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Université de Montréal

# The Effects of Stenting and Endothelial Denudation on

# Experimental Aneurysm Healing and Gene Expression

# Following Endovascular Treatment

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Université de Montréal

Faculté des études Supérieures

Ce mémoire intitulé :

**The Effects of Stenting and Endothelial Denudation on**

**Experimental Aneurysm Healing and Gene Expression**

**Following Endovascular Treatment**

Présenté par:

Tim E. Darsaut

A été évalué par un jury composé des personnes suivantes:

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Directeur de recherche: Docteur Jean Raymond

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Membre du Jury: Docteur Tom Marotta

Mémoire accepté le:

*I dedicate this work to all people affected by neurosurgical illness*

# SOMMAIRE

Les récidives sont communes après le traitement endovasculaire des anévrismes intra-crâniens. Une meilleure compréhension des mécanismes cellulaires et moléculaires de la trombose intra-anévrismale après traitement pourrait conduire à de meilleures stratégies pour réduire les récidives.

La première étude traite des effets de l'utilisation d'endoprothèse (ou stent) et de la dénudation endothéliale sur l'évolution des anévrismes et l'occlusion des vaisseaux adjacents. Des anévrismes expérimentaux ont été créés et traités uniquement par 'stenting' ou par 'stenting' avec ou sans dénudation endothéliale. Les résultats angiographiques et pathologiques des deux groupes ont été comparés. Le 'stenting' seul était insuffisant pour obtenir l'occlusion complète d'un anévrisme, mais les résultats se sont améliorés lorsque l'endoprothèse etait combinée avec la dénudation endothéliale.

La deuxième étude porte sur les effets de l'endoprothèse sur l'expression génique des cellules de la paroi de l'anévrisme et s'efforce d'établir une corrélation entre les changements d'expression et les résultats angiographiques et pathologiques. Le profil d'expression de 14 gènes d'intérêt potentiel a été analysé par la RT-PCR sur les tissus du collet des anévrismes bilatéraux de la carotide, dont l'un d'eux a été traité par un endoprothèse.

L'utilisation de l'endoprothèse à amélioré les resultats angiographiques. Les profils d'expression mRNA étaient bien en rapport avec les effets attendus sur la formation néointimale et l'organisation du thrombus et avec la recanalisation (ou formation d'un canal endothélialisé à l'intérieur du thrombus); mais les différences entre les groupes soumis au 'stenting' et les groupes contrôles n'ont pas atteint le seuil de signification statistique.

**Mots-clés** : anévrisme, endoprothèse (stent), endothélium, gène, modèle animal.

# SUMMARY

Recurrences are common after endovascular treatment of intracranial aneurysms. An understanding of the cellular and molecular biology of intra-aneurysmal thrombus evolution following treatment promises to lead to better strategies to reduce recurrences. The first paper studies the effects of stenting and endothelial denudation on aneurysm and branch vessel occlusion. Experimental aneurysms were created and treated with stenting alone or stenting and endothelial denudation. Angiographic and pathologic findings were compared between the two groups.

We found that stenting alone was insufficient to lead to complete aneurysm occlusion, but results improved when stenting was combined with endothelial denudation.

The second paper studies the effects of stenting on the genetic expression of cells of the aneurysm wall and attempts to correlate changes in expression with angiographic and pathologic findings. Bilateral carotid aneurysms were created; one was stented. Tissue from the aneurysm neck underwent RT-PCR at various times to create mRNA expression profiles for 14 potentially interesting genes.

Stenting led to angiographic improvement of aneurysm appearance. mRNA expression profiles were in keeping with expected effects on neointima formation and thrombus organization and with endothelialized channel formation within the thrombus, but differences between stented and control groups did not reach significance.

**Key words:** aneurysm, stent, endothelium, gene, animal model

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| | |
|---|---|
| ADP | Adenosine diphosphate |
| ADPKD | Autosomal dominant polycystic kidney disease |
| ASA | Acetylsalicylic acid |
| BM-MNC | Bone marrow-derived mononuclear cells |
| C | Celsius |
| CD | Cluster of differentiation |
| CT | Computerized tomography |
| DNA | Deoxyribonucleic acid |
| DVT | Deep venous thrombosis |
| EC | Endothelial cell |
| ECM | Extracellular matrix |
| eNOS | Endothelial nitric oxide synthase |
| EPC | Endothelial progenitor cell |
| E-selectin | Endothelial-selectin |
| F | French |
| G | gravity |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GDC | Guglielmi detachable coil |
| GSL | Griffonia simplicifolia lectin |
| HCl | Hydrochloric acid |
| HPS | Hematoxylin-phloxine-saffron |
| ICH | Intracerebral hemorrhage |
| ISAT | International subarachnoid aneurysm trial |
| ISUIA | International study of unruptured intracranial aneurysms |
| MC/MPh | Monocyte/macrophage |
| MCP-1 | Monocyte chemotactic protein-1 |
| MMP | Matrix metalloproteinase |
| MPC | Mesenchymal progenitor cell |
| MT-MMP | Membrane type matrix metalloproteinase |
| mRNA | Messenger ribonucleic acid |

| | |
|---|---|
| NaCl | Sodium chloride |
| NGS | Normal goat serum |
| NO | Nitric oxide |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PDGFR | Platelet-derived growth factor receptor |
| PE | Pulmonary embolus |
| PECAM | Platelet-endothelial cell adhesion molecule |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SAH | Subarachnoid hemorrhage |
| $\alpha$-SMA | Alpha-smooth muscle actin |
| Taq | Thermophilus aquaticus |
| TBT | Tris-Buffered saline-Tween |
| TEAM | Trial of endovascular aneurysm management |
| TGF | Tissue growth factor |
| TIMP | Tissue inhibitor of metalloproteinase |
| TNF | Tumour necrosis factor |
| VCAM | Vascular cell adhesion molecule |
| VE-cadherin | Vascular endothelial cadherin |
| VEGF | Vascular endothelial growth factor |
| VEGFR | Vascular endothelial growth factor receptor |
| VLA | Very late antigen |
| VSMC | Vascular smooth muscle cell |
| vWF | von Willebrand factor |

*The task of science is to stake out the limits of the knowable,*
*and to center consciousness within them.*

*Rudolf Virchow (1821-1902)*

*A life filled with toil and work is not a burden,*
*but a blessing.*


*Rudolf Virchow (1821-1902)*

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# 1. INTRODUCTION

This thesis comprises six chapters. The first provides relevant background regarding intracranial aneurysms and how they can be treated, followed by an introduction to the cellular and molecular factors likely involved in intracranial aneurysm healing. The second chapter explains the rationale, hypotheses, and goals of the experiments performed for this thesis, while the third chapter covers the materials and methodology used in completing and analyzing the experiments. The fourth chapter is made up of the two manuscripts produced as a result of this work. A general discussion of the material within the papers is covered in chapter five, with some concluding remarks. The sixth chapter provides references to the literature cited in this thesis.

## 1.1 INTRACRANIAL ANEURYSMS

### 1.1.1 Demographics

An aneurysm is an abnormal swelling along a blood vessel[1]. The reported prevalence of unruptured aneurysms ranges from 0.1-6%, but is likely considerably less than 5% [1]. Aneurysm rupture is the most common cause of spontaneous subarachnoid hemorrhage (SAH)[2], with an incidence estimated at 10/100 000 population per year[3, 4]. This devastating disease strikes women 1.6 times more than men[1], usually between the age of 30 and 70 years, with a peak incidence during the $6^{th}$ decade[5]. The consequences of SAH can be grave, with approximately 45% overall mortality and moderate to severe disability in 30%[6-8]. In adults, 85% of aneurysms are found in the anterior circulation, and most of these aneurysms are saccular[2]. Posterior circulation aneurysms comprise 15%, a greater proportion of which are fusiform.

Aneurysms are much less common in children. Childhood aneurysms often bear atypical etiologic or morphologic features, and are suspected to represent a distinct pathological entity[9].

## 1.1.2 Etiopathogenesis

The etiopathogenesis of most saccular aneurysms remains unknown. Cerebral blood vessels normally contain an outer adventitial layer, a middle layer of smooth muscle or media, an internal elastic lamina, and an innermost intimal layer. Cerebral vessels lack an external elastic lamina, which may predispose them to aneurysmal formation. Microscopically, aneurysms are found to have structural changes in the wall of the blood vessel, including mural atrophy, loss of the internal elastic lamina and media, and thinning of the adventitia[10].

Aneurysm etiologies are commonly classified into acquired or congenital causes. Support for the hypothesis that aneurysms are acquired lesions arises from the observation that most cerebral aneurysms arise at branch points or where arteries abruptly change curvature, implicating chronic hemodynamic stress as a etiologic agent [9]. In keeping with this theory, the risk factors most commonly linked to aneurysm formation and rupture are cigarette smoking and arterial hypertension[1, 11]. Furthermore, intracranial aneurysms are rarely found in children, and are found with increasing incidence until the 8th decade of life[11].

However, there is supporting evidence for congenital predispositions to aneurysm development. Several heritable disorders are associated with intracranial aneurysms, including Ehlers-Danlos type IV, Marfan's syndrome, neurofibromatosis type I, and autosomal dominant polycystic kidney disease (ADPKD)[1]. Although a mutation at a particular gene locus has not been discovered, intracranial aneurysms are known to run along familial lines[11]. Taken together, the evidence suggests that the causes of aneurysm formation and rupture are multiple and multifactorial, most likely resulting from a

combination of genetic factors which predispose the arterial wall to form pathological dilations in response to noxious environmental factors.

The remaining causes of aneurysm formation represent only a small minority of cases. Mycotic aneurysms are those that occur following infection, usually in association with bacterial endocarditis[2], while post-traumatic aneurysms are an uncommon sequelae of trauma, usually following a penetrating injury to the arterial vessel wall[2].

### 1.1.3 Clinical Manifestations

Unruptured aneurysms usually remain asymptomatic, although they may present with signs and symptoms of mass effect, cortical or meningeal irritation, or occasionally with ischemic events from emboli originating from within the aneurysm. The majority of aneurysms are diagnosed after rupture, which usually causes SAH, intracerebral hemorrhage (ICH) or subdural hematoma. The annual incidence of SAH in patients with unruptured aneurysms is still unknown, and is the topic of much current debate[12-14]. For ruptured aneurysms, clinical symptomatology depends on aneurysm size, location, bleeding severity, and the presence of associated hydrocephalus. Aneurysm rupture can result in sudden death (as high as 65% of patients, as reported in the prospective arm of ISUIA)[12], while the remainder typically seek medical attention for a spectrum of neurological signs and symptoms ranging from sudden headache to decreased level of consciousness[1, 2]. Typical clinical features include the sudden onset of severe headache, often with neurological deficits, associated nuchal pain and rigidity, a low-grade fever, and retinal hemorrhages. Figure 1.1 presents typical CT findings of aneurysmal SAH.
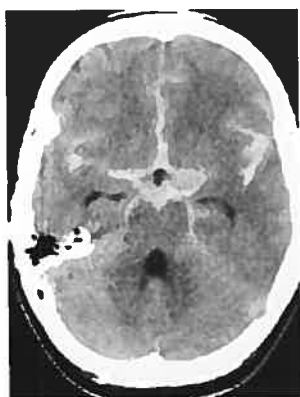


**FIGURE 1.1**

**CT head showing diffuse subarachnoid hemorrhage.**
Subsequent digital subtraction angiography revealed a left anterior choroidal artery aneurysm.

### 1.1.4 Complications of aneurysm rupture

30-50% of aneurysm ruptures are fatal prior to medical or surgical intervention[1]. In the absence of prompt aneurysm obliteration, approximately half of patients with aneurysm rupture will die within one month, usually due to re-hemorrhage[1]. For untreated patients who survive 6 months, there is a 2% rupture rate per year for ten years, which falls to 1% after a decade[1]. Rebleeding has an associated mortality of 80%[1]. Mortality for patients who survive until treatment is approximately 10%, with 60% of survivors having a favorable outcome[5]. Overall mortality from SAH is approximately 60-65%[12, 15]. Other potentially life-threatening early complications include acute hydrocephalus due to blockage of cerebrospinal fluid flow by thrombus formation within the ventricular system or basal cisterns[2]. Vasospasm of major intracranial vessels secondary to clot and clot breakdown products in the subarachnoid space can lead to cerebral ischemia or infarction. Other potential complications of aneurysm rupture include neurological deficits, treatment-related complications, systemic complications secondary to acute physiological stress, as well as complications secondary to immobility. Chronic hydrocephalus is a common sequel of aneurysmal SAH, often requiring shunting of cerebrospinal fluid.

## 1.2 TREATMENT OF INTRACRANIAL ANEURYSMS

While the treatment of unruptured aneurysms, without more complete knowledge of the natural history, remains a matter of debate, there is little controversy that ruptured aneurysms should be treated to prevent further rebleeding. In general there are two options for the treatment of intracranial aneurysms; open surgical or endovascular treatment.

### 1.2.1 Open surgical treatment

Current surgical treatment of saccular aneurysms typically consists of a craniotomy to place a metallic clip across the base of the aneurysm, effectively removing the aneurysm from the circulation and maintaining or recreating a normal arterial configuration (Figure 1.2). The aneurysm clip re-apposes the arterial mural structures between the clip blades, maintaining a continuous endothelial covering layer.
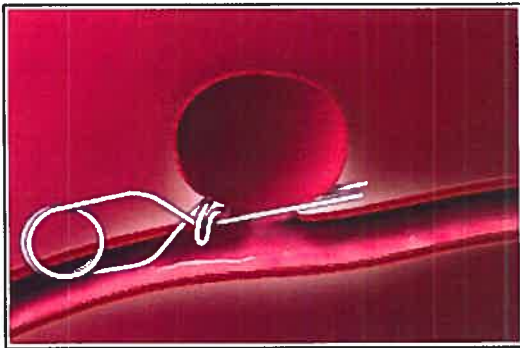


**Figure 1.2**

Aneurysm clip across the neck of a sidewall berry aneurysm, removing it from the circulation. (From Boston Scientific; www.bostonscientific.com)

Surgical rates of morbidity and mortality for unruptured aneurysms are typically reported in surgical case series to be <4% and <2%, respectively[16-18], a likely underestimate related to publication bias. However, the ISUIA investigators have repeatedly reported surgical morbidity and mortality rates for unruptured aneurysms ranging from 12.6-15.7%[12, 19]. For ruptured aneurysms, in cases deemed salvageable, surgical mortality rates range from 7-29%[1]. Morbidity due to surgery on ruptured aneurysms is difficult to separate from morbidity due to the aneurysm rupture itself.

Other surgical treatments for unclippable aneurysms depend on the nature, morphology, and location of the aneurysm, as well as the surgical exposure. Aneurysmorrhaphy with parent vessel reconstruction or surgical bypass following by parent vessel occlusion offer definitive treatment, but are more technically difficult and carry more risk[20]. Other alternatives, such as aneurysm wrapping with muscle or muslin gauze to reinforce the wall, are of dubious efficacy, being used only when there are no other options[20]. As the role of endovascular therapy is increasingly recognized, partial neck clipping followed by coiling is recognized as a viable alternative for difficult cases[20].

