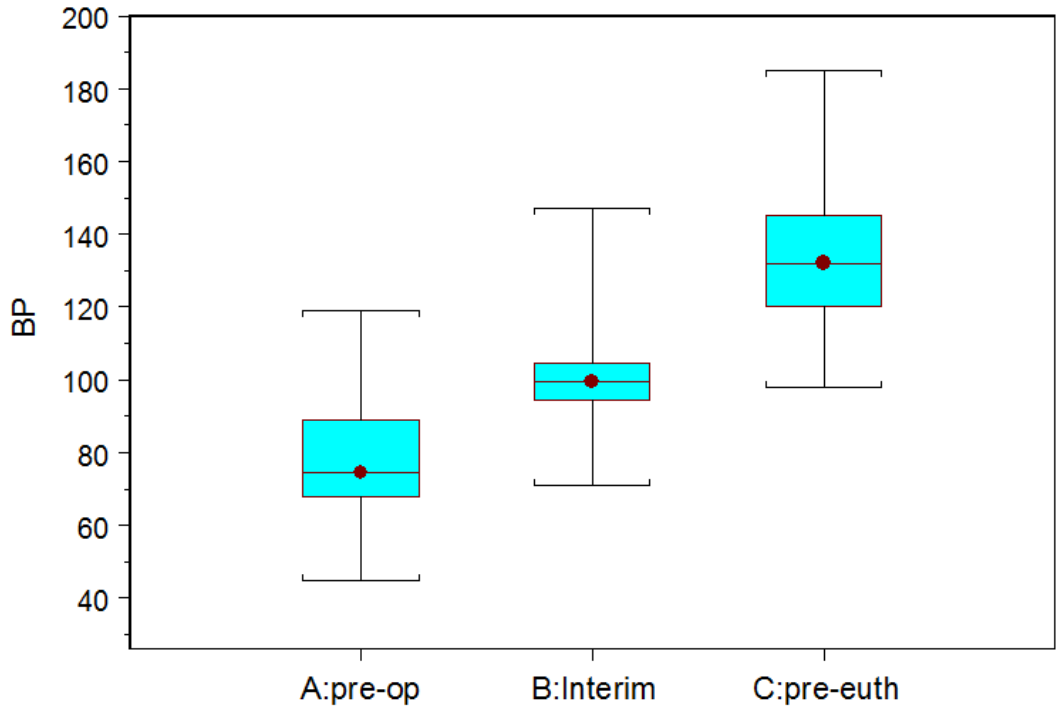

Figure 2. Preoperative, Interim and Pre-Euthanasia systolic blood pressure measurements in the experimental cohort

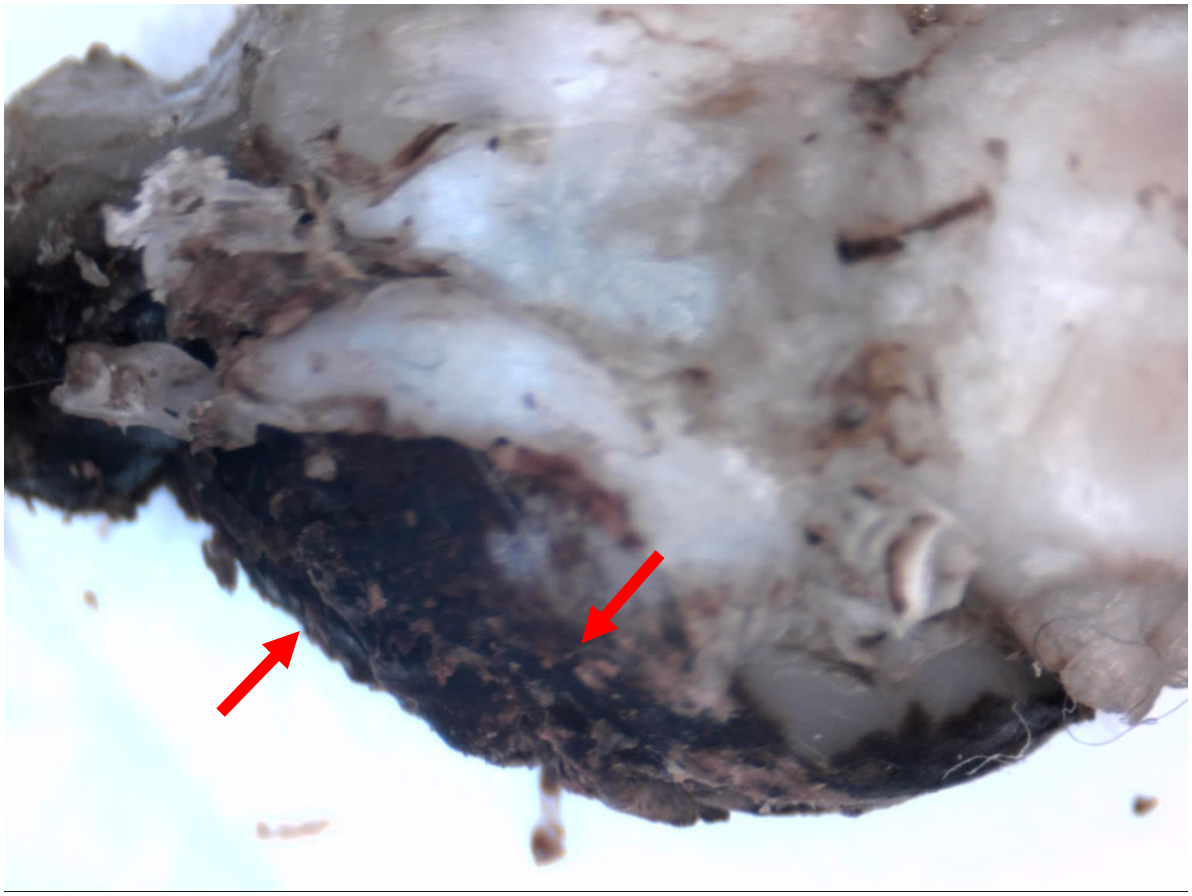

Figure 3. Formalin fixed specimen demonstrating a large volume haematoma in the subarachnoid space (red arrows) over the right brain convexity

Twelve brains from the 15 experimental mice were examined; the two mice that died during the procedure were excluded from this analysis. The brain of another mouse that died during the observation period was rendered unsuitable for analysis during processing and was also excluded. A total of 28 arterial bifurcations were harvested. One macroscopic cerebral aneurysm was observed (fig. 4).

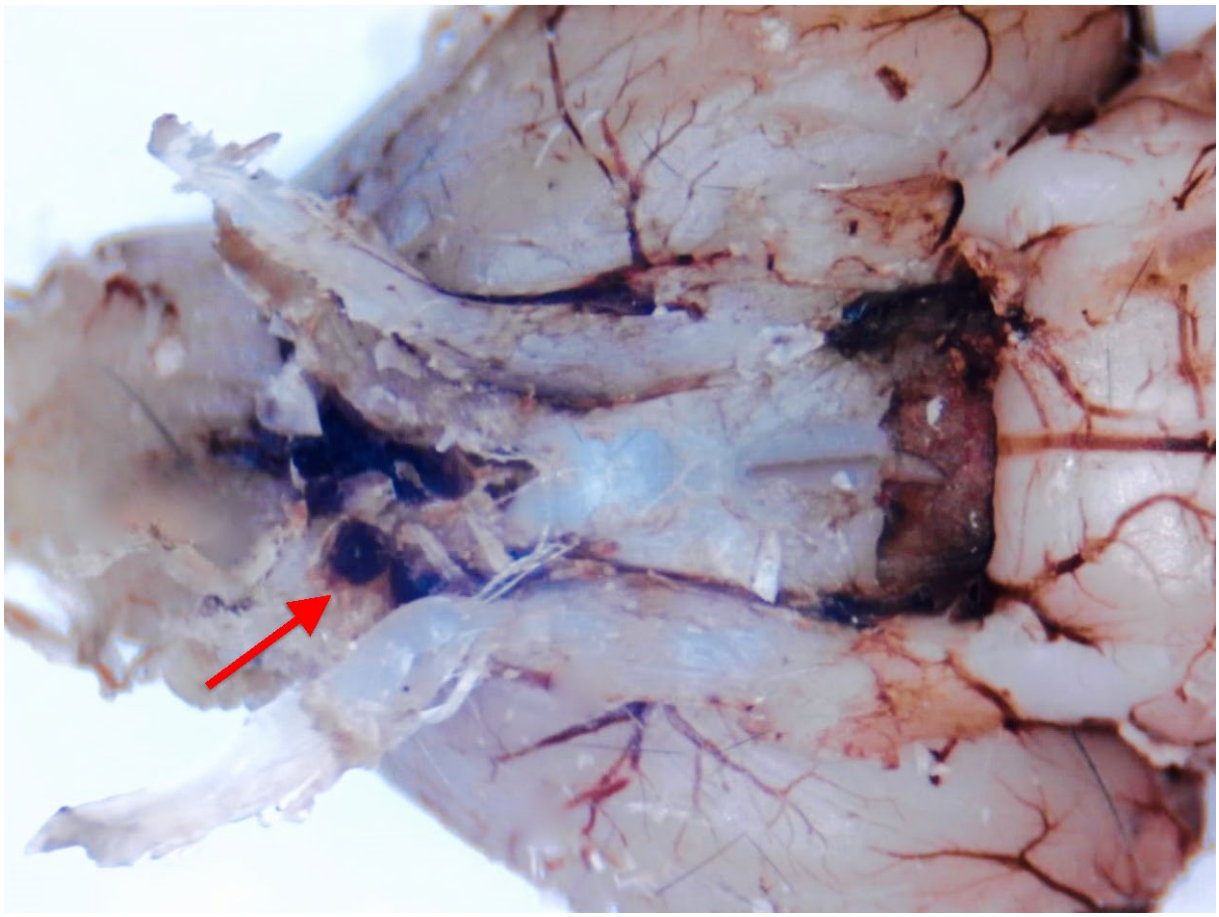

Figure 4. Unruptured macroscopic cerebral aneurysm. The aneurysm is observed in the anterior circulation (red arrow)

Histological assessment.

After removal of the brain from the vault, and prior to fixation, macroscopic aneurysm formation (i.e. a saccular aneurysm visible either to the naked eye, or under x3.5 magnification), and any associated SAH, were sought by direct vision. The brains were then fixed in 10% neutral buffered formalin. Following fixation, the brains were processed using a Leica Peloris tissue processor.

The total processing time was 87 h. Each stage, excluding the wax stages, was performed at 35°C. The tissue was kept under vacuum during processing. Following processing, the brain slices were blocked in paraffin wax and cooled on an ice plate. Blocked paraffin brains were sectioned at eight microns on a Leica RM2235 Manual Rotary Microtome. Sections were floated out on a water bath at 35°C,

collected on silanized activated slides and dried overnight. The tissue was kept under vacuum during processing.

All sections were examined, and arterial bifurcation points identified and analysed, by an experienced histopathologist blinded to the outcome of the index animal.

Macroscopic cerebral aneurysm formation (saccular cerebral aneurysms visible either to the naked eye, or under x3.5 magnification), and any associated SAH, were sought by direct vision or by dissecting microscopy. Macroscopically intact arterial bifurcation points were analysed by an experienced histopathologist (LK), blinded to the outcome of the index animal, for changes suggestive of early CA formation. Endothelial change was classified into three distinct stages as per the classification system proposed by Jamous et al[64, 88-90] (Table (2). We classified internal elastic lamina degeneration change using the classification system proposed by Aoki et al[91] (Table 3), in keeping with previous human and animal studies of aneurysm pathogenesis detailing progressive internal elastic lamina degeneration as a key feature of aneurysm pathogenesis[14, 92-95]

Analysis was limited to identification of endothelial change () (i.e. endothelial detachment, sub-intimal thickening, and inflammatory cell infiltration with or without arterial wall protrusion) and degeneration of the internal elastic lamina (internal elastic lamina degeneration).

endothelial change was classified into three distinct stages as per the classification system proposed by Jamous et al[89], with Stage 3 change representing macroscopic aneurysm formation, i.e.:

Categorisation of vessel wall endothelial changes		
Stage 1		Endothelial change only
Stage 2	a	Endothelial change with apical intimal pad elevation
	b	Late inflammatory change with destruction/protrusion of vessel wall
Stage 3		Macroscopic saccular aneurysm formation

Table 1: Histological categorisation of the vessel-wall endothelial change (EC) associated with early cerebral aneurysm (CA) formation. Modified after Jamous et al.[89]

Similarly, internal elastic lamina degeneration was classified using the system proposed by Aoki et al[91], which described internal elastic lamina degeneration relative to the surrounding elastic lamina (Table 3)

Categorisation of vessel wall Internal Elastic Lamina change	
Stage 1	Continuous
Stage 2	Fragmented
Stage 3	Complete disappearance

Table 3: Histological categorisation of vessel-wall internal elastic lamina degeneration (IELD) associated with early cerebral aneurysm formation. Modified after Aoki et al.[75]

Histopathological findings

In the experimental group, where n=28 bifurcations were harvested from n=12 brains, pre-aneurysmal changes were observed in 7/12 (58%) brains, amongst 12/28 (43%) bifurcations (figs 2 & 3). Type I change was observed in one bifurcation, Type 2a in n=9, Type 2b in n=2 and Type 3 (i.e. macroscopic cerebral aneurysm) in n=1. Internal elastic lamina degeneration was specifically observed in n=8. All bifurcations with internal elastic lamina degeneration also showed EC (figs 3, 4 & 5). One macroscopic cerebral aneurysm was identified at one bifurcation point (fig 4)

Of the sub-group of n=4/13 mice which died during the observation period (17-21 days), endothelial change was observed in n=3 (stage 1 in n=1, and Stage 2a in n=2), whilst concomitant degeneration of the internal elastic lamina was observed in one bifurcation. (Fig. 5).

One macroscopic aneurysm was identified at one bifurcation point (fig 3): seven bifurcations were harvested from this mouse. Both endothelial change-2b and internal elastic lamina degeneration-2

were observed in these bifurcations, whilst the internal elastic lamina degeneration-3 was observed within the aneurysm itself (fig 8,9).