### 1.2.2 Endovascular treatment

Endovascular treatment, an increasingly popular alternative to surgery, involves arterial puncture at a remote site, with navigation to and deposition of various materials within the circulation. The rise in popularity was initially due to the less invasive nature of endovascular treatment, which is now supported by the publication of the multicenter randomized ISAT trial of ruptured aneurysms, which showed decreased morbidity and mortality at 1 year for endovascular treatment (23.7%) compared to surgery (30.6%)[21].

In general, endovascular treatment strategies can be directed towards either the aneurysm or the parent vessel (Figure 1.3). When the strategy targets the aneurysm, thrombogenic material (typically platinum coils) are deposited within the aneurysm fundus, in hopes of causing a localized reaction with thrombus formation, followed by clot organization and neointimal formation to obliterate the aneurysm and restore normal blood flow patterns. The strategy of coil deposition within aneurysms was shown with ISAT to be of clinical benefit.

Treatment directed towards the parent artery can be either 'deconstructive', occluding the parent vessel as well as the aneurysm, or 'reconstructive', using stents to alter aneurysm hemodynamics, provoke thrombosis and promote neointima formation at the junction of the parent vessel and aneurysm neck. Strategies to treat aneurysms using stents have never been proven beneficial but are increasing in popularity[22]. I will start by introducing the well-accepted embolization procedure and review the limitations of this strategy. I will then discuss the potential benefits of stents as adjuncts to coil embolization, or as a primary mode of treating intracranial aneurysms.
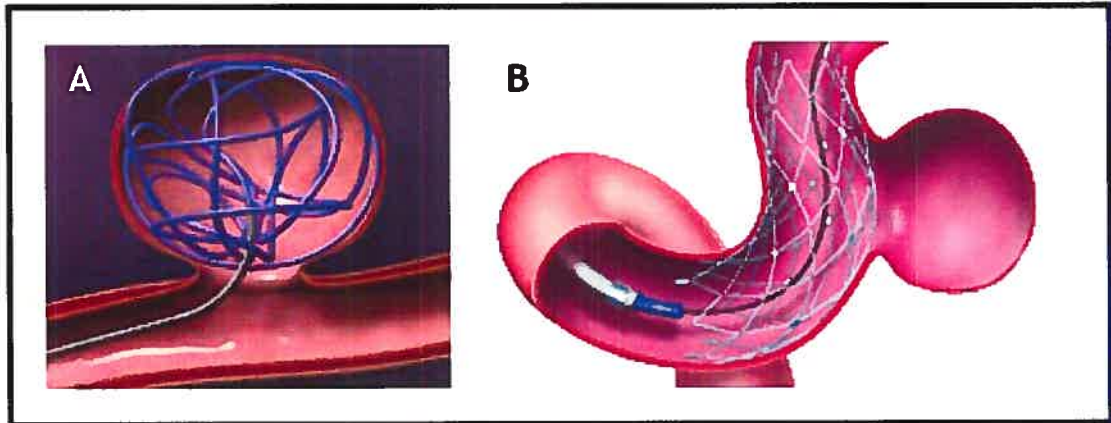
**Figure 1.3**

**Artist's rendition of different endovascular strategies to treat aneurysms**

A) Coils deposited within the aneurysm. B) Endoluminal stent deposited in the parent artery to bridge the neck of the aneurysm. (from Boston Scientific; www.bostonscientific.com)

### 1.2.2.1 Coils

Although embolization of aneurysms with various materials, including free coils, balloons, and glue had been possible for decades for rare lesions, its clinical use remained exceptional until the introduction of Guglielmi detachable coils (GDCs) in 1991. Briefly, endovascular treatment with GDCs involves packing multiple thrombogenic platinum coils into an aneurysm to prevent blood from entering the fundus. The coils adjust to the contours of the aneurysm, and once satisfactorily positioned, the operator separates the coil from the stainless steel delivery wire through an electrolytic process. The key feature of GDCs was this detachment system, which permitted the retrieval and repositioning of the coils when necessary, with detachment only when the coils were safely and stably positioned within the aneurysm. The improved safety profile imparted by the detachment system led to the increased popularity of endovascular treatment, with little concern for its long-term efficacy. Although coiling can lead to good outcomes, there are several important drawbacks with this technique. One occasionally encountered limitation of

endovascular therapy is difficult intravascular access due to atherosclerosis, which prevents proper negotiation of wires and catheters to access the aneurysm. This difficulty is exacerbated when the aneurysm is located in tortuous or distal vasculature. Complications at the arterial puncture site in the groin can also lead to clinically significant hemorrhages, occasionally requiring peripheral vessel stent-grafting. However, the most important drawback associated with endovascular coiling remains the angiographic recurrence rate, which is reported to approach 20-40%[23]. In a most recent case series the retreatment rate following endovascular coiling reached 15% after two years, but the clinical significance of these recurrences in terms of rehemorrhage was very modest [24]. Although the risk of rupture of aneurysm recurrences is still not known, the fear of SAH from an aneurysm recurrence continues to lead some neurosurgeons to treat aneurysms with surgical clipping. Attempts to decrease recurrence rates have focused mostly on coil modification; coatings to promote thrombosis[25], to increase aneurysm packing density[26], emit radiation[27], or biologically active coatings[28] have been introduced, but none have successfully decreased recurrence rates. Liquid embolic agents such as Onyx have also proven ineffective in this regard[29]. Finally, endovascular techniques are not suitable for all aneurysm morphologies; coils deposited within aneurysms with wide necks or a dome:neck ratio of <2 are at risk of herniating into the parent vessel, leading to thrombo-embolic complications. Technical innovations, such as the balloon-remodeling technique have been developed to address this, but this adds complexity and procedural risk[30]. The notion to use stents in the intracranial circulation originated from the need to prevent coil herniation in wide-necked aneurysms.

### 1.2.2.2 Stents

Stents are made of tubular metallic mesh, and were originally designed to treat acute or subacute coronary artery occlusion following balloon angioplasty, aiming to prevent recoil of the arterial wall after balloon dilatation. Balloon-expandable stents were introduced into atherosclerotic vessel segments and the balloon forcibly expanded to dilate the lesion, leaving the stent in situ in order to maintain vessel patency. Neuroendovascular therapists

have benefited from the cardiology experience regarding ischemic complications and vessel re-stenosis from neointimal hyperplasia following stenting, but the cardiology literature is not always applicable to intracranial disease processes. Important pathological differences between diseased atherosclerotic blood vessels and vessels bearing aneurysms may alter the arterial response to stenting.

The use of stents for intracranial applications has been hampered by the accessibility to the tortuous intracranial circulation. Initial stenting attempts for intracranial pathologies used coronary stents as an 'off-label' indication[31]. The balloon-expandable coronary stents are designed to exert large radial forces to force open atherosclerotic stenoses and then maintain vessel patency. The coronary stents available on the market were quite rigid, and neuroendovascular therapists found them difficult to navigate through the carotid siphon at the skull base[32]. Balloon-expandable stents are limited by their poor flexibility and 'pushability', as compared to self-expandable stents[33]. Nonetheless, in the less tortuous posterior circulation, balloon-expandable coronary stents have been successfully used to treat aneurysms[34]. More recently, self-expandable stents designed specifically for intracranial applications have been developed. These devices and their deployment systems are more flexible, enabling intracranial navigation. In contrast to their coronary counterparts, intracranial stents do not need to open stenotic segments, and self-expandable stents, which exert smaller radial forces but adapt more closely to the contours of the parent vessel, are more commonly used than balloon-expandable stents. Self-expandable stents must nonetheless exert sufficient radial force to properly anchor the stent within the vessel, to prevent stent migration, or to prevent the herniation of coils out of aneurysms[30].

In the treatment of intracranial aneurysms, stents are typically used in conjunction with coils or other embolic agents, although they can be used alone (Figure 1.4). As previously mentioned, the main utility of the stent in conjunction with endovascular treatments is to prevent the herniation of embolic material into the parent vessel, especially for wide neck or fusiform aneurysms [35, 36]. However, the use of stents for routine non-wide necked aneurysms has been suggested, due to theoretical effects of the stent on aneurysm healing. Stents are thought to act primarily by redirecting blood flow along the parent vessel,

altering aneurysmal hemodynamics and promoting thrombosis [37, 38]. Redirecting blood flow forces may also promote more complete aneurysm healing and decrease recurrence rates by re-establishing a new endoluminal boundary that excludes the aneurysm. The stent may also buffer the hemodynamic forces implicated in recurrence, or alter the fluid dynamics in the area of the stent, thereby promoting neointima formation [39]. The effectiveness of the stent at achieving these goals depends primarily on the stent design.



**Figure 1.4**

Artist's rendition of endovascular coiling in conjunction with stenting. Note how the stent prevents coil herniation into the parent vessel lumen.

The choice of stent should be based on the site and morphology of the aneurysm, the stent design, as well as the delivery system. The relevant morphology of the aneurysm/parent artery complex includes the caliber of the parent vessel, the desired length of "landing zone" for the stent, the width of the aneurysm neck, and the presence of branch or perforator vessels[30]. Stents are usually chosen to match the caliber of the recipient vessel[40]. Intentional attempts to over-dilate cerebral vessels has led to extremely high morbidity and mortality rates[41]. Manufacturers recommend a "landing zone" of 4-5 mm on either side of the aneurysm neck[32]. Longer stents present a larger thrombogenic surface area, and carry a greater theoretical risk to branch or perforating vessels. At sites of tight vessel curvatures, a more flexible stent is required to conform to the parent artery.

The design of the stent includes the size, composition, and structure, including the number of struts per circumference. Clinicians can choose from a variety of lengths and diameters, but most stents in current clinical use are made of nitinol, a nickel-titanium alloy. The ideal stent composition is one that renders the stent biologically inert and non-

thrombogenic, and nitinol has been shown to be superior to other metals with respect to neointimal hyperplasia and in-stent stenosis[42]. For nitinol stents, radio-opaque markers must be added to guide proper deployment, because nitinol is radiolucent.

A properly chosen stent structure is paramount to avoid complications[43]. Stent strut width and cell size dictate the porosity of the stent, which is the ability for blood to continue to flow outside the confines of the stent mesh (ie: into the aneurysm or adjacent branch vessels) [30]. The ideal stent porosity alters blood flow sufficiently to provoke a localized thrombosis within the aneurysm while sparing all small vessels originating from under the stent[30]. These opposing goals may not always be attainable with the stents currently available, and compromise is often necessary. In cases devoid of critical perforators, where thrombosis is desired anywhere outside the confines of the stent, the ideal stent would be a stent covered with an impermeable membrane with a non-thrombogenic internal surface. Covered stents such as these are useful to treat fusiform aneurysms with no perforators arising from the parent vessel[44], and are also used to treat abdominal aortic aneurysms[45]. Stents with a greater number of struts (lower porosity) are able to exert a greater amount of radial force. Radial force is an important consideration in atherosclerotic disease, where the main goal of the stent is to maintain vessel diameter, but for intracranial aneurysms, a minimum of radial force is required to prevent coil herniation or stent migration. The number of struts correlates with the amount of potentially thrombogenic surface area, and may pose a greater risk of parent vessel thrombosis. The number and type of linkages between stent cells further dictates the flexibility of the stent. These considerations have led stent manufacturers to create highly flexible, high porosity stents with few stent struts per cross-sectional diameter.

The stent deployment system is also of crucial importance to a successful endovascular procedure. The two most important features are navigability and method of delivery (self- or balloon-expandable). Stents designed for intracranial use are now sufficiently navigable to access most aneurysms of the posterior and anterior circulation. Self-expandable stents offer several advantages compared to balloon-expandable stents. These stents are deployed by pulling back the sheath covering the spring-loaded stent, which expands to fill the contours of the parent vessel. The low radial force exerted by a self-expandable

stent is sufficient to anchor the stent within the lumen, as well as to prevent coil herniation into the parent vessel in most cases if necessary. This method of delivery does not cause angioplasty-type injuries when deployed, which may reduce the incidence of neointimal hyperplasia and in-stent stenosis[46]. Balloon-expandable stents are designed to expand to a fixed volume, and are more prone to migration within the parent vessel if they are undersized.

The risks of stenting can be classified as acute or chronic. Thrombogenic risks comprise the majority of acute and subacute risks due to stenting[32]. The thrombogenicity of intracranial stents is almost indisputable given the cardiology experience with coronary stents[47], and as all stenting procedures are now performed using vigorous anti-platelet regimens, it is ethically impossible to determine the risk of thrombosis due to stents by themselves. The best anti-platelet regimen has not been decided upon, but several have been suggested for intracranial stents under various circumstances [32, 35, 48]. Stent deployment also carries ischemic risks to perforating vessels, by partially occluding vessel ostia originating under the stent, or causing embolization of fragments of atheroma [49]. It is important to note the difference in thrombotic risks between the conventional coiling strategies that target aneurysms by depositing coils *outside* the normal circulation and stenting strategies that deploy devices within the parent vessel. The vessel trauma caused by the stent and the permanent deployment of a foreign body creates a risk of thrombosis to the exact vessel we aim to preserve. Thrombotic complications can also occur in a delayed fashion, requiring long-term anti-platelet therapy. The use of these agents after SAH may be a source of additional hemorrhagic complications, including aneurysm rebleeding, or following other procedures, such as external ventricular drainage. Other risks of stenting include suboptimal placement, migration, fracture, or prolapse into the potential spaces outside the vessel lumen, such as a branch ostium or aneurysm [33, 50]. Catastrophic arterial perforation may also occur[33]. Finally, the arterial response to stenting can also lead to neointimal hyperplasia and in-stent stenosis, which is well-characterized in the cardiology literature. Although the nature of the aneurysmal disease process differs from the atherosclerotic coronary situation, intracranial in-stent stenosis

due to exuberant neointima deposition with hemodynamically significant flow reduction has been reported[51].

### 1.2.3 Aneurysm recurrences following treatment

Following complete surgical clipping as determined on angiography, aneurysm recurrence rates range from 1.5 to 3%[52]. For surgically clipped lesions with known residua, the recurrence rate is 7-25%[52, 53]. Endovascular treatment is either incomplete or is associated with aneurysm recurrence in 20-40% of cases[23]. Engineering and biomedical strategies to decrease recurrence rates through novel device designs and biological coil modifications have not solved the problem of recurrences. Most developments were proposed by manufacturers and developed by engineers, with little concerns for the biological mechanisms involved in healing or recurrences following endovascular treatment. There is a need for better characterization of the molecular mechanisms occurring following aneurysm treatment to help engender and guide further attempts to decrease recurrence rates.