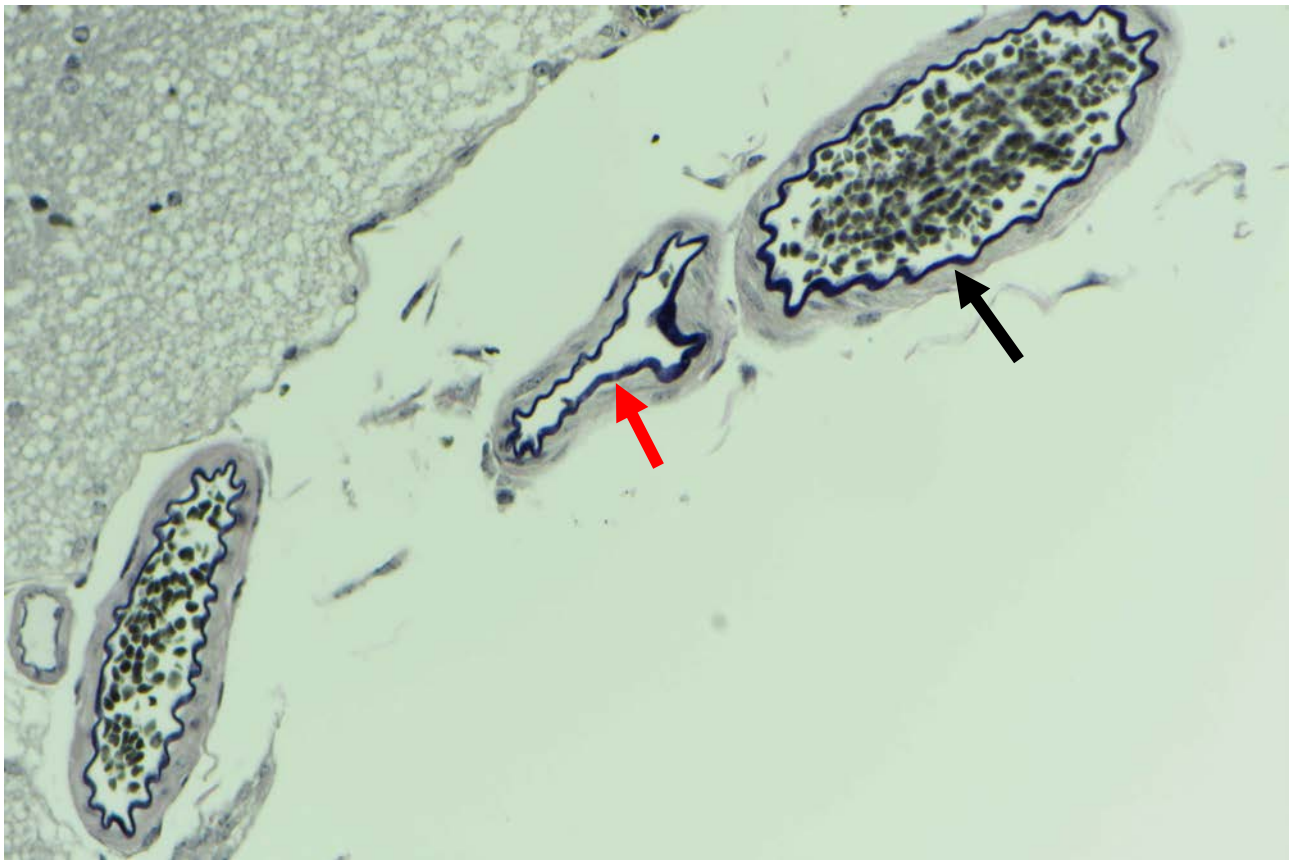

Figure 4. VerHoeff's Van Gieson stain is useful in demonstrating atrophy of elastic tissue. This slide shows EvG staining of a control vessel wall. The endothelium (red arrow) and internal elastic lamina (black arrow) are intact. The vessel is of normal thickness.

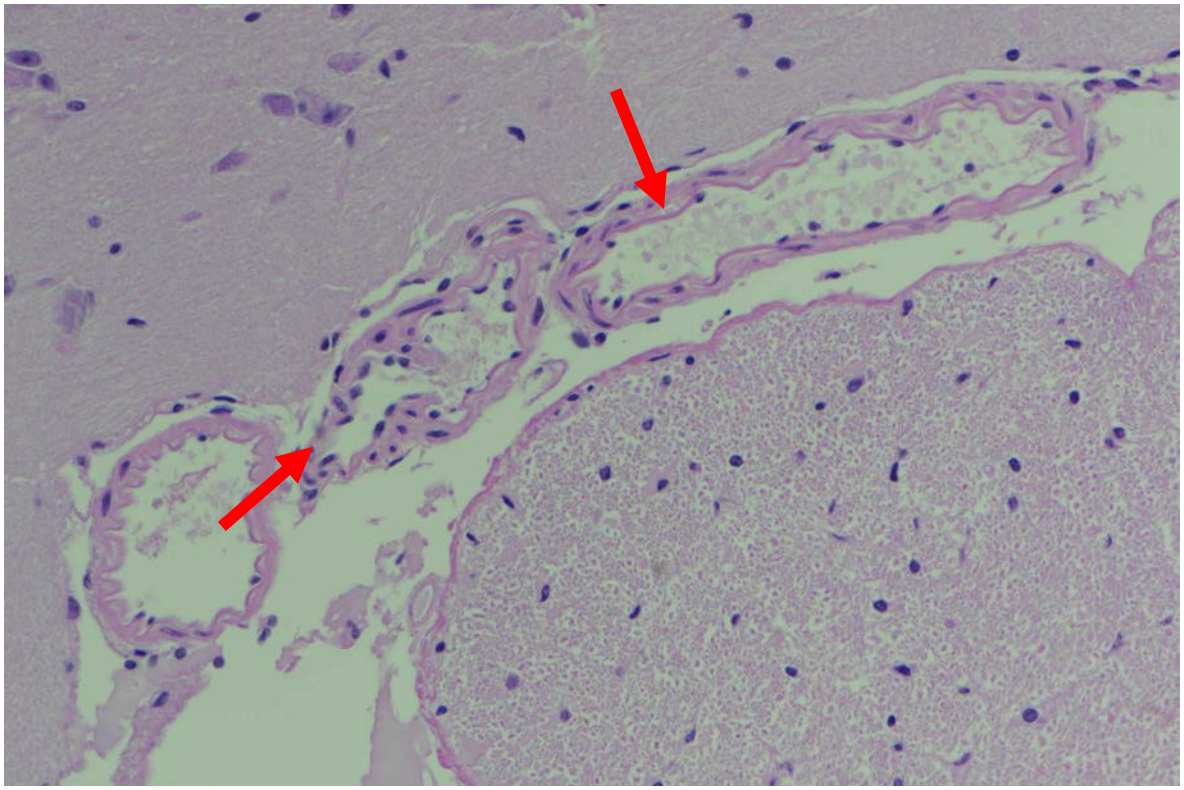

Figure 5. Haematoxylin and Eosin stain (H&E Staining) is a combination of two histological stains; haematoxylin and eosin. Haematoxylin stains cell nuclei blue, and eosin stains cytoplasm and extracellular matrix pink. This H&E stain of an arterial bifurcation demonstrates mild pathological features associated with early cerebral aneurysm formation (i.e. mild endothelial change and mild internal elastic lamina degeneration [internal elastic lamina degeneration]) on H&E staining, HP. Both red arrows depict loss of endothelium (endothelial change-1). See Table 1 and Table 2 for histological classifications.