## 1.3 MECHANICAL AND HEMODYNAMIC FORCES ACTING ON ANEURYSMS

### 1.3.1 Overview

Blood flow is a result of repetitive coordinated myocardial contractions which propel blood through the vascular tree. In vivo hemodynamic forces are complex; they vary with cardiac cycle, heart rate, blood pressure, blood viscosity, vessel architecture, and viscoelastic properties of the artery[54]. Forces generated by pulsatile blood flow are attenuated primarily by two processes: elastic stretching of the arterial media, and friction between the elements of blood and the vessel wall. Chronic pathological elevation of these forces has been implicated in the formation and rupture of intracranial aneurysms[1]. The nature and magnitude of hemodynamic force acting on an aneurysm depend on the location and morphology of the aneurysm. Following endovascular treatment, the

organizing thrombus is subjected to continued pulsatile blood flow, which potentially impacts many of the ongoing biological processes.

Mechanotransduction was initially thought to be due to activation of specific mechanoreceptors, such as a mechano-sensitive ion channels or cell surface adhesion molecules, but there is also evidence for the entire endothelial cell cytoskeleton to act as a mechanoreceptor [55, 56]. Eukaryotic cells are able to maintain cytoskeletal rigidity through cytosolic filaments that generate and resist mechanical loads[55, 57]. These intracellular scaffolds allow the cell to respond to mechanical signals from the environment, by transducing cellular deformations through cellular signaling pathways, resulting in physiologic or pathologic responses[58]. Forces of lesser magnitude that would not normally deform the cell soma are able to trigger mechanotransduction signaling pathways through the deformation of cell surface receptors, such as integrins[59, 60]. The establishment of appropriate cell-cell and cell-matrix contacts is critical for transduction of mechanical forces in smooth muscle tissue[61]. Here, I will discuss the hemodynamics of lateral wall aneurysms, followed by a description of the two types of mechanical forces acting on vascular cells.

## 1.3.2 Aneurysm hemodynamics

Aneurysm hemodynamics studies reveal that flow within an aneurysm depends primarily on the morphology of the aneurysm and the relationship to the parent artery[62]. For lateral wall aneurysms, three distinct zones have been described: 1) an inflow zone entering the aneurysm at the distal portion of the ostium, 2) an outflow zone exiting the aneurysm at the proximal end of the ostium, and 3) a central slow flow vortex [63]. Modification of the inflow zone may provoke aneurysm thrombosis while maintaining parent vessel patency [63]. The ability of stents to modify the inflow zones of an aneurysm are well documented[62], and this effect of stents likely contributes to the obliteration of lateral wall aneurysms (Figure 1.5) [42].
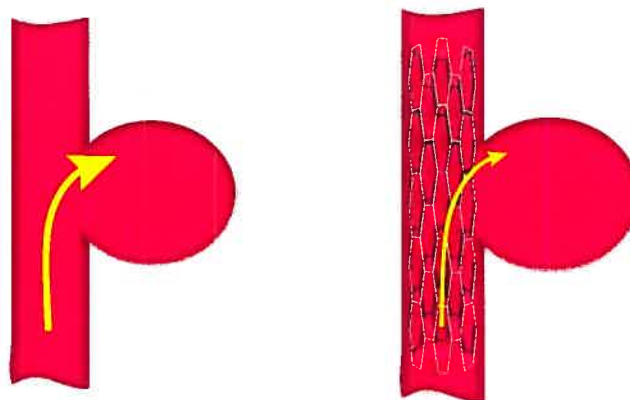
**FIGURE 1.5** Schematic comparing flow dynamics of non-stented versus stented aneurysms

Hemodynamic forces have long been implicated in the initiation of atherosclerosis, with greater levels of laminar flow conferring an atheroprotective phenotype[64]. There is an increased incidence of atheroma formation on the proximal portion of diseased branch vessels, purportedly due to decreased laminar shear stress or increased [64]. Shear stress is known to modulate gene expression (see section 1.3.3.2) , and the loss of a protective genetic expression profile may trigger cellular and molecular events that account for atheroma formation localized to vessel bifurcations[65].

Blood flow into a lateral wall aneurysm conceptually resembles that into a branch vessel; the distal portion of the ostium acts as a flow divider, and is exposed to higher levels of shear stress compared to the proximal aneurysm ostium [66]. The observed increased neointima formation at the proximal ostium [67] likely reflects this difference in laminar shear stress between proximal and distal ostia.

## 1.3.3 Mechanical forces acting on vascular cells

Stretch and shear stresses are important physiologic stimuli known to trigger cell signaling in vascular cells[56, 68, 69]. Although endothelial cells (ECs) and vascular smooth muscle

cells (VSMCs) are exposed to both types of mechanical force, the shear stress from blood flow is borne primarily by ECs, while VSMCs are primarily subjected to cyclic stretch from pulsatile pressure[69]. The response to mechanical forces may be important to the occurrence of aneurysm recurrences following endovascular treatment.

### 1.3.3.1 Cyclic Wall Stress

Cyclic wall stress is the tensile stress vector acting perpendicular to the blood vessel lumen that occurs as the blood vessel lumen is expanded with systole[70]. This force, which primarily affects vascular smooth muscle cells, results in 5-6% wall excursion at peak systole under normal physiological conditions, and can be as high as 10% under hypertensive conditions[71]. Mechanical stretching of cultured VSMCs has several effects, including reorientation of cells, changing protein and DNA synthesis, and increased production of extracellular matrix components[61]. Mechanical stretch also promotes stem cells to differentiate into VSMCs, and may also lead to transdifferentiation of ECs into VSMCs[70]. Chronically elevated cyclic wall stress is a feature of hypertension, which induces VSMC hypertrophy and hyperplasia [72]. Mechanical strain also leads to increased production of proteoglycans[73], and promotes extracellular matrix remodeling[70]. This cellular response aims to counterbalance the applied force by forming more extracellular matrix to buttress the vessel wall. Stent placement likely decreases the net cyclic strain sensed by the arterial wall by absorbing some of the radially directed force of pulsatile blood.

### 1.3.3.2 Shear Stress

Shear stress is the frictional force exerted at the endothelial apices caused by the flowing elements of the blood. The velocity of flowing blood is maximal in the center of the vessel lumen, and decreases as it approaches the wall[74]. The fluid can be viewed as divided into a series of adjacent layers moving at different velocities with respect to each other. The velocity differential creates the shearing flow. Blood flow is disturbed at

branch points and curved areas of the vascular tree, compared to the simple laminar flow in straight parts of the aorta[69]. The endothelium acts as a sensing surface to transduce shear stress forces, using surface integrins, intercellular adhesion molecules, VEGFR-2, ion channels, G-protein coupled receptors, and trimeric G proteins[69]. Endothelial cells change their genetic expression profiles with the different types of shear forces, i.e.: laminar, disturbed, or oscillatory flow[69]. Laminar flow results in the upregulation of anti-proliferative, anti-inflammatory, and differentiative properties, with down-regulation of genes associated with cell cycle progression and proliferation[69], which is of particular importance to atherosclerosis and localized neointima formation.

Abrupt reductions in fluid shear stress has been shown to induce VSMC proliferation in experimental prosthetic grafts[75]. Stenting likely disrupts the laminar shear of blood entering the aneurysm, leading to turbulent blood flow. We hypothesize that the stent-related disruption in blood flow will result in a change in genetic expression at the level of cells of the aneurysm wall, which we hope to correlate with angiographic and pathologic indicators of aneurysm healing.

## 1.4 ENDOVASCULAR TREATMENT RESPONSE

### 1.4.1 Overview

The primary goal of therapy is to protect patients from rupture or re-rupture; because of historical evidence with surgical clipping and balloon embolization, it is generally thought that stable, long-term angiographic exclusion of the aneurysm is the surrogate endpoint to aim for. To confirm this hypothesis, observational study of thousands of patients for many years would be necessary to determine clinical efficacy, something which is incompatible with the steady technical and clinical advances in this field. An alternative hypothesis, from pathological studies in animal models, suggests that a thick, complete neointima is the signature of a stable occlusion. Further refinements suggest that a thick neointima completely closing the neck of the aneurysm is the result of normalized flow patterns, with the intra-aneurysmal thrombus fully organized, without recanalization.

Endovascular therapy relies on the vascular biological response to lead to a successful outcome. Short term goals of therapy include occlusion of the aneurysm. While some methods can achieve this by purely mechanical means (surgical clipping, onyx glue or balloon embolization), endovascular treatment usually relies on thrombus to achieve this goal. Subsequent evolution of the formed intravascular thrombus is crucial to the long-term outcome of aneurysm treatment, which is dependent on the dominance of one of two phenomena: a) organization of the thrombus and neointima formation at the neck and b) recanalization [76]. The most important long-term outcome is protection from aneurysm rupture, but it is unknown what degree of protection, if any, is conferred by partial aneurysm occlusion. Given the paucity of natural history data regarding angiographic recurrences, an ideal result would also produce stable, non-threatening angiographic results. The anticipated pathological correlates of this angiographic endpoint would require the formation of a new, stable mural structure completely occluding the aneurysm, and covered by a continuous sheet of endothelium.

The molecular response to endovascular therapy is a complex and incompletely understood process that may be conceived of as a reaction to a form of vascular injury. The universal response to vascular injury involves neointima formation, as documented after a wide array of physical or chemical injuries to the inner (endothelial) or outer (adventitial) layers of the vessel wall. The molecular response to vascular injury depends on the nature and severity of the injury, but generally involves every type of cell in the vicinity (vascular smooth muscle cells, myofibroblasts, endothelial cells, platelets and leukocytes) as well as circulating cells, elements in the blood, and the extracellular matrix[56, 77, 78]. Because the response to embolic treatment is a relatively new area of investigation, many observed phenomena are interpreted in light of mechanisms and concepts previously evoked in other more mature research areas, such as re-stenosis following arterial balloon injury with or without stenting, angiogenesis, atherosclerosis, thrombosis, organization, inflammation, recanalization, and endothelialization. Most pertinent to this thesis are thrombosis and thrombus organization, and the processes of neointima formation, re-endothelialization, and recanalization.

## 1.4.1.1 Thrombosis and Inflammation

Clotting is a requisite component for circulatory homeostasis, and is involved in the recovery of vascular integrity following injury[79]. For inappropriate intravascular clotting, such as that triggered by pathological mechanisms, blood flow may be restored early, by thrombolysis, or late, through the process of recanalization[79]. Endovascular aneurysm treatment aims to form a thrombus localized within the aneurysm; the thrombotic reaction to the introduction of an intravascular foreign body, as well as the tissue reaction to the clot, involves mediators classically considered as part of inflammation. There is a link between an excessively vigorous inflammatory reaction and neointima formation, at least in terms of clinical models of in-stent restenosis[80]. A more detailed molecular explanation of the vast subjects of thrombosis and inflammation are considered beyond the scope of this thesis.

## 1.4.1.2 Thrombus Organization

Thrombus organization is the process that converts a thrombus into tissue. In a successfully organized thrombus, pathologists distinguish two sets of vessels: a) vasa vasorum of the outer and middle coats of the organized thrombus, and b) vessels that form channels within the thrombus[79, 81]. The organization of intravascular clots relies on several cellular events: i) the portion of the clot exposed to flowing blood is coated by monocytes, which subsequently penetrate the clot; ii) the flow-exposed surface of the clot is re-endothelialized, iii) myofibroblasts and neo-capillaries appear or develop within the tissues[79]. Occasionally, the neo-capillaries destined to provide blood supply to the newly formed tissue filling the aneurysm can re-join the parent vessel, contributing to thrombus recanalization (also see section 1.4.1.5).

In treated experimental aneurysm specimens, the tissue found within the aneurysmal sac continues as a 'neointima' at the neck, closing the entrance to the sac, and there is no clear demarcation between the so-called neointimal layer and the organized thrombus.

### 1.4.1.3 Neointima Formation

An integral aspect of aneurysm healing is neointima formation at the neck, representing a universal response to vascular injury, characterized by the formation of myxoid tissue with stellate-shaped smooth muscle cells in a loose extracellular matrix[82]. Traditionally, neointima formation was thought to occur in three phases: i) an acute phase, characterized by interactions of platelets, thrombin, and leukocytes which release biologically active mediators and activate medial smooth muscle cells; ii) an intermediate phase, where smooth muscle cells divide and migrate to the area of vessel injury; and iii) a chronic phase, where large amounts of extracellular matrix are produced and subsequently undergo a process of remodeling[77]. It is important to note that the classic 'neointima' was described in atherosclerosis models devoid of intima, or in endothelial injury models, leaving all other parietal layers to participate in the pathophysiological process; the classical theory emphasized VSMCs migrating from the media to the sub-endothelium[77], but others, using large animal models, described the importance of thrombosis, followed by endothelialization, with VSMC infiltrating the thrombus as a key feature in neointima formation[83-85]. In aneurysms, after endovascular treatment, we wish to see 'neointima' replacing and closing the space where blood was flowing. Hence, a provisional matrix (clot) is a sine qua non pre-requisite, and the neointima formed in fact represents replacement of the clot by a new parietal layer composed of 'organized clot'. The new parietal layer delimits the space with residual blood flow (as defined by the endothelium) from the contents of the occluded aneurysm, which subsequently fills with connective tissue. In this perspective, there is a continuum between clot organization (within the occluded sac) and neointima formation (closing the neck of a completely treated aneurysm).

The classical paradigm of neointima formation has been challenged by the discovery and implication of circulating blood elements in vascular repair. Circulating cells, in particular endothelial progenitor cells (EPCs), as well as dendritic cells, form part of a ever-expanding list of cells involved in this complex biology[86, 87]. The cellular milieu in which vascular healing occurs consists of many different cell types, producing and

responding to various chemical and mechanical stimuli. Some of the regulatory factors likely involved in this process will be discussed in section 1.5.

### 1.4.1.4 Re-endothelialization

The endothelial lining regenerates to cover the new mural structure filling the aneurysm orifice. Regenerated endothelial cells on the neointimal surface may migrate from adjacent healthy vascular wall, from the adventitial vasa vasorum, or arrive from the bloodstream[86, 88]. Following stent implantation in animal models, 20% of endothelialization occurs by 4 days, <40% at 7 days, while endothelialization is typically complete by 28 days[89]. However, the importance of the process and timing of re-endothelialization is a controversial topic[90]. One strategy in the cardiologists' battle against restenosis aims to reduce neointimal hyperplasia by promoting early re-endothelialization using growth factors such as VEGF [89]. Efforts within the neurovascular arena to diminish thrombo-embolic complications originating from the thrombus around the coil mass have also targeted early re-endothelialization as a therapeutic goal[28, 91]. However, our laboratory has shown an association between the integrity of the endothelial lining and recurrences; early re-endothelialization may counter-balance thrombus organization and promote the formation of endothelialized channels within the thrombus[92, 93].

### 1.4.1.5 Recanalization

Recanalization is a process that potentially compromises the long-term occlusion of endovascularly treated aneurysms. In general, circulatory homeostasis has two lines of defense against intra-vascular thrombosis, which in most cases is a pathological occurrence. Acutely, thrombolysis occasionally succeeds in opening up occluded vessels. However, when thrombolysis fails, the more chronic process of recanalization attempts to create new channels through or beside the clot to rejoin the patent portions of the vasculature. Much of the evidence for recanalization comes from work done on deep

venous thrombosis, although certain aspects of this process, such as the growth of progressively maturing channels through a matrix share conceptual similarities with angiogenesis and arteriogenesis. The theoretical importance of mechanical signaling is detailed in section 1.3.3.