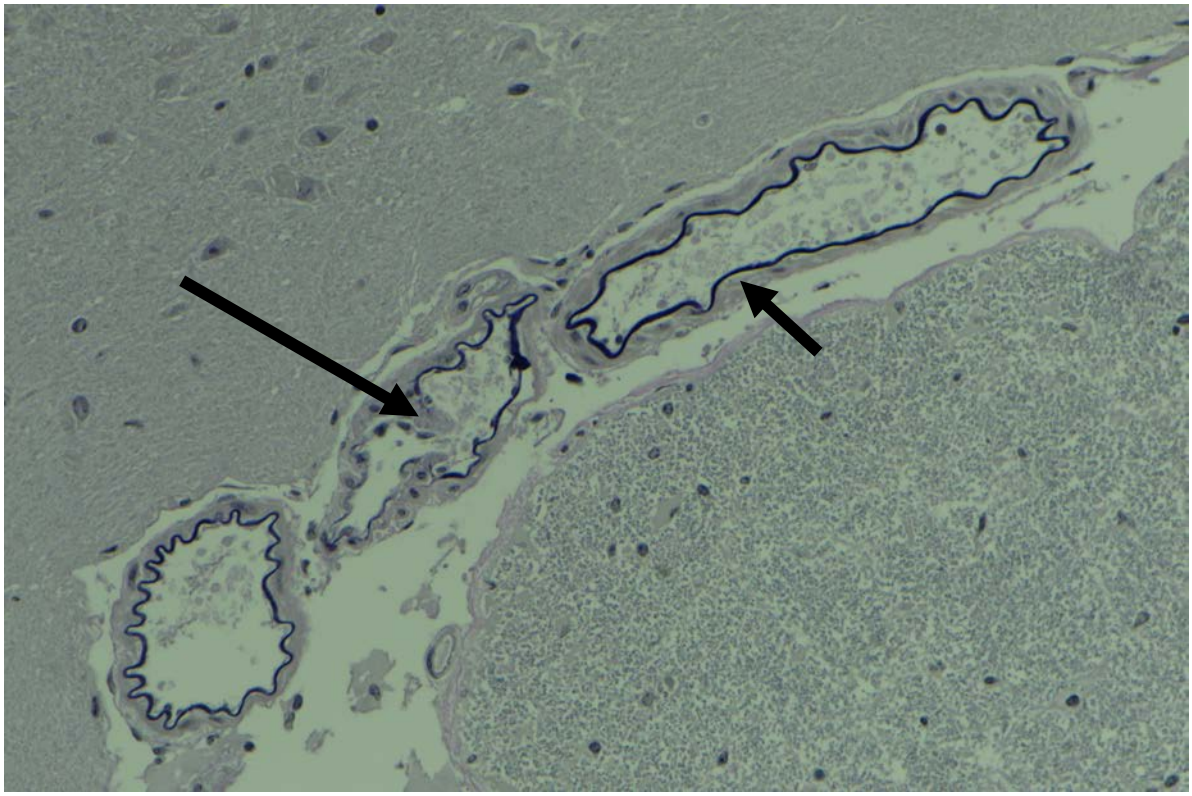

Figure 7. The same bifurcation point seen in Fig.6, this time stained with EvG. The slide shows moderate pathological features associated with early aneurysm formation. Example of internal elastica fragmentation ('Reuterwall's tear', IELD-3). Short black arrow depicts flattening of IEL corrugations (IELD-2). Long black arrow depicts IEL fragmentation ('Reuterwall's tear', IELD-3) combined with endothelial cell detachment and sub-intimal thickening/apical pad elevation (endothelial change-2A

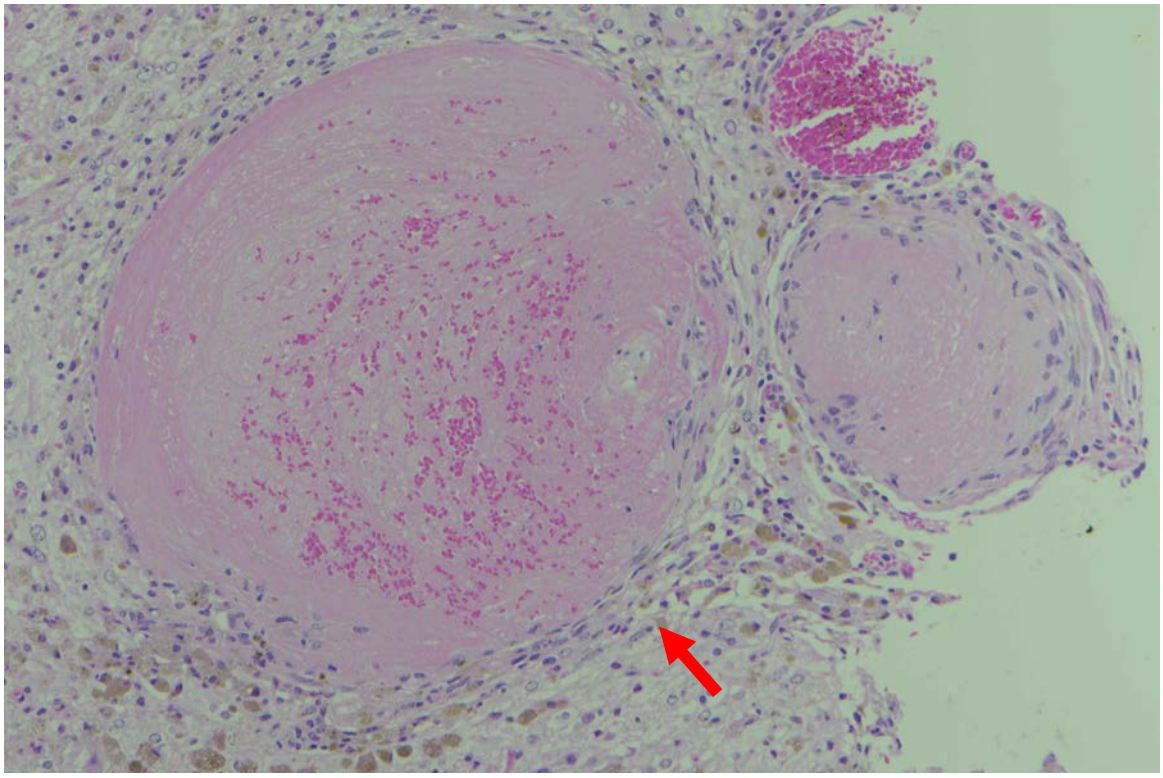

Figure 6. H&E staining of the thickened arterial wall (right) in the vicinity of a macroscopic aneurysm (left). There is extensive inflammatory infiltration and protrusion of the vessel wall (red arrow).

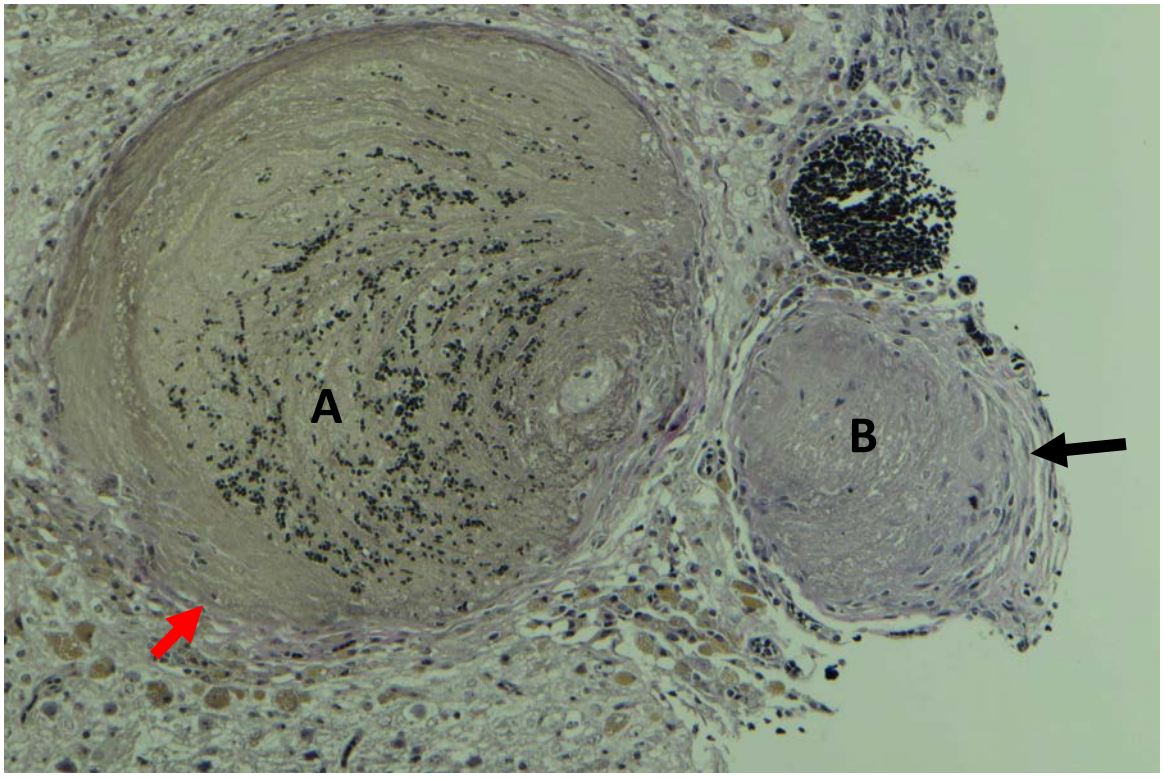

Figure 7. Severe pathological changes associated with macroscopic cerebral aneurysm formation on Silver staining: unruptured cerebral aneurysm (HP). There is complete absence of the media and internal elastic lamina (IELD-4) within the

unruptured aneurysm (A) wall, and replacement by amorphous connective tissue. Inflammatory cells are seen below the aneurysm (red arrow). The associated bifurcation (B) demonstrates an area of medial thickening (black arrow). The bifurcation B generally exhibits advanced endothelial change (endothelial change-2B) and advanced IELD (complete absence, IELD-4). See Table 1, 2 for histological classification, H&E = haematoxylin and eosin, HP = high power, IELD = internal elastica degeneration.

Of the sub-group of 4/13 mice that died during the observation period, two brains were processed. Endothelial change was observed in 2/2 (endothelial change-1, internal elastic lamina degeneration-2 in a mouse dying at Day 8/21, and endothelial change2a, internal elastic lamina degeneration-2 in a mouse dying at Day 6/21). One bifurcation harvested from one mouse that died in the immediate post-procedural period contained no abnormalities.

Of the n=12 brains successfully processed, one mouse showed macroscopic cerebral aneurysm formation, with pre-aneurysmal changes seen at the bifurcation points of n=7 mice. A total of n=28 arterial bifurcation points were harvested, showing varying degrees of degeneration and structural abnormalities. SAH without cerebral aneurysm was observed in n=1 brain. This sample showed massive disruptive parenchymal haemorrhage with subarachnoid extension, however on microscopy and histopathological analysis an obvious arterial aetiology was not visualized, and this brain was excluded from analysis. Macroscopic cerebral aneurysm without SAH was observed in one brain. The endothelium in this sample exhibited primarily type 2b change, with type 2 and 3 internal elastic lamina degeneration in the area of the cerebral aneurysm. The cerebral aneurysm wall was notably thinner than the surrounding arterial wall.

Pre-cerebral aneurysm changes were observed in n=12 bifurcations. Of these, Type I change was observed in n=1, Type 2a change in n=9, type 2b change in n=2 and Type 3 change (i.e. macroscopic cerebral aneurysm formation) in n=1. internal elastic lamina degeneration was specifically observed in n=8; all bifurcations with internal elastic lamina degeneration also showed endothelial change.

Mouse Number	Endothelial Change	Internal Elastic Lamina Degeneration	Bifurcations seen
1	None	IELD-1	2
2	EC-2a	IELD-2	1
	EC-2b	IELD-3	1
	None	IELD-1	1
3	None	IELD-1	3
4	None	IELD-1	3
5	EC-1	IELD-2	2
	EC-2a	IELD-3	2
6	EC-2b	IELD-3	2
7	EC-2b	IELD-3	2
	EC-2b	IELD-4	1
	EC-3	IELD-4	1
8	EC-2b	IELD-3	1
9	EC-1	IELD-3	1
	EC-2a	IELD-3	1
10	EC-2a	IELD-3	1
	EC-2b	IELD-3	2
11	EC-2a	IELD-3	1

Table 4. Pathological features associated with early cerebral aneurysm formation observed. Pathological features of early cerebral aneurysm formation were observed in 8 of 11 (73%) brains, and in 18 of 27 (67%) bifurcations. All cerebral artery bifurcations with internal elastic lamina degeneration (IELD) also demonstrated endothelial change (EC). Where EC was absent, IELD was also absent: i.e. in 3 mice (Nos. 1, 3 and 4) no endothelial abnormalities were found amongst the bifurcations harvested

Discussion

Prior studies have successfully incorporated systemic hypertension and intracranial elastase infusion to produce cerebral aneurysm formation in rodents [7, 8, 10-14]. The cerebral aneurysms formed in such studies have typically been rapidly produced: i.e. within a period of 2-6 weeks. This questions their representativeness with regard to many, if not most, human cerebral aneurysms observed clinically. The studies of Hassler [25, 53, 96-98] and others [99, 100] [101] have previously suggested that most cerebral aneurysms initially develop from vessels with pre-aneurysmal changes; and that, in many such vessels, pre-aneurysmal change leading to cerebral aneurysm leading to subarachnoid haemorrhage may be delayed [16, 46, 102].

A further feature of current models is that the sizes of the cerebral aneurysms so formed have been relatively large. This further questions their representativeness to human cerebral aneurysm pathogenesis since numerous clinical studies have shown that most cerebral aneurysms, either those that remain unruptured for variable periods of time, or those that present acutely with rupture and subarachnoid haemorrhage, are relatively small in size [23, 103]. Moreover, disparately large cerebral aneurysm size may also, at least in part, explain a further aberrant feature of extant murine models: i.e. that all exhibit a high incidence of rupture [2, 7-11]. Both ISUIA I and ISUIA II demonstrated that cerebral aneurysm rupture risk is correlated with aneurysm size[15] .

We therefore sought to produce pre-aneurysmal changes, to more closely mimic the slower time course presumed in cerebral aneurysm development [16, 46, 102]. Such a model would therefore be more appropriate for the investigation of factors which either stimulate or inhibit pre-aneurysmal development in previously normal vessels, or which stimulate or inhibit subsequent pre-aneurysmal → cerebral aneurysm development (± rupture). Extant models have hitherto not been structured to address each of these sequences separately.

The methods used in our study therefore differed slightly from those of prior studies: in particular, regarding both the amount, and mode, of elastase delivered. Whilst previous studies have incorporated a micro-infusion pump to deliver elastase; we, by contrast, incorporated a semi-automated dispenser and microliter syringe to manually deliver elastase into the right basal cistern. The efficacy of latter had been proven in our pilot study (subsequently used the control arm in our main study) in which we substituted methylene blue for elastase. Furthermore, the dosage of elastase administration administered in our study was also at the lower limit of that formerly used by Nuki [14]. The manual technique used in our study removed some of the need for specialised equipment and training which would otherwise have been required.

The higher doses of elastase used in prior studies[14] certainly achieved a higher incidence of cerebral aneurysm formation than in our study. Although Hosaka et al[10] reported a stereotactic elastase/Angiotensin-II mouse model of cerebral aneurysm induction female C57/BL6 mice using identical incubation times, stereotactic co-ordinates and elastase and Angiotensin-II dosages to our study, their model also incorporated renovascular hypertension and common carotid artery ligation. The exaggeration of the haemodynamic insult required for the initiation of such accelerated cerebral aneurysm pathogenesis may, as previous authors have themselves indeed acknowledged [14], 'skip' key events in the sequence [normal artery → pre-aneurysmal → cerebral aneurysm ± SAH] which may play a key role in clinical cases. Notwithstanding, the dose, rate and mode of human angiotensin II delivered in our study (0.11µl/h, subcutaneously) conformed to that of prior studies.