Briefly, the restoration of blood flow across occluded blood vessels seems to depend primarily on the activity of two cell population, monocyte/macrophages (MC/MPh), and circulating progenitor cells[79]. MC/MPhs, under the influence of the chemokine MCP-1, act to penetrate the extracellular matrix formed by the mass of platelets and fibrin, and create tubular spaces, or 'tunnels'[94]. Matrix metalloproteinases are further known to be important to this process[94]. These tunnels are subsequently seeded with circulating progenitor cells. In turn, these (at least in some hypothesized paradigms) will differentiate into different types of cells depending on the depth of penetration. On the surface of the thrombus, they would differentiate into endothelial cells, while cells adopt a myofibroblast or smooth muscle character in the deeper layers[79, 95]. Ultimately, the capillaries formed in the deeper layers may form communications with the endothelium lined clefts at the surface of the thrombus. For pathological thrombi with a blood vessel, this can serve to re-establish blood flow across occluded vessels; in endovascularly treated aneurysms, this potentially leads to recurrences.

### 1.4.1.6 A more integrated view of aneurysm healing

Because research domains tend to develop along existing concepts, attempts to understand the mechanisms of aneurysm healing usually follow one of two paradigms. The first paradigm relies on similarities between the formation of neointima in models of arterial restenosis and at the neck of treated aneurysms, while the second emphasizes the similarities between angiogenesis and the formation of endothelialized clefts in recurrences. We have a tendency to conceive of the biological reaction to the presence of the endoluminal clot as consisting of two opposing phenomena; one assuring a permanent occlusion (organization/neointima formation) and the other promoting re-establishment of the lumen (recanalization). Both mechanisms, however, are in fact synchronous and

collaborative in restoring lumen patency and blood flow. It would be less efficient to attempt to remove the entire volume of the clot initially. Recanalization and re-endothelialization of channels permits the rapid re-establishment of some blood flow. Infiltration of the clot with myofibroblasts will allow the formation of an interconnected network of α-actin positive cells and extracellular matrix that can retract the organizing clot (Figure 1.6). As obstructing tissues are being 'contracted', the endothelialized spaces correspondingly expand, leading to a vessel lumen of increasing diameter and more efficient blood flow. Seen in this manner, both phenomena contribute to recovery of the integrity of the vessel. In this integrating perspective, recanalization leads to blood flow, and blood flow promotes recanalization.
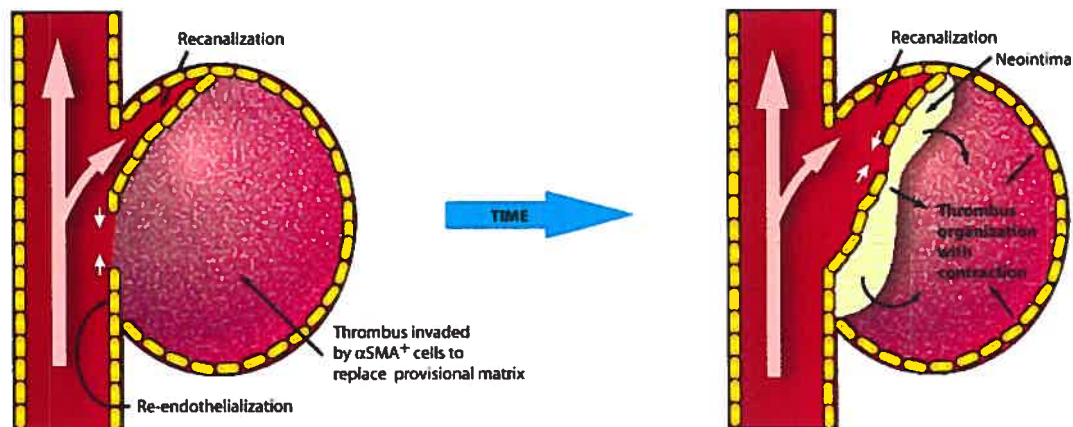


**FIGURE 1.6**

Schematic showing process of recanalization following endovascular treatment, with thrombus invasion by α-SMA cells and formation of small endothelialized channels between thrombus and aneurysm wall. Subsequent clot contraction, with continued hemodynamic forces, leads to channel expansion and aneurysm recurrence. (Illustration by G. Gevry)

One could also note that endothelialization, neointima formation, organization, and recanalization are the names or labels used to focus on one or another aspect of the vascular healing phenomenon, which in fact is a more complex and dynamic reaction than these concepts would suggest. These terms permit us to orient research into aneurysm

healing, but they have also been the origin of much confusion and misconception. Better characterization of the cells and molecular mechanisms involved in thrombus organization / neointima formation and recanalization promises to provide clues which can be used to decrease the rate of aneurysm recurrence. One of the goals of this work was to gain insight into the establishment of endothelialized channels that we have found to form within some treated aneurysms. For example, by examining the expression profile of the endothelial marker CD34 over time, we hoped to gain insight into the kinetics of endothelialization of these channels, which may represent the precursors to recurrences.

## 1.4.2 Cells involved in the response to endovascular treatment

Traditionally, cells were identified and classified on the basis of microscopic appearance, relying on differential stain uptake or typical topographical localizations to distinguish cell types. Advances in molecular diagnostics have added a level of complexity to cell typologies, which now rely on antibody recognition of cell surface markers with various degrees of specificity. The difficulty in exact determination of cell nature and origin comes from the fact that typical, characteristic, or so-called 'specific' markers can be described only when cells occupy their typical location and acquired their destined function. When anatomy and physiology are disturbed by some event, the transient, migrating, and proliferating cell types harbour intermediate phenotypes appropriate to their momentary functions and can no longer be identified with certainty using these 'specific' markers.

Initially, the cells that form the neointima were thought to be of smooth muscle cell origin because of α-smooth muscle actin staining, but subsequent investigation has led to uncertainty regarding the nature and origin of α-SMA+ cells. This has led to the adoption of multiple paradigms, depending on the pathobiologic process under study. For example, α-SMA+ cells found within the neointima have been assigned a medial VSMC origin in the cardiology restenosis and atherosclerosis literature, while the vascular wound healing and clot organization literature implicates the adventitial myofibroblast as the progenitor of α-SMA+ cells. More recently, circulating precursor cells have been

described, as well as a thesis involving the transformation of monocytes into myofibroblasts[79].

Similarly, classical theory taught that endothelial cells covering an intravascular thrombus migrated from adjacent healthy endothelial wall. With the recent discovery of circulating progenitor cells, both the origin of the cells that repopulate to coat the surface of the thrombus, as well as line the channels within the thrombus is now under considerable scrutiny. Both vessel-wall derived as well as circulating endothelial cells express endothelial-specific markers, including CD34, VEGFR2, Tie-1, Tie-2, VE-cadherin, E-selectin[96], and currently it is not possible to distinguish parietal from circulating endothelial progenitor cells.

In this thesis, I duly acknowledge the controversy surrounding the origins of α-SMA+ and endothelial cells, and categorize the cells involved in the endovascular treatment response into endothelial, neointimal, and circulating cells.

### 1.4.2.1 Endothelial cells

Endothelial cells are specialized epithelial cells that form a monolayer barrier between flowing blood and the blood vessel wall. The endothelium is supported by a basal lamina, which rests on a thin layer of fibro-collagenous support tissue separating the intima from the vascular smooth muscle cells. The endothelium plays a key role in vascular homeostasis, modulating vascular tone, caliber, and blood flow in response to humoral, neural, and mechanical signals.

In general, the function of the endothelium varies, depending on the state of the endothelial cells. In the basal resting state, the endothelium forms a non-thrombogenic layer, and secretes various vasoactive substances, including nitric oxide (NO) [97]. Nitric oxide, in addition to its vasodilatory effects, also modulates inflammation, platelet activation, and thrombosis[97]. Furthermore, NO counteracts leukocyte-endothelium adhesions, VSMC proliferation, and platelet aggregation[97]. In response to appropriate

chemical or mechanical signals (usually signaling disruption of endothelial integrity), endothelial cells adopt an activated state by altering their genetic expression. There are several consequences of this phenotype switch: endothelial cells secrete growth factors and trigger signaling cascades affecting adjacent endothelial cells and VSMCs, resulting in cell migration and proliferation. These signals further lead to extracellular matrix degradation to facilitate cell migration. Finally, endothelial cells alter their surface molecular expression profile. Upregulated surface adhesion molecules serve to arrest circulating cells, including platelets and leukocytes, to participate in the process.

The intravascular thrombus formed within an aneurysm (usually following endovascular treatment, although spontaneous aneurysm thrombosis can occur) unclenches a series of chemical signals that results in, among other things, endothelial cell activation. The presence of endothelial cells within the ensuing chemical milieu of the maturing clot is associated with incomplete aneurysm healing and recurrences[93, 98]. The expression of non-thrombogenic and thrombolytic surface molecules by endothelial cells within the clot has been suggested to contribute to the process of recanalization and aneurysm recurrence[93]. Following endovascular therapy, the non-endothelialized portion of the maturing clot exposed to blood flow presents a potentially thrombogenic surface. Efforts to promote early endothelial coverage of the clot have been suggested as a strategy to decrease thromboembolic complications[28, 91]. However, strategies that promote early re-endothelialization may also increase recurrence rates by prematurely populating the clot with endothelialized channels.

In our publication, we used CD34 as a marker of cells of endothelial lineage, hoping to follow the process of formation of endothelialized channels within the thrombus. We hypothesized that CD34 expression would increase steadily with time as channels became lined, and then plateau. We further hypothesized that the effects of stenting on mechanotransduction would decrease the ability of these channels to form, a difference that we hoped would be reflected in CD34 expression.

### 1.4.2.2 Neointimal cells

The origin of the α-smooth muscle actin (α-SMA) positive cells found within the neointima remains unclear (see section 1.4.2). Vascular smooth muscle cells and myofibroblasts are the most widely accepted candidates, although there is evidence that monocytes may also transdifferentiate into α-SMA+ cells[79]. One explanation is that following vessel injury, different types of local cells as well as arrested circulating cells respond to local signals and revert to a 'default' cell type best suited to the repair process[99]. Under this concept, it is possible for all three cell types to contribute to the body of α-SMA + cells observed within the neointima.

In this work, we assayed for the expression of α-SMA mRNA. In an arterial balloon injury model, smooth muscle cells are known to begin to proliferate in the media after approximately 24 hours [100]. After 4 days, they migrate into the intima, where they continue to proliferate and form extracellular matrix, a process which continues until steady-state is reached at 3 months, at which point the intima is estimated to comprise 20% cells and 80% matrix [101]. Recent work suggests that other cell types may also play a significant role in this process [102, 103]. In a porcine vascular thermal injury model that quantified macrophage and myofibroblast cells at the site of injury, the appearance of these other cells types was found at 14 days, and continued to increase until 28 days[78].

In our work, we hypothesized that stent deployment would lead to an increase in α-SMA+ cells within the neointima that would peak at 4 days and continue to steadily increase over time, thereby resembling an arterial balloon-injury response.

### 1.4.2.2.1 Vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) are highly specialized cells whose principal function is contraction and regulation of blood vessel tone, diameter, blood pressure, and blood flow distribution[104]. In the cardiology restenosis and atherosclerosis literature, VSMCs are typically described in terms of two phenotypes, contractile or synthetic. However, VSMCs have many different functional states, each with varied molecular expression profiles. VSMC plasticity further contributes to the difficulty of accurately determining cell of origin within neointimal tissue[104]. According to one paradigm, VSMCs change phenotype in response to local environmental cues, including growth factors/inhibitors, mechanical influences, cell-cell and cell-matrix interactions, and various inflammatory mediators[104]. In response to these cues, VSMCs "switch" to a more synthetic phenotype and secrete extracellular matrix, and migrate towards the vessel lumen. VSMCs thus serve to repair the injured vessel and to confer more rigidity to the vessel wall. According to another paradigm, the presence of specific growth factors, such as vascular endothelial growth factor (VEGF) or platelet-derived growth factor (PDGF) can lead adventitial fibroblasts, endothelial cells, or arrested circulating progenitor cells to differentiate into VSMCs[99].

It seems that for an aneurysm to become completely occluded, the fundus and neck of the aneurysm must be completely filled with extracellular matrix prior to re-endothelialization of the clot surface. Precise control of VSMC synthesis of extracellular matrix may facilitate the formation of the correct amount of organized tissue to completely obliterate aneurysms.

### 1.4.2.2.2 Myofibroblasts

Myofibroblasts are highly specialized mesenchymal cells that play a central role in tissue repair[105]. Current thinking is that myofibroblasts are derived from fibroblasts, which populate the arterial adventitia[99, 105]. One chemical signal triggering the phenotypic transformation from fibroblast to myofibroblast has been shown to be TGF-$\beta_1$, although

the mechanism remains obscure[105]. Myofibroblasts also express α-SMA, contributing to the controversy surrounding the origin of cells found within the neointima (see section 1.4.2). The primary role of these cells is to close the open space within the aneurysm body through the production of extracellular matrix and contraction of the organizing thrombus to contribute to the process of re-establishing the arterial lumen.

### 1.4.2.3  Circulating cells

Platelets and leukocytes are the classic circulating cells involved in the response to vascular injury. However, recent animal and some human evidence is accumulating that stem cells derived from mesenchymal or bone-marrow sources may seed tissues and contribute to the maturation of intravascular thrombus, to angiogenesis, endothelialization, or recanalization.

### 1.4.2.3.1  Platelets

Platelets are released from the bone marrow into the bloodstream to survey the integrity of the vascular system, and respond to breaches by becoming activated, degranulating, and forming aggregates adjacent to injured endothelial cells or exposed extracellular matrix elements[74]. During the process of activation, platelets form a procoagulant surface, stimulating the formation of thrombin and fibrin[74, 106].

Platelet degranulation releases multiple factors, including preformed ADP, serotonin, and thromboxane, which further promote thrombosis[107]. The ensuing molecular cascade signals leukocytes to roll and arrest on endothelial cells and migrate into tissues to repair injury, contributing to low-grade inflammation in the area.

The role of platelets is not solely pro-thrombotic; platelets and endothelial cells also release nitric oxide which prevents platelet adherence to the vessel wall, thereby providing a necessary negative feedback mechanism [107]. Platelets also release PDGF, which

further serves to inhibit thrombosis [93, 98], and plays an important role in VSMC migration and neointima formation. Platelets are also a source of TGF-$\beta_1$, which is involved in both neointima formation and angiogenesis.