In our study, n=15/15 brains were harvested, of which 12/15 could have feasibly yielded pre-aneurysmal or cerebral aneurysm changes (i.e. from mice which had survived beyond the total procedural time*).* From these n=12 brains, n=28 arterial bifurcations were harvested. Massive sub-arachnoid haemorrhage without cerebral aneurysm formation was observed in one brain: unfortunately, due to massive tissue disruption, neither cerebral aneurysm, nor any bifurcations, could be harvested here. One macroscopic saccular cerebral aneurysm without subarachnoid haemorrhage

(fig 4) was observed in one other case. Taken together, these results suggest that, despite having used lower doses of elastase, the sequence [normal artery → pre-aneurysmal → cerebral aneurysm ± SAH] may still remain accelerated. Hypothetically, by lowering the dose of elastase further still, the sequence progression [normal artery → pre-aneurysmal → cerebral aneurysm ± SAH] may be further retarded.

The role of hypertension and haemodynamic stress on cerebral vessels was first investigated by Forbus[99] who demonstrated haemodynamic pressure to be maximal at arterial branch points, correlating with the origin site of many aneurysms. This propensity to aneurysmal pathogenesis was attributed to small defects in the tunica media ("*loci minoris resistentiae*"); critically, however, Forbus also found many media defects without apparent cerebral aneurysm formation. Stehbens[102, 104-106] noted medial defects to be common around the apex of arterial bifurcations, supporting the hypothesis that these may have been produced by greater wall stresses produced. Carmichael[100] proposed cerebral aneurysm formation be dependent on the combination of both a medial defect and a superimposed insult to the internal elastic lamina. Hassler[98] noted minor defects in all age groups, but major defects mostly in older adults, concluding that minor defects progress to major defects with age. This correlated with his other observation that micro-aneurysms (<2mm) progress to macroscopic aneurysms (>2mm) over a protracted time period[98].

The ability to sense and transduce local haemodynamic forces is unique to endothelial cells [107]. Chronic derangement of haemodynamic forces initiates an inflammatory response resulting in adaptive alterations in vessel wall shape and composition[108]. These morphological alterations are thought to represent a critical step at the earliest stages of aneurysm formation. Using renovascular hypertension and common carotid artery ligation, Jamous et al[89] induced cerebral aneurysms in a

rat model with a three month incubation time. Based on their findings, the authors proposed a novel classification of endothelial change leading to cerebral aneurysm formation (Table 1, fig 3). Inflammation is a known critical factor in the development of atherosclerosis[109-111] and abdominal aortic aneurysm formation[112-115]; however its role in human cerebral aneurysm initiation, development, and ultimately rupture remains to be more completely understood[7, 11, 116-120]

Degeneration or disruption of the internal elastic lamina of intracranial arteries is similarly accepted to be a component of human aneurysm pathogenesis[121-125]. Histological analysis of cerebral aneurysm walls often exhibits a continuum of internal elastic lamina degeneration, ranging from almost intact to severely degenerate to absent[10, 14, 36, 121-124]. The aneurysm wall itself typically lacks an internal elastic lamina. Human studies suggest that serum elastase and collagenase levels are elevated in patients with cerebral aneurysms[71-74], consistent with histological studies of expression of collagenase and elastase activity in cerebral aneurysm tissue in both human studies and animal models[74, 75].

Pre-cerebral aneurysm changes were sought in our study as EC or internal elastic lamina degeneration at major arterial bifurcations [108, 126-134]. Compared to control mice, mice with pre-aneurysmal changes revealed thickened vascular walls, with endothelial and internal elastic lamina disruption at arterial bifurcations. In our study, internal elastic lamina degeneration was specifically observed in n=8. All bifurcations with internal elastic lamina degeneration also showed EC. Such findings are consistent with previous histopathological studies of both animal-model and human cerebral aneurysm histopathology [1, 7, 10, 14, 36, 53, 116, 118, 135-143]. Interestingly, similar pre-aneurysmal changes were seen in rats, with renal hypertension without elastase use (see below)[63, 64, 88, 89].

The peri-procedural mortality observed in our study was within previously reported limits [2, 8, 81]. Mice were observed for 2-3 weeks prior to euthanasia: n=6 died during this interval, including n=2 which did not survive GA. Operative times (25min and 20min) and total procedural times (30 and 25 min) for both these mice were comparable with those of the rest of the experimental group. Autopsy of one of these mice revealed multiple haemorrhagic lesions at large arterial aortic branch points, in addition to a large intracerebral haemorrhage. No arterial bifurcations were successfully harvested from this mouse. Autopsy of the second mouse which died prior to recovery from anaesthesia revealed no obvious intracranial or systemic pathology. One cerebral artery bifurcation was harvested from this mouse. Histological of this cerebral artery bifurcation did not reveal either EC or internal elastic lamina degeneration. Notwithstanding, survival duration beyond the total procedural time did not represent an obvious factor in pre-aneurysmal development in our study. Thus, 3/4 mice which died during the observation period demonstrated pre-aneurysmal changes. By contrast, 4/9 who survived the observation period failed to demonstrate pre-aneurysmal changes at necropsy. Notwithstanding, the second mouse which died prior to emergence from anaesthesia revealed no obvious intracranial, nor systemic, pathology. Whilst n=1 bifurcation was harvested from this mouse it did not reveal either EC or internal elastic lamina degeneration. This suggests that a certain minimum time period is required following the experimental procedure for pre-aneurysmal changes to occur.

It is possible that Angiotensin-II use may have directly caused some of the changes in the sequence [normal artery → pre-aneurysmal → cerebral aneurysm ± SAH] observed in our, and others, study. For example, Angiotensin-II is associated with various non-haemodynamic effects, such as the promotion of inflammation, and the induction of reactive oxygen species [7, 112, 144]. Angiotensin-II also exerts several direct effects potentially relevant to cerebral aneurysm pathogenesis, including stimulation of monocyte recruitment [145], activation of macrophages[109] and enhanced oxidative stress[110]: all potentially capable of contributing to cerebral aneurysm formation independent of systemic hypertensive stress. Angiotensin-II is also involved in “outside-in” signalling on endothelial cells [146], and increases the binding of various inflammatory cells to endothelium [145]: these may be

contributory factors towards the endothelial dysfunction that is associated with cerebral aneurysm initiation [146].

Previous studies have therefore suggested that the non-haemodynamic effects of Angiotensin-II are a major causative factor in progression of lesions in murine models toward aortic arterial aneurysm formation [147]: notwithstanding, their relative contribution toward cerebral aneurysm pathogenesis is currently unknown. The possibility that Angiotensin-II use may have directly caused some of the changes in the sequence [normal artery → pre-aneurysmal → cerebral aneurysm ± SAH] observed could be tested by using alternative methods of inducing hypertension [9, 64, 67, 88, 89, 93]. However, as aforementioned, Jamous produced similar pre-aneurysmal changes were seen in rats, with renal hypertension without elastase use[88, 89]. Notwithstanding, since our model reproduced pre-aneurysmal and cerebral aneurysm formation, it remains valid (and retains experimental utility) irrespective of the precise mechanism of pre-aneurysmal or cerebral aneurysm pathogenesis.

Conclusion

Evidence of pre-aneurysmal, cerebral aneurysm or SAH was observed in 69% od mice: pre-aneurysmal alone in 58%. We have described a simpler model than hitherto, which is more cost effective, and which specifically lends itself towards studying pre-aneurysmal changes in mice. This model could be used to study factors which potentially advance or impair the progression pre-aneurysmal changes toward subsequent cerebral aneurysm formation and/or rupture.

To our knowledge, this is the first description of stereotactic injection of elastase using a manual technique. The rate of administration was similar to that described in previous models using automated pumps; our peri-procedural mortality was also within reported limits. Our method may represent an equally efficacious and potentially more cost-effective method of stereotactic injection of substrate, without the requirement for specialised equipment.

Using lower dose elastase solution than previously employed we developed a model of early CA pathology. Our model demonstrated that the spectrum of known early cerebral aneurysm pathology can be created at multiple bifurcations in mice, with the degree of endothelial change appearing to correlate with severity of degeneration of the internal elastic lamina. This model may permit the study of factors which could potentially advance or slow the progression of cerebral aneurysm formation.