The formation of thrombus is the sine qua non of a good endovascular outcome. An aggregated mass of platelets and fibrin is required to form the initial provisional matrix for subsequent cellular invasion to form neointima at the neck and organization within the body of the aneurysm. However, platelets are also the main culprit in thrombotic complications following the introduction of intravascular devices. Thus, in clinical practice, the use of indwelling intravascular devices such as stents require the use of two antiplatelet agents, acetylsalicylic acid (ASA) and clopidogrel (Plavix) to irreversibly inhibit platelet function. ASA irreversibly blocks the formation of platelet thromboxane A2, which inhibits platelet aggregation, while clopidogrel leads to irreversible blockade of ADP receptors on the platelet surface, inhibiting platelet aggregation, fibrin cross-linking on platelets, as well as inhibiting the glycoprotein IIb/IIIa pathway. While these agents may minimize initial thrombo-embolic complications, they could conceivably modulate subsequent healing or recanalization.

## 1.4.2.3.2 Leukocytes

Following vessel injury, circulating leukocytes respond to released chemokines and localize to the site of injury, where they become activated. Leukocytes, particularly neutrophils, contribute to the inflammatory component of tissue healing by upregulating expression of adhesion molecules and releasing vasoactive substances such as oxygen-derived free radicals, proteolytic enzymes, growth factors, cytokines and chemokines[77]. These substances can play a nefarious role by perpetuating further injury, both directly and by further activating leukocytes. However, they can also contribute to healing by stimulating VSMC proliferation and neointima formation[77].

Monocytes (CD14$^+$) are immune cells that differentiate into macrophages following their migration into tissue, and are commonly found within the substance of the neointima[78].

Monocytic/Macrophage (MC/Mph) cells participate in neointimal formation by releasing cytokines, metalloproteinases, and growth factors[86]. In the presence of high levels of MCP-1, MC/Mph cells have been shown to form "tunnels" within thrombus[94], creating conditions necessary for recanalization of arterial or venous clot. The formation of these tunnels through organizing clot allows for the re-establishment of blood flow following intravascular occlusion. In the case of formed clot inside a treated aneurysm, the tunnels thus formed may be the beginning of an aneurysm recurrence, requiring appropriate hemodynamic and chemical stimuli for further growth and development. The process of channel formation within the thrombus, termed recanalization, shares conceptual and theoretical similarities with angiogenesis; clues to the molecular mechanisms occurring during aneurysm recurrence may be found by looking for known angiogenetic factors.

More recently, these cells have been hypothesized to have the capacity to transdifferentiate into either myofibroblasts or endothelial cells, reviving the old pathological paradigm of bone-marrow origin for these cells[108].

### 1.4.2.3.3 Progenitor cells

Following endovascular treatment, circulating progenitor cells may arrest at the treatment site and differentiate into endothelial cells to contribute to the process of re-endothelialization/recanalization, or into α-SMA+ cells that populate the neointima and contribute to the formation of extracellular matrix. Mesenchymal progenitor cells (CD34$^+$) can differentiate into both neointimal and endothelial cells, while recently, a class of CD34$^-$CD14$^+$ cells derived from bone marrow mononuclear cells have been identified that are also able to differentiate into endothelial cells.

### 1.4.2.3.3.1 Mesenchymal progenitor cells

Mesenchymal progenitor cells (MPCs) exhibit a high degree of plasticity, and can differentiate into several tissue types, including VSMCs and endothelial cells[109]. MPCs

can be isolated from bone marrow, umbilical cord and peripheral blood[109, 110]. Differentiation of MPCs depends on the presence of appropriate physiologic signals, which includes PDGF and proper cell-cell couplings[109]. Circulating MPCs may seed the tunnels formed by monocytes and differentiate into endothelial cells to line the channels found within maturing thrombi[79].

## 1.4.2.3.3.2 Endothelial progenitor cells

There are at least two classes of circulating endothelial progenitor cells (EPCs), CD34$^+$ (derived from MPCs) and CD34$^-$CD14$^+$ cells (derived from bone-marrow mononuclear cells (BM-MNCs)). These cells are able to integrate into the vasculature of tissue undergoing repair, participate in angiogenesis, and may contribute to the process of recanalization[68]. As with MPCs, the differentiation and behaviour of these EPCs into endothelial cells is determined by their cellular milieu[68]. The regulation of differentiation of these cells is complex, involving cell-matrix interactions, secreted soluble factors, resident vascular cells, and peripheral blood mononuclear cells[68]. These regulatory factors interact and modulate the effects of one another and are themselves further regulated by physiologic stimuli, including mechanical forces.

Because both parietal and circulating endothelial progenitor cells express the same cellular markers, there is no recognized means to distinguish these cell types[96]. This is further complicated by the fact that subsets of hematopoietic cells express markers similar to those of endothelial cells such as CD34, PECAM, vWF, VEGFR2, Tie-1 and Tie-2. Therefore, the relative contribution of endothelial progenitor cells to the process of re-endothelialization/recanalization following endovascular treatment remains unclear, but offers an alternative hypothesis to the origin of the endothelial cells found within the maturing thrombus.

### 1.4.3 Extracellular matrix

The extracellular matrix (ECM) is made up of collagens, structural glycoproteins, and proteoglycans[111], and is primarily produced by VSMCs and fibroblasts[112]. The ECM makes up an estimated 80% of the neointimal volume[101], and ECM components also form basement membranes and the internal elastic lamina. These layers serve as structural support for cells, but also form barriers between tissue compartments regulating the cellular migration. The ECM acts as a substrate for cell adhesion, spreading, and migration, and is important for the growth, differentiation, and survival of adherent cells[60, 113, 114]. As evoked in section 1.4.2.3.1, the pertinent matrix at the level of the lumen of the aneurysm as well as at the ostium is continuously evolving as a result of cellular migration from the provisional platelet-fibrin clot to the more mature matrix found in the neointima at the neck and the organizing clot in the fundus of the aneurysm. Following endovascular treatment, an injury repair program is initiated, which leads to migration and proliferation of cells to and within the neointima, along with active secretion of extracellular matrix.

The newly secreted extracellular matrix can be covalently linked to neointimal cells via cell surface integrins, which sense the integrity of the surrounding matrix. In this manner, cells can be provided with directional guidance clues for migration[60]. The linkage provided by the 'α-SMA+ cells-integrins-collagen' network also allows matrix contraction, as shown in fibrin gel and collagen gel cultures of VSMCs[115]. The fluidity of the matrix can be modulated by the balance of activity of a class of proteases known as matrix metalloproteinases and their inhibitors.

### 1.4.3.1 Matrix metalloproteinases and their inhibitors

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that degrade ECM components under physiological and pathological conditions[116]. There are 24 identified members of the MMP family, classified into 4 groups, including the collagenases, gelatinases, stromelysins, and membrane types (MT-MMPs) [117]. MMPs, particularly

MT-MMPs, are also essential to endothelial cell migration into fibrin gels[118]. Of these, the gelatinases MMP-2 and MMP-9 are most important to us, because these enzymes degrade denatured fibrillar collagens (gelatin), including elastin [119]. These potent proteolytic enzymes are secreted by a variety of cell types, including endothelial cells, VSMCs and macrophages[116]. MMPs activity is countered by another group of enzymes known as the tissue inhibitors of metalloproteinases (TIMPs)[116, 120]. The balance of MMP and TIMP activity controls the diffusion of substances and the migration of cells through the ECM. These proteinases also modulate signal transduction pathways by acting on a wide variety of substrates, including inflammatory mediators, growth factors, and growth factor receptors[120].

Considerable work has been done to elucidate the kinetics and expression of MMP-2 and MMP-9 under various experimental conditions. In an in vivo model of a surgically created arteriovenous fistula resulting in increased shear stress, MMP-2 and MMP-9 levels were found to be elevated; MMP-2 levels peaked at 2 days and remained elevated, whereas MMP-9 levels peaked at 2 days and then quickly decreased[121]. This result was interpreted as a response of MMPs to degrade the basement membrane and both permit ECs to migrate into a larger area and to permit VSMCs to migrate and re-orient themselves to adapt to arterial enlargement, with MMP-2 playing a more lasting role, and MMP-9 having more transient effects[121]. Subsequent in vitro work has shown MMP-9 expression to be responsive to the shear pattern, with oscillatory or turbulent blood flow resulting in greater, sustained expression compared to unidirectional shear, which demonstrated a lesser, more limited increased expression [122]. Arterial balloon injury and subsequent remodeling increase the production and activation of MMP-2 and MMP-9 [123, 124]. MMP-9 has also been localized to atherosclerotic plaques in humans[125]. Another model, testing the effects of transient low flow on a background of chronically elevated high flow, showed increased MMP-2 expression while MMP-9 expression decreased[126]. Perhaps the model most similar to ours is the variable flow model of Bassiouny et al., where animals with experimentally decreased blood flow showed increased MMP-2 expression [127].

MMP-9 is also essential to angiogenesis. Deficient angiogenesis in MMP-9 -/- mice can be rescued by bone marrow transplant of MMP-9 +/+ cells, supporting a role for both MMP-9 and circulating cells in the formation of new vessels, or even recanalization[128].

We hypothesize that stenting will lead to decreased flow entering the aneurysm, with subsequent increases in MMP-2 and MMP-9 expression. An increase in TGF-$\beta_1$, which is known to increase MMP-2 and MMP-9 expression[129], may further increase the levels of these MMPs. As observed with the high shear models, we anticipate that the increase in MMP-9 will be of brief duration, whereas that of MMP-2 will be more sustained.

The expression of the TIMPs is closely related to MMP expression; tight regulation is essential for these potentially highly destructive enzymes. In response to appropriate stimuli, including mechanical stress, the balance between MMP and TIMP activity can be altered, resulting in increased matrix degradation. We hypothesize that the changes in mechanical force caused by the stent, in conjunction with the released molecular mediators, will lead to either changes in TIMP expression in the same direction, but at a lesser magnitude, than MMP expression, or increased MMP expression and decreased TIMP expression.

## 1.5  REGULATION OF THE VASCULAR RESPONSE TO INJURY

The vascular response to injury is a series of coordinated events involving many different cell types, driven and regulated by locally released mediators, cell-cell and cell-matrix adhesions, and transduced mechanical forces.

### 1.4.1  Soluble factors

There are a plethora of soluble factors potentially involved in the localized response to endovascular therapy, which function by altering the molecular expression of cells exposed to their effects. Binding and expression kinetics of these cytokines are further

dependent on the affinity of the receptor for the soluble factor, which in turn can also be influenced by other cytokines [130]. The differences in pleiotropy, dose-dependence, binding kinetics, and potential multiple interactions of the soluble factors produced by the different cell types of this in vivo aneurysm model (described in more detail in section 3.3.1) amounts to considerable molecular complexity. For purposes of simplification, I chose to focus on the soluble factors PDGF-BB, TGF-$\beta_1$, MCP-1, and TNF-$\alpha$, which are known to be important to vascular injury repair, clot organization, and neointima formation[131-133].

In the molecular portion of this thesis, we attempt to quantitate and compare the level of mRNA expression of these cytokines by cells within aneurysms in the experimental (stented) and control groups. It is difficult to predict the effects of stenting on mRNA expression due to the number of potential confounders in this macro translational research model. Achieving experimental isolation is difficult at best; controlling for potential sources of bias is also difficult. Yet experiments such as these offer the promise of demonstrating which cytokines are the best candidates for future experimental manipulation in hopes of improving outcomes following endovascular therapy. What follows is a brief description of four soluble factors we thought were most likely to be involved in regulating the endovascular treatment response.

### 1.5.1.1 Platelet derived growth factor-BB

Platelet derived growth factor (PDGF) is a potent chemoattractant and mitogen produced by activated platelets and macrophages that stimulates VSMC proliferation and migration[104]. PDGF is a composed of variable combination of A and B chains linked by a disulfide bridge, resulting in three different isoforms (AA, BB, AB). PDGF initiates a signaling cascade following binding to their tyrosine kinase receptors PDGFR-$\alpha$ or PDGFR-$\beta$ [104]. PDGF stimulates monocytes/macrophages to produce other cytokines, including TGF-$\beta_1$[134]. PDGFR-$\beta$ signaling, mediated by PDGF-BB, is important to neointima formation, because it stimulates VSMC migration and proliferation[104, 135].

Experiments to surgically manipulate blood flow in vivo show that the levels of PDGF-BB expression are increased in response to reduced flow [136]. In vitro, cultured endothelial cells increase their expression of PDGF-BB in response to acute increases in shear stress or turbulent flow [137]. However, chronic exposure to shear stress suppresses PDGF expression[138]. Cyclic strain (section 1.3.3.1) also increases PDGF expression[71]. We hypothesize that stent deployment across the aneurysm neck will acutely disrupt blood flow into the aneurysm, leading to turbulent flow and increased PDGF-BB expression. Stenting likely also decreases cyclic strain, by limiting the radial force applied to the vessel wall, which we hypothesize will decrease PDGF-BB expression. The net effect of these conflicting stimuli on the genetic expression and formation of neointima awaits experimentation.

## 1.5.1.2 Transforming growth factor-$\beta_1$

Transforming growth factor-$\beta_1$ (TGF-$\beta_1$) has been described as a multifunctional regulator; its actions are dependent on species, cell phenotype, growth conditions, and interaction with other growth factors[121, 139]. It plays a central role in normal development, tissue repair, as well as some pathological processes. Depending on the type of model being studied, TGF-$\beta_1$ exerts different effects: in normal arteries, TGF-$\beta_1$ is atheroprotective, stimulating proteoglycan formation and inhibiting VSMC proliferation[140, 141], whereas in the settings of atherosclerosis and restenosis, it promotes lesion formation, by promoting VSMC proliferation and inducing ECM fibrosis[142, 143]. TGF-$\beta_1$ expression changes occur within 24 hours[144] in response to many different stimuli, including changes in shear stress [126]. In general, increased shear stress leads to increased TGF-$\beta_1$ expression, although intermittent decreases in shear stress in a arteriovenous fistula model chronically exposed to high shear stress also caused an increase in TGF-$\beta_1$[126].

TGF-$\beta_1$ can exert its effects by stimulating VSMC proliferation, increasing ECM protein synthesis, and alter activity of MMPs to facilitate the migration of fibroblasts and

macrophages[105, 145]. TGF-$\beta_1$ can also promote endothelial cell and VSMC differentiation[104, 144].

Cells recovered from the necks of treated experimental aneurysms have been shown to be TGF-$\beta_1$ sensitive, and local delivery of TGF-$\beta_1$ can increase neointimal thickness[146]. TGF-$\beta_1$ may promote aneurysm healing by increasing extracellular matrix deposition and over-expression of TGF-$\beta_1$ has been considered as a treatment strategy[147].

In particular, we seek to characterize the effects of TGF-$\beta_1$ on neointima formation within experimental aneurysms. Because both increases and decreases in shear stress increased TGF-$\beta_1$ levels, we hypothesize that stenting will increase TGF-$\beta_1$ expression and augment neointima formation.

### 1.5.1.3 Monocyte chemotactic protein-1

Monocyte chemotactic protein-1 (MCP-1) is a potent and specific activator of monocytes and basophils that promotes monocyte migration[148]. MCP-1 is secreted by vascular cells, including endothelial cells, VSMCs, and fibroblasts, to attract circulating monocytes to the arterial wall[148, 149], where they promote neointima formation[150, 151]. MCP-1 is implicated in the initiation and progression of atherosclerotic lesions, as well as in neointimal hyperplasia following balloon injury[152].

The role of MCP-1 in the evolution of intra-vascular thrombus is of particular interest to our work. In the presence of high levels of MCP-1, monocyte/macrophage cells have been shown to form "tunnels" within thrombus, creating conditions necessary for the seeding of circulating progenitor cells which subsequently organize and potentially recanalize maturing clots[94] (see section 1.4.2.3.3.2). Factors which increase MCP-1 may not only increase neointima formation, but also promote clot recanalization and predispose endovascularly treated aneurysms to recurrences.

The expression of MCP-1 is known to be responsive to shear stress[153]. The introduction of laminar shear stress to cultured endothelial cells induces a biphasic

response of MCP-1 expression, peaking at 1.5 hours and then returning to baseline by 4 hours[154]. Steady laminar shear stress continued for greater than 5 hours leads MCP-1 expression to become completely quiescent. Continued laminar shear stress is thus thought to be atheroprotective by reducing MCP-1 expression [153]. The complexity of the molecular milieu is further increased by in vitro work which demonstrates variability of MCP-1 expression in response to changes in temporal gradient of the flow profile to which endothelial cells are exposed[155].

In our aneurysm model, the blood flow entering the aneurysm is not laminar [66]. We hypothesize that MCP-1 expression will be increased by the disruption of blood flow caused by stent placement across the aneurysm ostium. We anticipate that the rise in MCP-1 will correlate with increased neointimal formation within the aneurysm. Also likely affecting MCP-1 expression at the level of the aneurysm in our in vivo model will be the local cytokines TNF-α and PDGF, which have been shown to upregulate MCP-1 expression in cultured endothelial cells [156].

### 1.5.1.4 Tumour necrosis factor-α

Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine produced by macrophages, neutrophils, endothelial cells, and VSMCs [157, 158]. TNF-α acts on a variety of cell types and exerts multiple biological effects. TNF-α is known to upregulate the expression of the adhesion molecules, including VCAM-1, and MCP-1 which further attract leukocytes to the area. TNF-α can also alter the expression of growth factors, modulate endothelial cell apoptosis, and stimulate MMP activity[158, 159].

Circumstantial evidence from balloon-injury models of restenosis also implicate TNF-α activity in formation of neointima[160]. TNF-α expression by medial VSMCs precedes their proliferation [161], and TNF-α levels have been found to be elevated in atherosclerotic artery segments [162]. Finally, the addition of a TNF-α antagonist has been found to diminish neointima formation in rabbits [163]. Although the precise mechanism of action remains obscure, the downstream effects of TNF-α mediated

signaling are beginning to be understood [164, 165]. Laminar or high shear stress is known to inhibit production of this cytokine[160], while areas of turbulence or low shear stress, where atheroma forms, have high TNF-α levels[130].

Concerning experimental aneurysms, turbulent blood flow or low shear stress augments TNF-α expression, and we hypothesize that stenting will increase the turbulence of blood flow into the aneurysm and increase TNF-α expression. We further anticipate the rise in TNF-α will increase VCAM-1 expression.

## 1.5.2   Adhesion Molecules

## 1.5.2.1 Cell-cell interactions

Cellular interactions are critical events in vascular biology, resulting in intercellular adhesions and transfer of information through signaling pathways. Specific molecular mechanisms govern these interactions, the simplest of which involves an adhesion molecule of one cell binding the counterligand of another[166]. This binding of cells together can transmit nuclear signals which alter cellular function. Many intercellular adhesion molecules have been identified, but for the purpose of this thesis I will focus on those assayed experimentally, which are VCAM-1 and PECAM-1 (CD31).

Integrins also mediate adhesions between cells[167]; however, the majority of their interactions are with the extracellular matrix; integrins are discussed in section 1.5.2.2.1.

## 1.5.2.1.1 Platelet endothelial cell adhesion molecule-1

Platelet endothelial cell adhesion molecule-1 (PECAM-1), also known as CD31, is a glycoprotein expressed on the surface of platelets, monocytes, neutrophils, macrophages, and some T lymphocytes[168], and is involved in leukocyte aggregation and endothelial-leukocyte interactions [69]. In solitary cells, PECAM-1 is diffusely distributed across the cell surface, but once a cell-cell contact is made, PECAM-1 localizes to the cell-cell

junction[169], which has been shown to be the site of greatest tension within the membrane when shear stress is applied [170]. At the cell membrane, PECAM-1 is reported to participate in at least two important mechanosensory complexes; first, a trimolecular complex with VEGFR2 and vascular endothelial cell cadherin, which responds to shear stress via the phosphorylation of a PECAM-1-associated kinase, initiating nuclear signaling[171]; and second, a complex with endothelial nitric oxide synthase (eNOS), which responds to high levels of shear stress by producing NO to vasodilate the blood vessel[172].

PECAM-1 is thus both an essential component of endothelial cell junctions and a modulator of various biological processes, including wound healing, thrombosis, inflammation, cell adhesion, and signal transduction[168]. However, PECAM-1 expression does not change in response to shear stress[173, 174], and the expression levels were used as an internal control in our stenting experiments.

## 1.5.2.1.2 Vascular cell adhesion molecule-1

Vascular cell adhesion molecule-1 (VCAM-1) is expressed on endothelial, epithelial, macrophage, and dendritic cells[175], where it serves to bind those cells expressing the integrin very late antigen-4 (VLA-4): monocytes, lymphocytes, basophils, and eosinophils[176]. VCAM-1 expression is increased following rat arterial balloon injury, and is implicated in the formation of neointima [152]. VCAM-1 expression is induced by pro-inflammatory cytokines including TNF-$\alpha$, however, shear stress downregulates the expression of VCAM-1[177-179]. At areas of laminar blood flow, this would confer an atheroprotective effect, by decreasing leukocyte tethering.

With respect to aneurysms, we anticipated that stenting would further reduce the shear of the flow of blood entering the aneurysm, increasing VCAM-1 levels. We hypothesized that the increased tethering of leukocytes might promote more complete neointima formation within the aneurysmal sac and around the stent.

## 1.5.2.2 Cell-matrix interactions

Cell-matrix interactions enable several important biological processes. First, they allow for cell movement by providing traction for migration, by assembling extracellular matrices to serve as tracks for migration, and by transmitting guidance signals that serve to direct cells to targets[180]. Second, they form adhesions which allow for tissues to withstand mechanical loads, and are essential to tissue integrity[180]. Finally, these interactions influence cell proliferation and differentiation through signaling pathways[61]. The majority of cell-matrix interactions are mediated by cell surface receptors known as integrins. Integrin expression is regulated by growth factor signaling, through interactions with the matrix and other cells, as well as through mechanical signaling pathways[60, 181].

## 1.5.2.2.1 Integrins

Integrins are a family of cell surface receptors that mediate the binding of the cell to specific components of the extracellular matrix. There are at least 24 known integrins, each recognizing several matrix proteins; conversely, most matrix proteins bind more than one integrin[182]. The significance of this cellular tether is that integrin-mediated matrix binding can initiate an increasingly recognized number of intracellular signaling cascades, with multiple downstream effects[69]. The dynamic environment of the extracellular matrix is thus communicated to the intracellular machinery via integrins.

Integrins are involved in multiple physiological and pathological processes, including apoptosis, tumour growth and metastasis, aneurysm formation and are likely involved in the response to endovascular treatment [183]. Integrin signaling is known to modulate MMP activity[184], and may exert their effects through this pathway.

We chose to assay for mRNA expression of one integrin, $\alpha_v\beta_{III}$, which is expressed on platelets, VSMCs, and endothelial cells. Like the other integrins, it mediates intercellular adhesions and cell-ECM adhesions[185], but integrin $\alpha_v\beta_{III}$ is responsive to shear stress.

Shear stimulates a conformational change in the integrin, leading to increased binding of ECM ligands, which initiates nuclear signaling via multiple pathways[171, 186, 187]. Integrin $\alpha_v\beta_{III}$ expression by SMCs in vitro is not responsive to alterations in shear stress[188], but because integrin $\alpha_v\beta_{III}$ is elevated in atherosclerotic plaques compared to controls[189], we hypothesize that the increased nuclear signaling or migration of cells through the ECM following endovascular treatment might increase integrin $\alpha_v\beta_{III}$ expression. Table 1 summarizes the hypothesized and observed effects of stenting on the expression of the potential genes of interest.

**Table 1.** Genes potentially involved in the molecular mechanisms of aneurysm healing, with the hypothesized expression changes obtained from a survey of the literature of other vascular processes, and our experimental observations.

| | Expression Changes due to Stent | |
|---|---|---|
| GENE | Hypothesized | Observed* |
| | | |
| TGF-β | ↑ | ↑ |
| PDGF-BB | ↑ | ↑ |
| MCP-1 | ↑ | ↓ |
| TNF-α | ↑ | ↓ |
| | | |
| VCAM-1 | ↑ | - |
| | | |
| MMP-2 | ↑ | ↑ |
| MMP-9 | ↑ | ↑ |
| TIMP-1 | ↓ | ↓ |
| TIMP-3 | ↓ | ↓ |
| TIMP-4 | ↓ | ↓ |

# 2. RESEARCH PRESENTATION

## 2.1 PROJECT FRAMEWORK AND CONCEPTUAL LINKS

This thesis is built around a central idea; to discover novel ways to promote healing after endovascular treatment, inspired by Rudolph Virchow's triad. Following work done by the prominent German pathologist, three risk factors for DVT/PE (deep venous thrombosis / pulmonary embolism) formation were identified and popularized as Virchow's Triad: 1) venous stasis, 2) traumatic injury to the blood vessel, and 3) hypercoagulable state. These factors have been widely recognized to predispose patients to morbidity or mortality from the formation of intra-vascular clot in the deep veins, particularly in the lower extremity, of which portions can break off and embolize to the lungs.

The parallels between DVT formation and endovascular aneurysm treatment begin with the iatrogenic use of intravascular devices in an attempt to form a localized thrombus within the aneurysm. We hypothesized that factors important to the formation of venous clot might also apply to the formation and favorable evolution of intra-aneurysmal clot. In this thesis, we performed experiments to manipulate two of the Virchow's factors: First, we used stents to alter blood flow patterns, both within the parent vessel as well as into the aneurysm. Because intra-aneurysmal thrombosis is the sine qua non of endovascular therapy, our hope was that the stent would lead to sufficient blood stasis within the aneurysm to trigger thrombosis, and possibly mitigate any hemodynamic effects leading to aneurysm recurrence. Second, we mechanically removed the endothelial lining of the experimental aneurysms, akin to a traumatic injury to the blood vessel, to further incite localized thrombus formation. Removal of the endothelial cells exposes a thrombotic surface, removes the anti-thrombotic and thrombolytic character of the endothelial cells, and further removes the signaling contribution of this cell type to the evolving aneurysm thrombus. Although we did not experimentally manipulate the third

risk factor, hypercoagulability, it is interesting to note that localized efforts to promote coagulation have been attempted by coating coils with thrombogenic material[25].

By using experimental aneurysms, we were able to observe the effects of modifying flow (with stenting) and traumatizing the blood vessel (with endothelial denudation) on the formation and evolution of intra-vascular thrombus, using a clinically important surrogate end-point, angiography, but also, to study the cellular and biochemical evolution using pathology and modern molecular techniques.

## 2.2   HYPOTHESES

1. The addition of endothelial denudation to the endoluminal stenting of experimental aneurysms can improve angiographic and pathologic results.

2. Endoluminal stenting poses a risk to branch vessels that are covered by the stent.

3. Stenting will alter the levels of mRNA expression of key molecules likely involved in the molecular response to endovascular treatment.

4. Endothelial denudation will drastically modify mRNA expression after stenting (future work).

## 2.3 RESEARCH GOALS

### 2.3.1 General Objective

We seek to improve the clinical and angiographic outcomes of endovascular treatment of intracranial aneurysms, by furthering the comprehension of the biological processes involved in the formation, organization, and evolution of intra-aneurysmal thrombus. Identification of key cells or molecular cascades involved the formation of stable, durable intra-aneurysmal tissue, or conversely, those involved in undesirable effects, such as recanalization, will allow us to develop new technical or molecular strategies to reduce the morbidity and mortality of intracranial aneurysms.

### 2.3.2 Specific Objectives

A specific goal of this project is to understand the nature, involvement, and relative contributions of various key molecules and cells involved in thrombus formation, organization, and evolution following endovascular treatment. Our endeavours focus on the influence of stenting on cellular signaling pathways, cell-cell and cell-matrix interactions, as well as the effects of stents on enzymes that modify the consistency of the extracellular matrix.

Additional goals include insights into the process and kinetics of intra-arterial thrombus organization and the formation of endothelialized channels within an evolving thrombus exposed to hemodynamic forces.

We also studied the effects of combining endothelial denudation with stenting to see how this cell type altered angiographic and pathologic outcomes.

On a clinical note, stents are increasingly used to treat intracranial aneurysms, in spite of the lack of experimental support for their efficacy. A final goal is to raise clinician awareness of the factors to consider when using intracranial stents to treat aneurysms.

# 3. MATERIALS AND METHODS

## 3.1 *IN VIVO* METHODOLOGY - SURGICAL MODELS

Our laboratory has developed an experimental canine carotid lateral wall vein pouch aneurysm model. We used beagles weighing 8-12 kg. Protocols were approved by the Institutional Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. All procedures were performed under general anaesthesia. Animals were sedated with acepromazine (0.1 mg/kg), glycopyrrolate (0.01 mg/kg), and butorphanol (0.1 mg/kg), and anaesthesetized with intravenous thiopental (15 mg/kg). Animals were ventilated artificially and maintained under anaesthesia with 2% isoflurane. Post-operative analgesia was provided for three days with a 50 μg fentanyl patch.

### 3.1.1 Lateral carotid aneurysm model

Lateral carotid aneurysms were created according to the model of German and Black, as modified by Graves[190, 191]. In 25 beagles, through a midline incision, a portion of the external jugular vein is harvested and divided into two segments of equivalent length. Following exposure of the carotid artery, temporary vascular clips are placed to interrupt circulation. An arteriotomy is made and the vein is anastomosed to the artery in an end to side fashion using running 7.0 Prolene suture. The fundus of the vein pouch is secured with hemostatic clips and circulation is restored.