The use and further development of this model may serve as a simple, low-cost and reproducible method of induction pf pre-aneurysmal change in future studies using various inhibitors, knockout or transgenic mice to test the roles of specific pathways in aneurysm pathogenesis.

Although requiring further refinement, or model offers an opportunity to study the molecular mechanisms of cerebral aneurysm pathogenesis. Future development of these models may result in further identification and definition of pathways of cerebral aneurysm formation, Screening of specific

molecular targets may become feasible and practical, allowing the development of pharmacological agents capable of modification of the processes leading to cerebral aneurysm formation and rupture.

By building on previous studies, we have described a small animal model of cerebral aneurysm induction, which validates previous works and appears to recapitulate the key pathological processes leading to cerebral aneurysm induction and formation in humans.

Appendix:

Observations and Technical Considerations

Measurement of blood pressure

One of the requirements of this model was induction and maintenance of a hypertensive state to facilitate pre-aneurysmal induction. The method of achieving this aim (placement of a subcutaneous micro-osmotic pump delivering a constant dose of Angiotensin II) has been described.

Systolic, diastolic and mean blood pressure (BP) was measured in all mice undergoing the elastase protocol on three occasions; pre-operatively, pre-euthanasia, and at an interim point approximately halfway through the observation period. BP was measured using the non-invasive tail cuff method. This involves utilisation of a tail-cuff placed on the tail to occlude blood flow. Upon deflation, a second sensor placed distal to the cuff monitors the blood pressure. The distal sensor measures BP by detecting the first appearance of the pulse whilst deflating or disappearance of the pulse on inflation of the cuff. This may be achieved via light emitting technology (i.e. use of a light-based/LED sensor) (photoplethysmography), use of a piezoelectric current (piezoplethysmography), or by use of a specifically designed differential pressure transducer to measure blood volume in the tail. This Volume Pressure Recording method utilises a volumetric analysis of both blood flow and volume in the tail. It has a number of distinct advantages over photoplethysmography or piezoplethysmography; it relies upon a volumetric method of measuring blood flow so is not prone to measurement artefact related to ambient light. It is independent of the animal's skin pigmentation. In addition, movement artefact is greatly reduced.

In our study, all measurements were taken via tail-cuff plethysmography using a volume pressure plethysmograper and infra-red warming blanket (Kent Scientific). This method was chosen for a number of reasons. Its accuracy and dependability when compared with invasive methods has previously been validated in BP measurement mice[148] and has been used successfully in previously described rodent models of CA formation[7, 8, 14, 63, 64, 88, 90, 149-151]. It does not require a

surgical procedure or insertion of an invasive pressure-monitoring device and is more cost effective than more invasive methods. In addition, it is the preferred method of BP measurement in rodents at the JCU VBU.

Standard operating protocol of BP measurement in mice was followed. Initial attempts to measure BP under isoflurane anaesthesia were unsuccessful, therefore an awake method was used. Animals were restrained using a proprietary rodent holder incorporating a darkened nose cone, allowing constant observation of the mouse whilst simultaneously limiting the animal's view and creating a low-stress environment. The mouse's muzzle was allowed to protrude through the front of the nose cone to facilitate breathing. The tail of the mouse was fully extended through the rear hatch opening of the holder. A brief period of acclimatisation approximating 15 minutes allowed for each mouse prior to obtaining BP measurements. During BP measurement, the holder was rested on an infra-red warming blanket to maintain proper core body temperature. All measurements were taken at room temperature as per standard operating procedures.

A total of ten readings were recorded for each mouse, with both the highest and lowest recorded measurements omitted prior to statistical analysis.

Although this method has been validated in mice as small as 8 gm, in our study difficulties were encountered in obtaining consistent, consecutive BP measurements in mice weighing less than 24g. This typically manifested as a requirement for multiple insufflation/deflation cycles of the tail cuff to obtain a single BP value. Although initially ascribed to a lower tail blood volume secondary to the stress experienced by the mice during the procedure, similar difficulties in obtaining BP were encountered in otherwise outwardly calm mice. In addition, these issues were more consistently noted in lighter mice (i.e. those weighing below the median weight) and were encountered during preoperative, interim and pre-euthanasia BP measurements.

Thus, it could be inferred from this data that the size of the mouse was a determining factor in successful BP measurement. Although measurement of BP via the tail-cuff method using the Ceva system has been validated in low weight juvenile mice, our experience with this system is that more rapid measurements are obtained with mice over 25 g.

Angiotensin-II induced hypertension

Hypertension was induced via constant subcutaneous infusion of a weight-determined dose of Angiotensin-II via an osmotic minipump. This method was selected as it is routinely used in the JCU VBU for this purpose. Angiotensin-II dosage was determined in a weight-dependent manner (see appendix for table). This necessitated accurate weighing of the mouse, the empty micro-osmotic pump, the volume of angiotensin to be placed in the pump, and the post-fill pump to ensure accuracy of the angiotensin-II dose. Although technically straightforward, the time taken to achieve this for each cohort of five mice added considerably to the set-up time of the procedure. Obtaining an accurate weight for each mouse initially proved problematic due to the activity of the animals; this was best facilitated by weighing the mouse after very brief spell (<30 sec) in the anaesthesia induction chamber. Although not sufficient to induce full anaesthesia, this method sufficiently sedated the mouse to allow placement on a scale and an accurate weight to be obtained. The mouse was then allowed to recover from this prior to induction of full anaesthesia and placement in the stereotactic apparatus. Due to the time taken to weigh and fill the Alzet pump with angiotensin-II (the accuracy of the dosage of which was dependent on obtaining an accurate weight of the mouse into which it was to be implanted), this method was felt preferable to a prolonged period under full anaesthesia to which the mouse would have been subjected.

The method by which the Alzet micro-osmotic pump was implanted has been described in a previous chapter. Previously described models describe placement of the Alzet pump via a 1cm, parasagittal incision 1cm cephalad from the base of the tail, and fashioning a small subcutaneous pocket between the skin and muscle layers using a pair of forceps [8]. Our method of placement differed slightly in that the incision used for subcutaneous placement of the was made at a point remote from the eventual placement site of the pump (i.e. intrascapular, with the pump placed just cephalad to the base of the tail. To create a pocket, the skin at the caudad end of the incision was grasped with forceps and tented away from the underlying muscle layers. A small, blunt tipped scissors was then inserted and opened

to create a subcutaneous defect whilst leaving the underlying pannniculous carnosus and vascular bed intact. The pump was then inserted and using fingers gently manipulated subcutaneously to its final position. This aspect of the procedure took no more than 5 minutes for any mouse. Wounds were checked on a twice daily basis; all healed in a satisfactory manner within 3-4 days and appeared well tolerated by the mouse. No mouse exhibited signs of stress or irritation at the wound site. Unexpected explantation of the pump due to wound dehiscence is a recognised complication of this aspect of the procedure, however this was not observed in our study.

The use of a weight determined dose of Angiotensin-II via constant infusion leads to a measurable rise in blood pressure as early as one week, and may maintain a hypertensive state for the duration of the infusion, typically up to 28 days[8, 80, 144, 152-154]. In addition to its hypertensive effects, angiotensin-II infusion can induce a number of non-haemodynamic effects such as induction of ROS and promotion of inflammation[7, 8, 80, 145]. In addition, Angiotensin-II may significantly increase adhesion of monocytes to endothelium, probably via Angiotensin-II type II receptors[145]. Whether this factor in and of itself may facilitate pre-aneurysmal changes by augmenting the monocyte/endothelial interaction required for cerebral aneurysm induction remains uncertain. Although pharmacological hypertension may also be induced via alternative methods such as norepinephrine infusion or dietary DOCA administration, the overall hypertensive effect of these methods tends to be of a lesser magnitude and of less significance over time than that induced by Angiotensin-II infusion. The experience of the VBU is that Angiotensin-II infusion induced hypertension via the subcutaneous method tends to consistent, reproducible and sustained for the duration of the project.