Following a period of 4-6 weeks to allow the anastomoses to heal, the endovascular procedure was performed. Transfemoral access was obtained, and following angiography, a balloon-expandable stent (BX Velocity, Cordis, Miami, Florida, USA or Herculink Plus, Guidant, Santa Clara, California, USA) ranging in length from 13-33 mm and in diameter from 3.0-4.5 mm were positioned within the parent vessel to bridge the aneurysm neck and deployed. The contralateral aneurysm underwent balloon angioplasty using the same

balloon used to expand the stent to serve as a control. Dogs have a more active fibrinolytic system than humans, and therefore did not receive any antiplatelet agents or anticoagulants, as is common practice with this experimental model[42]. Angiograms were performed prior to and immediately following the stenting procedure, and prior to sacrifice. Twenty animals underwent a separate harvesting procedure at 4 hours (n=3), 1 day (n=3), 4 days (n=6), 7 days (n=3) and 14 days (n=5) post-stenting, where the portion of the carotid artery bearing the aneurysm was removed under anaesthesia. This was performed to minimize the risk of post-mortem artifacts on mRNA quality and quantity. Following successful harvesting, the animal was sacrificed. The aneurysm was cut from the parent vessel at the neck, and two sections were taken; a 2 mm axial section of the neck region of the aneurysm, and a 3 mm section of the dome of the fundus. This allowed for comparison of gene expression at the neck, where we hypothesized mechanical signals would be greatest, with that at the fundus. At the outset of our study, samples were directly frozen in liquid nitrogen, but following the high rate of mRNA degradation with this technique, we began to immerse the tissue in Trizol (Invitrogen, Canada) prior to freezing until biochemical analysis. An additional 5 dogs did not undergo this harvesting procedure; their aneurysms were designated for pathological analysis. Following the initial stenting procedure, they underwent angiography and sacrifice at 12 weeks and their carotid-aneurysm complexes were removed and placed in formalin for pathological analysis.

### 3.1.2 Lingual aneurysm model

The lingual aneurysm model is a modification of the lateral wall carotid model, where the aneurysm is constructed more distally along the carotid artery near the origin of the lingual artery[192]. The proximity of the branches to the aneurysm allows for the study of the effects of stenting on branch occlusion and flow alterations in the small vessels covered by the stent.

In eight dogs, through a midline incision, the external jugular vein was harvested and divided into two equal parts to form vein pouches. One vein pouch was temporarily

inverted and mechanically scraped to remove the endothelial lining prior to anastomosis. On each side, the lingual nerve was identified and mobilized, and temporary aneurysm clips were applied to branch vessels. An arteriotomy was created proximal to the site of the lingual artery origin, which represents a modification from the original, where the aneurysm was created distal to the branch. End-to-side anastomoses of the vein segment to the artery were fashioned, with normal vein on one side, and denuded vein on the other. The suture line was sealed with running 7.0 Prolene suture, and the aneurysm pouches sealed with hemostatic clips. Circulation was then restored.

The endovascular procedure was performed immediately following closure of the surgical site, as there was no perceived advantage to await aneurysm maturation for this type of study, as well as to minimize the number of anaesthetics administered to the animals. Transfemoral access was obtained, and following angiography, bilateral balloon-expandable stents (BX Velocity and BX Sonic, Cordis, Miami, Florida) of lengths 18-28 mm and diameters 2.5-3.5 mm were positioned to bridge the aneurysm neck and lingual artery, and deployed. Animals did not receive any antiplatelet agents or anticoagulants. Angiograms were performed immediately following stent deployment, and prior to sacrifice at 10 days (n=2), 10 weeks (n=4), and 20 weeks (n=2) post-stenting. Following femoral access with catheters larger than 5F, we performed cutdown over the femoral artery, which was identified and tied off. For endovascular procedures with catheters 5F or smaller, simple compression of the puncture site was used.

### 3.1.3 Carotid bifurcation aneurysm model

This surgical construct features a wide-neck aneurysm at the site of bifurcation of a major blood vessel[193]. This model more closely mimics a basilar tip aneurysm, which is exposed to significantly greater hemodynamic forces than are sidewall aneurysms.

In four dogs, through a midline incision, both carotid arteries were exposed and temporarily clipped with aneurysm clips. The left carotid artery was tied off at the origin, mobilized under the trachea, and anastomosed to the contralateral carotid artery. A

portion of harvested external jugular vein, either normal (n=2) or denuded (n=2), had two slits cut longitudinally on one side, and was anastomosed to the junction of the two carotid arteries, forming a bifurcation aneurysm (see Figure 3.1 for details). The dome of the vein pouch aneurysm was then sealed with hemostatic clips, and circulation was restored. The endovascular procedure was performed immediately following closure of the surgical site. Using transfemoral access, angiography was performed, and a single balloon-expandable stent (Herculink Plus, Guidant, Santa Clara, CA) of length 18 mm and diameters 4.0-4.5 mm were positioned within the parent vessel to bridge the aneurysm neck and deployed. Animals did not receive any antiplatelet agents or anticoagulants. Angiograms were performed immediately following stent deployment, and prior to sacrifice at 5 (n=2) and 10 weeks (n=2). Following femoral access with catheters larger than 5F, we performed cutdown over the femoral artery, which was identified and tied off. For endovascular procedures with catheters 5F or smaller, simple compression of the puncture site was used.
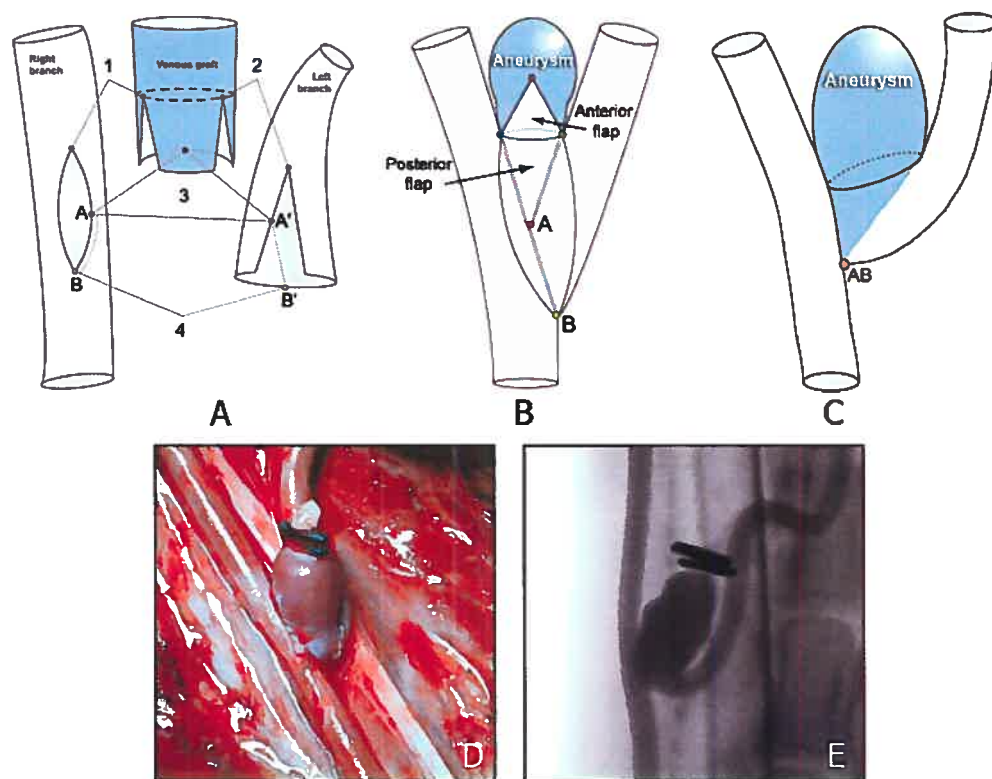


**Figure 3.1**

**Construction of the wide neck aneurysm model.** A, Artistic representation of the preparation of arterial and venous components. B, Construction of the Y-type carotid bifurcation model. C, Wide neck aneurysm model. To obtain a wide neck incorporating the origin of the left branch (left carotid artery), the distance between the proximal and distal contact points of the left and right carotid arteries is reduced to 0 (see text for details). D, Intraoperative photograph of the resulting wide neck aneurysm model. E, Angiogram of the carotid artery, obtained immediately after surgery. (Illustration by G. Gevry and I. Salazkin. Modified from Reference [194]).

## 3.2 ANGIOGRAPHY

### 3.2.1 Lateral carotid aneurysm model

The results of treatment were compared using angiography. Each aneurysm was compared from time of initial to final angiography and scored as better, same, or worse, where better implied less filling of the aneurysm with contrast material. In this manner, stented aneurysms were compared to non-stented aneurysms with respect to the extent of occlusion following angioplasty with stenting versus angioplasty alone. A typical angiogram is presented in Figure 3.2.
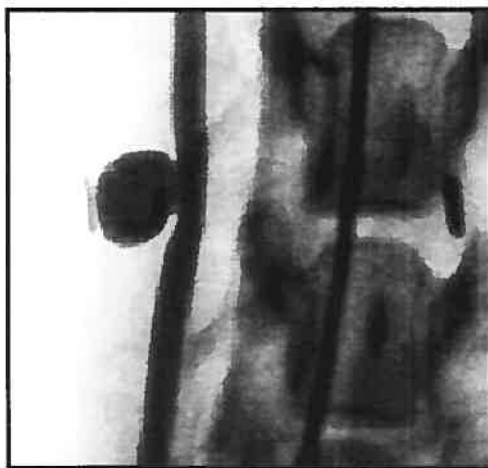


**Figure 3.2**

Angiogram of typical canine carotid lateral wall vein pouch aneurysm at four weeks.

### 3.2.2 Lingual aneurysm model

Aneurysms were scored with a nominal binary scoring system, where 0 indicated a completely occluded aneurysm, and 1 indicated residual aneurysm opacification. The incidence of branch occlusion was recorded by counting the number of branches covered by the stent following deployment, and comparing to the number of branches visible at final angiography. The development of altered flow dynamics due to the stent was also recorded. Stent-related occlusion or hemodynamic alterations were compared from denuded to non-denuded sides. In addition, the rate and extent of in-stent stenosis was recorded, by measuring the ratio of stent to angiographic lumen on the same angiogram frame. Significant stenosis was considered to be a stenosis encroaching on the luminal diameter by greater than 50%, as commonly defined in the cardiology literature[195]. A typical angiographic image of a lingual aneurysm is presented in Figure 3.3.
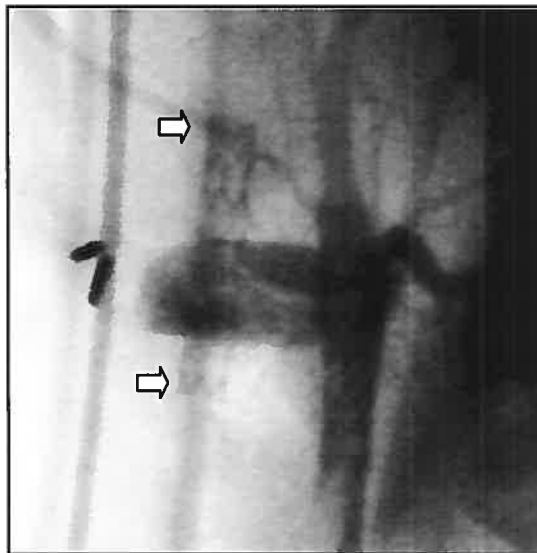


### Figure 3.3

Angiogram of typical stented canine lingual aneurysm immediately following surgical creation. Note the stented contralateral artery in the background (arrows).

### 3.2.3 Carotid bifurcation model

Aneurysms were evaluated at initial and final angiography, and given the designation better, same, or worse, with more occluded aneurysms considered better, as with the lateral wall aneurysm model.

### 3.2.4 Efficacy of endothelial denudation

Finally, to ensure that the segments of external jugular vein that were submitted to mechanical scraping to remove the endothelium were in fact de-endothelialized, we harvested several additional external jugular vein segments from separate animals and performed the standard denudation procedure, followed by fixation in formalin for staining with Verhoeff and hematoxylin-phloxine-saffron (HPS) stain, and factor VIII immunostaining. A segment of non-denuded vein was harvested and preserved in the same manner for comparison purposes.

## 3.3 PATHOLOGY

### 3.3.1 Lateral carotid aneurysm model

The five animals designated for pathological analysis had their aneurysm-artery complexes fixed in formalin. Pathological specimens were prepared by cutting the carotid artery longitudinally, followed by "en face" microphotography of the aneurysm (Clemex, Quebec, Canada) (Figure 3.4). For arteries bearing the stent, this included cutting through the stent. Following photography, arteries with incorporated stents had the stents removed to allow for thin sectioning and staining. This process unfortunately disrupts the layer of neointima over the stent, but was deemed to be an unavoidable step to permit further analysis. The macroscopic photographs of the aneurysm orifices were not scored, as there were not enough samples to reach statistical significance. The images were examined and used for two purposes: to validate the use of angiography as a surrogate measure of aneurysm occlusion, and to search for correlations between the extent of

pathologically confirmed aneurysm organization and biochemical results. The samples were cut into serial 5 μm sections spaced 100 μm apart, and stained with HPS, Movat's pentachrome, and blank slides for immunohistochemistry. Immunohistochemistry was performed using monoclonal antibodies for smooth muscle actin and endothelial cells. As with the macroscopic photography, the microscopic results were used to seek correlations between the extent of aneurysm occlusion and biochemical profile.
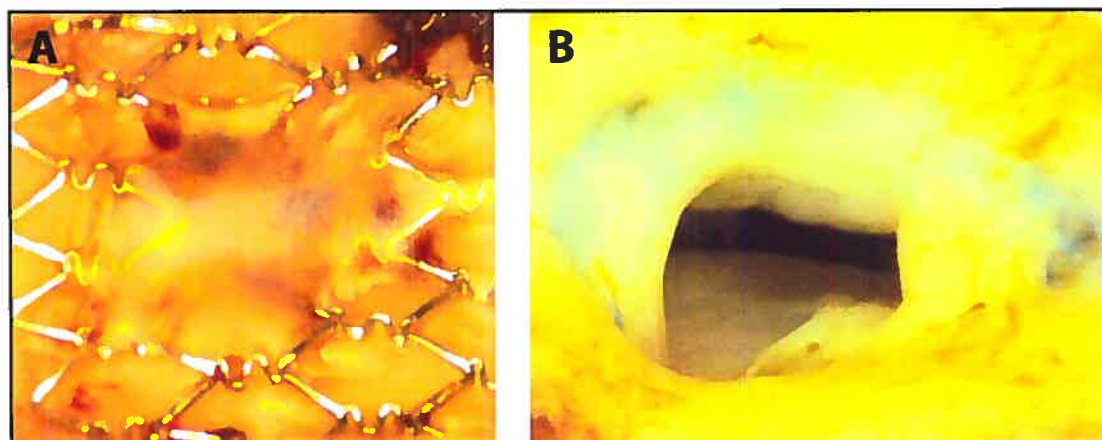


**Figure 3.4**

Photographs of a stented lateral wall aneurysm (on left) and an unstented control. Note the complete neointima formation up to and incorporating the stent struts on the left, and the widely patent aneurysm lumen on the right.

## 3.3.2 Lingual aneurysm model

Pathological analysis was performed in all eight animals as in the lateral carotid aneurysm model. In the animals sacrificed at 10 days, the stents were easily removed, permitting coronal sectioning of the aneurysms. The macroscopic and microscopic photography were used to confirm the veracity of angiography in determining aneurysm occlusion.

### 3.3.3 Carotid bifurcation model

In all four animals, the stented artery was cut longitudinally and photographed, as above. A second longitudinal cut was made, following the arterial branch formed by the anastomosed carotid. This further revealed the aneurysm ostium for photography. Axial sections through the aneurysm dome were subsequently stained with HPS and Movat's pentachrome stain, as above.