Stereotactic injection of elastase - Validation of stereotactic co-ordinates

The method by which the stereotactic injection of elastase was performed has been described in previous chapters. Briefly, a small right sided cranial burr-hole was made using a 2mm fine-tipped burr attached to an electric drill at a point 1.2mm rostral and 0.7mm lateral right of the bregma. These co-ordinates were obtained from both the Mouse Brain Atlas and had been validated by a previously described model. In a pilot study using these co-ordinates, Hosaka et al[10] reported a 100% incidence of successful infusion of bromophenol blue into the right basal cisterns surrounding the Circle of Willis (n=10). This compared favourably with the authors' alternative co-ordinates (1.2mm rostral and 1.0mm lateral of bregma) and those of previously described models (2.5mm posterior to bregma and 1.0mm lateral to midline)[14], which resulted in successful administration of substrate to the basal cisterns in 30% and 60% respectively. These results were broadly in keeping with our pilot study to assess the feasibility of stereotactic injection of a substrate (methylene blue) into the cerebrospinal fluid space using the co-ordinates described by Nuki et al (i.e. 2.5mm posterior to bregma, 1.0mm lateral of Bregma and 5.0mm ventral to the skull surface), in which we demonstrated a 60% incidence of successful administration of dye to the cerebrospinal fluid space (n=3/5). A notable consideration in using these co-ordinates, however, was an apparent variability in the accuracy of needle placement when using these co-ordinates which appeared dependent on the operator and age of the mouse. To account for this, the authors advised a "series of test injections using dye… was extremely important to ensure the correct placement of the needle tip at the right basal cistern" and "the co-ordinates described (we) described in the original paper[14] should be regarded as a guideline"[8]. However, their recommendation that "…at least 10 test injections are needed before each operator establishes the appropriate coordinates that yield stable aneurysm induction", was neither financially or ethically feasible for our model. Although our pilot study used the co-ordinates described by Nuki et al, for the purposes of our elastase study stereotactic co-ordinates with the highest reported incidence of successful administration of substrate to the cerebrospinal fluid spaces surrounding the Circle of Willis were used.

Depth of needle placement was likewise a critical factor in ensuring successful administration of substrate; too deep and the substrate would not be able to leave the needle due to physical obstruction at the skull base, whereas too shallow a placement ran the risk of elastase injection into the brain parenchyma and subsequent induction of an intracerebral haemorrhage. Even when the needle had breached the brain parenchyma, and in the subarachnoid space, injection of elastase when the tip was too close to the brain surface often resulted in diffuse haemorrhage from the surface of the brain as reported in previous models[8]. To minimise the risk of this occurrence, and with reference to cross sectional imaging of the Mouse Brain Atlas and co-ordinates and technical advice provided by previous models, an initial depth of 5mm of the blunt-tipped needle ventral to the brain parenchyma was achieved, then depth of placement was slowly increased in 0.1mm increments until slight resistance to further advancement due to contact with the skull base was encountered. This typically resulted in a slight bowing of the 26G needle, visible to the naked eye. At this point, and again using 0.1mm increments, the needle tip was withdrawn until the needle appeared straight, then withdrawn by a further 0.3mm immediately prior to elastase infusion. The average and median depths of needle tip at point of resistance were 4.93 and 5.0mm respectively. Although these measurements were not validated using dye, and further test or calibration procedures performed, 13 of our cohort of 15 mice survived the procedure, and no stigmata of needle misplacement (collagenase-induced intraparenchymal haematoma, extensive haemorrhage in the subdural space, etc.) felt to be attributable to the procedure were demonstrated on necropsy of these 13 mice. In addition, no breach of the skull base due to the needle tip was seen on dissection under loupe magnification or light microscopy.

Of note, however, of the two mice who died in the peri-operative period, needle tip placement was to a depth of 6.1mm and 5.0mm respectively. In the case of the mouse with the deeper tip placement, this would suggest that intraparenchymal injection of elastase was not the cause of periprocedural mortality. The depth of tip placement of the other mouse was at the median point of the other 13 mice, all of which tolerated the injection without incident.

Method of Elastase infusion

In previously described models, the stereotactic injection of substrate has been performed using a narrow calibre needle (usually a 26G Hamilton syringe Model 701 with a blunt tip) and an ultramicro-infusion pump, typically at a rate of between 0.2-2ul/min[7, 8, 14, 81]. Our laboratory did not have access to this equipment; therefore an alternative method of injection of elastase was conceived.

A semi-automated repeating dispenser (Model PB600-1, Hamilton Instruments, Nevada, LV, USA) was attached to the stereotactic apparatus via a detachable arm. The PB600 dispenser is a versatile, semi-automated dispenser designed to deliver liquid volumes of 0.5-50uL repeatedly up to 50 times. The dispenser is designed to achieve this by mechanically advancing the syringe plunger 0.047" with each depression of the dispenser button. Based on a 6cm scale (i.e. the length of the Hamilton syringe barrel at full volume), this action advances a fluid column within the syringe 2% of the syringe's volume with each click (i.e. 1/50 the total volume of an attached syringe was dispensed with each push of the dispenser button). Although designed for hand-held use, we found that the dispenser was easily incorporated into the stereotactic frame. A 10ul Hamilton blunt tipped microliter syringe (Model 701N, Hamilton Instruments, Nevada, LV, USA) was installed into the dispenser, and the whole apparatus attached to the stereotactic apparatus. A 10ul syringe was chosen as not only was this the desired volume of elastase to be infused in our model, but the dispensing rate of 0.2uL per click also facilitated easy manual control of the rate of infusion to within the limits reported in previous models (i.e. 0.2-2uL/min)

Verification of the needle tip location in response to adjustment of the stereotactic co-ordinates was confirmed using graph paper prior to commencement of the project. Once the burr-hole had been performed, the drill was removed from the stereotactic frame. The base of the burr-hole was inspected to confirm complete breach of the calvarium, and the dura carefully punctured using a

sharp tipped 26G needle. The dispenser/needle apparatus was at this point attached to the system, and the desired co-ordinates for injection attained by zeroing the needle tip placement relative to the bregma and positioning the needle along the XY axis in the desired location (i.e. 1.2mm rostral and 0.7mm lateral right of bregma). This invariably resulted in the needle tip being placed in the centre of the burr-hole prior to intracranial advancement. The location of the needle tip was then zeroed on the brain surface prior to ventral advancement as described. Once satisfactory intracranial placement of the needle tip had been achieved as described above, 10ul of Elastase solution (1.0u/ml) was then injected manually into the right basal cistern at a rate of 2ul/min over a period of 5 minutes (i.e. 0.2uL per click at six second intervals). Upon completion of the infusion, the needle was slowly withdrawn, and the brain surface carefully inspected for signs of trauma or haemorrhage. The cranial wound was then closed in a single layer under loupe magnification with a clear monofilament suture as described in the operative chapter.

The volume of elastase infused with each click was dependent on the length of the plunger arm relative to the index rod of the dispenser. To ensure accuracy, this was calibrated when each syringe was inserted into the device. Calibration was a quick and straightforward procedure; the plunger assembly was retracted fully, then the dispenser button repeatedly depressed, counting the number of clicks, until the index rod no longer advanced. The index rod/plunger lengths were then adjusted appropriately, and the test repeated until 50 dispenses was achieved. Calibration took between 2-4 minutes approximately for each procedure. As a new syringe was used for each mouse per cohort, this was performed prior to induction of anaesthesia for each mouse to ensure any impact on operative duration was minimised.

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