## 3.4   IN VITRO METHODOLOGY

### 3.4.1   RNA extraction

The isolation of total cellular RNA was performed according to the manufacturer's protocol for Trizol reagent (Invitrogen). The specimens harvested from the lateral wall model were placed in Eppendorf tubes and immersed in liquid nitrogen. As the experiments proceeded, to minimize loss of RNA, we began to immerse the sample in Trizol prior to freezing. When ready for biochemical analysis, samples were thawed and sectioned into small pieces with a scalpel. The tissue sample was placed in a 1 mm diameter tube with 1 ml Trizol and metal spheres, and homogenized by shaking for 4 minutes, 3 times per tube. Following mechanical cellular disruption, samples were placed on ice. Phase separation proceeded by incubating the homogenized samples at 15°C for 5 minutes. 0.2 ml of chloroform was added to the sample tubes, shaken vigorously, and incubated at 15° for 3 minutes. Samples were centrifuged at 12000 x g for 15 minutes at 2°C. Following centrifugation, the phases were separated, with RNA remaining in the upper aqueous phase.

The aqueous phase was transferred into a fresh tube and RNA was precipitated by mixing with 0.5 ml isopropyl alcohol. Samples were incubated at 15°C for 10 minutes and centrifuged at 12000 x g for 10 minutes at 2°C. The RNA precipitate formed a gel-like pellet on the bottom of the tube. The supernatant was removed, and the RNA washed with 1 ml 75% ethanol. The sample was mixed by vortexing and centrifuging at 7500 x g

for 5 minutes at 2°C. Following air-drying for 5 minutes, RNA was re-dissolved in RNAase-free water and incubated at 55°C for 10 minutes.

### 3.4.2 Reverse transcription of RNA to DNA

Aliquots of 1 to 3 μg of RNA were used for first strand cDNA synthesis using Superscript II reverse transcriptase according to manufacturer's instructions (Invitrogen, Canada) followed by PCR amplifications.

### 3.4.3 PCR amplification

The amplification of the genes in question was performed using the primers listed in Table 2 (at the end of this chapter). PCR amplifications were performed with platinum Taq DNA polymerase (Invitrogen) according to manufacturer's instructions, on an Eppendorf Mastercycler gradient, with the following program: step 1, 94°C for 2 minutes; step 2, 52°C to 64°C for 1 minute; and step 3, 72°C for 1 minute. Forty cycles were performed for amplification of genes of interest, as well as for GAPDH. The amplification for each gene was in the linear curve. PCR products were visualized on 1.5% agarose gel stained with ethidium bromide and ultraviolet transillumination. Quantitative analysis was carried out with a computerized densitometric imager (ImageQuant) to obtain gene to GAPDH ratios.

### 3.4.3 Immunohistochemistry

Five-micrometer-thick tissue sections were deparaffinized and rehydrated by passage through xylene and graded ethanol and then washed twice with 0.05 mol/l Tris·HCl, 0.15 mol/l NaCl, and 0.03% Tween 20 (TBT). Endogenous peroxidase was quenched by incubation of sections in 0.3% $H_2O_2$ in methanol for 15 min. Cell membrane α-methyl-D-galactopyranosyl groups specific to endothelial cells were detected by an anti–GSL I

antibody biotinylated *Griffonia simplicifolia* lectin 1 (GSL-1) (Vector Laboratories, Burlingame, CA). An antibody to α-smooth muscle actin (1:400; clone 1A4; Sigma, Aldrich) was used to distinguish vascular smooth muscles cells, myofibroblasts, arterioles and venules from capillaries. Nonspecific binding was blocked by incubation in 10% normal goat serum (NGS) in TBT. The primary antibodies were incubated overnight in TBT containing 10% NGS in a humidified chamber and revealed by a biotin-avidin-peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Peroxidase activity was detected with 1 mg/ml diaminobenzidine tetrahydrochloride and 0.1% $H_2O_2$ as substrates. Sections were counterstained with hematoxylin (Vector Laboratories) and subsequently dehydrated in graded ethanol solutions, cleared in xylene, and mounted in Permount. Slides were visualized with a computerized imaging system (Vision PE, Clemex Technologies Inc., Longueuil, Quebec, Canada). Nonspecific staining was verified by omission of the primary antibody or by using non-immune normal serum.

## 3.5 STATISTICAL ANALYSIS

Statistical analysis of the lingual model lateral wall aneurysm angiographic scores was performed using Fisher's exact test. The lateral wall carotid model was scored using McNemar's test for paired variables. For the latter case, results were scored as 'better', and 'same or worse'. Better was implied to mean a decrease in the amount of contrast filling.

Statistical analysis of the angiographic branch occlusion rates was performed using Wilcoxon's signed ranks test, and the in-stent stenosis data was examined with McNemar's test.

Statistical significance for all tests was considered when P<0.05.

| | Gene | Tm | NT(bp) | Foward primer | Reverse primer | Type |
|---|---|---|---|---|---|---|
| **markers** | CD-31 | 52 | 687 | 5'-CCCAGGGTGACACTGGACAA-3' | 5'-CCTTCTGGATGGTGAAGTTGG-3' | Consensus human/mice |
| | CD-34 | 64 | 434 | 5'-GCCCAGTCTGAGGTGAGGCCTCA-3' | 5'-CAGGTGTTGTCTTGCTGAATGG-3' | Dog (NM 001003341.1) |
| | α$_v$ | 64 | 622 | 5'-GTCAAGGAGGATTCAGCATTGAT-3' | 5'-AGCAGCAATTGCAATATCATTGA-3' | Consensus human/mice |
| | VCAM | 58 | 694 | 5'-CTCTTGGAGAACCCAGATAGACAGTC-3' | 5'-ATGTTCCAGAATCTTCCAGCCTCATAGCAATTA-3' | Dog (NM 001003298.1) |
| **Reporter** | GAPDH | 64 | 500 | 5'-GCCAAAAGGGTCATCATCTC-3' | 5'-GCCCATCCACAGTCTTCT-3' | Consensus human/mice |
| **MMPs** | MMP-2 | 58 | 580 | 5'-ATGCAGAAGTTCTTTGGGCTGC-3' | 5'-TTGCCATCCTTCTCAAAGTTGTA-3' | Dog (AF 177217) |
| | MMP-9 | 64 | 476 | 5'-TATGACACCGACCGTCGGTTC-3' | 5'-GTACATGAGCGCTTCTGGCAC-3' | Dog (AF 169244) |
| **TIMPs** | TIMP-1 | 52 | 341 | 5'-GCGTTATGAGATCAAGATGAC-3' | 5'-CTGGTCCGTCCACAAGCA-3' | Dog (NM 001003182.1) |
| | TIMP-3 | 52 | 260 | 5'-TACCAGTACCTGCTGACAGG-3' | 5'-GCCCATCCTCGGTACCAGCT-3' | Dog (XM 53840.2) |
| | TIMP-4 | 58 | 331 | 5'-ATCTCCAGTGAGAAGGTAGT-3' | 5'-GTGGTGATTTGGCAGCCACAGTTC-3' | Consensus human/mice |
| **Growth Factor** | TGFβ1 | 64 | 366 | 5'-TTCCTGCTCCTCATGGCCAC-3' | 5'-GCAGGAGCGCACGATCATGT-3' | Dog (NM 001003309) |
| | PDGFbb | 64 | 393 | 5'-ATGAATCGCTGCTGGGCGCTCTTCCT-3' | 5'-GGAGCAGCGCTGCACCTCCAC-3' | Dog (NM-001003383) |
| | TNFα | 64 | 436 | 5'-CCTCCAACTAATCAGCCCTCT-3' | 5'-TCTCAGCGCTGAGTCGATCA-3' | Dog (NM 001003244.1) |
| | MCP1 | 64 | 305 | 5'-ATGAAGGTCTCCGCAGCGCTC-3' | 5'-TCATGGCTTTGCAGTTTGGGTTTG-3' | Consensus human/mice |

**TABLE 2:** Sequences of primers of selected genes chosen for RT-PCR.

# 4. RESULTS

## 4.1 THE EFFECTS OF STENTING AND ENDOTHELIAL DENUDATION ON ANEURYSM AND BRANCH OCCLUSION IN EXPERIMENTAL ANEURYSM MODELS

My contribution to the article entitled: The Effects of Stenting and Endothelial Denudation on Aneurysm and Branch Occlusion in Experimental Aneurysm Models consisted of planning and executing the experiments, as well as writing the manuscript. During this process, I benefited from the assistance of Igor Salazkin for the surgical creation of the lingual artery aneurysms, as well as for the macroscopic and microscopic photography of the aneurysm/stent complexes and histology slides. I performed the angiography and endovascular procedures in all animals, and performed the necropsies and tissue harvesting. I organized, prepared, and interpreted the angiographic and pathologic data. I benefited from the assistance of Sophie Lerouge and Fatiha Bouzeghrane for the immunohistochemistry of the vein and aneurysm samples. Guylaine Gevry assisted with the formatting of images and text for online submission. Statistical analysis was done by a dedicated biostatistician, Miguel Chagnon. Finally, Gilles Soulez and Jean Raymond provided critical review of the manuscript.

**The Effects of Stenting and Endothelial Denudation on Aneurysm and Branch Occlusion in Experimental Aneurysm Models**

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| | |

# The Effects of Stenting and Endothelial Denudation on Aneurysm and Branch Occlusion in Experimental Aneurysm Models

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# The Effects of Stenting and Endothelial Denudation on Aneurysm and Branch Occlusion in Experimental Aneurysm Models

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# Abstract

## Background and Purpose

Stents are increasingly used in the endovascular treatment of intracranial aneurysms. We studied the effects of stenting and endothelial denudation on aneurysm and branch vessel occlusion.

## Methods

Bilateral external carotid vein pouch aneurysms were created proximal to the lingual artery origin in 8 dogs, scraping the aneurysmal endothelial lining on one side. Both arteries were immediately stented using balloon-expandable stents. Angiography was performed at time of stenting and immediately prior to sacrifice, at 10 days (n=2), 10 weeks (n=4) and 20 weeks (n=2). In 4 other dogs, a wide-neck carotid bifurcation aneurysm was created, with the vein pouch denuded or not (n=2 each), followed by immediate stenting. Angiography was performed at time of stenting and prior to sacrifice at 5 (n=2) and 10 weeks (n=2). Branch occlusion between initial and final angiograms was recorded. Pathological evaluation of aneurysm tissue filling was studied, with attention to neointima formation at the aneurysm ostium and around branch vessel origins.

## Results

Seven of 8 stented and denuded lingual aneurysms were completely obliterated compared to 2 of 7 aneurysms that were stented alone (P<0.04). None of the carotid bifurcation aneurysms became obliterated, but denuded aneurysms showed partial thrombosis. Of 68 total stent-covered branches, 5 (7%) were occluded and 17 (27%) had altered angiographic flow.

## Conclusion

Stenting led to suboptimal results in the presence of an intact endothelial layer. Endothelial denudation can promote aneurysm occlusion when combined with stenting.

# Introduction

Endovascular therapy of aneurysms can improve the outcome of patients treated after subarachnoid hemorrhage (SAH) as compared to surgical clipping [1]. However, this treatment modality is associated with recurrences and a concern for future hemorrhage [2]. Attempts to decrease the recurrence rate have led to several technical and bioengineering innovations [3-7]. One strategy involves treating the aneurysm as a symptom of the diseased parent vessel, with deployment of a stent to bridge the aneurysm ostium. Currently, stenting is often combined with coil occlusion, hopefully to modify intra-aneurysmal hemodynamics, provoke aneurysm thrombosis, re-establish normal blood flow patterns within the parent vessel, and perhaps buffer the hemodynamic forces implicated in recurrences [8-12]]. In addition, the stent may provide a supportive scaffold for neointima deposition at the junction of the parent vessel and aneurysm neck. The major concern regarding this treatment strategy is the risk of stent-related occlusion of branch or perforating vessels, and the excessive formation of neointima with secondary parent vessel stenosis [13]. Furthermore, the use of stents alone, particularly if they are designed to minimally affect branches and perforators, may not sufficiently alter hemodynamics to initiate thrombosis and lead to aneurysm healing [14].

The defining boundary of the endoluminal space is the endothelial lining, which is comprised of a single layer of specialized cells with anti-thrombotic and thrombolytic properties important for the maintenance of vessel patency [15]. The endothelial lining is responsible for the persistence of experimentally constructed aneurysms, for residual flow within the neck of incompletely treated aneurysms, and is involved in recanalization following arterial occlusion [16, 17]. Endothelial denudation could be considered as a therapeutic strategy to improve the results of endovascular treatment of aneurysms [17]. However, endothelial denudation in the continued presence of blood flow leads to re-endothelialization [16, 18, 19]. After denudation, hemodynamic changes provoked by stent struts may alter re-endothelialization and promote aneurysm thrombosis, organization, and healing in such a fashion as to redefine the boundary of the endoluminal space to one defined by the stent, thereby excluding the aneurysm. The stent may also

lead to increased neointima formation at the site crucial for aneurysm obliteration, at the parent vessel-aneurysm neck interface.

The present work sought to examine the relationship of stenting and endothelial denudation to aneurysm and branch vessel occlusion, as assessed by angiography and pathology, and to explore the potential value of a novel approach combining both strategies to improve results of endovascular treatment.

## Methods

Protocols were approved by the Institutional Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. All procedures were performed under general anesthesia. Twelve beagles weighing 8-12 kg were sedated with acepromazine (0.1 mg/kg), glycopyrrolate (0.01 mg/kg), and butorphanol (0.1 mg/kg), and anesthetized with intravenous thiopental (15 mg/kg). Animals were ventilated artificially and maintained under anesthesia with 2% isoflurane. Post-operative analgesia was provided for three days with a 50 μg fentanyl patch.

For this work, the lingual aneurysm [20] and carotid bifurcation aneurysm models [21, 22] were used, with a modification to the lingual model. Briefly, for the lingual model (n=8), the region of the lingual artery was accessed through a midline incision. A portion of the left external jugular vein was harvested and divided in half to form venous pouches. One of the vein segments was temporarily inverted and scraped with a scalpel blade to cause endothelial denudation, as previously described [17]. To evaluate the extent of denudation and damage to the jugular vein segments with this technique, a portion of jugular vein of four other dogs was harvested and scraped in a similar fashion, fixed in formalin and processed for factor VIII immunostaining, Verhoeff and hematoxylin-phloxine-saffron (HPS) staining. Control non-denuded vein was also harvested for comparison.

On each side, the lingual nerve was identified and mobilized, and temporary aneurysm clips were applied to branch vessels. A 4mm arteriotomy was created proximal to the site of the lingual artery origin, which presents a modification from the distally located aneurysm in the originally described model [20]. End-to-side anastomoses of the vein segment to the artery were fashioned, with normal vein on one side, and denuded vein on the other. The suture line was sealed with running 7.0 Prolene suture, and the aneurysm pouches sealed with hemostatic clips.

The endovascular procedure was performed immediately following closure of the surgical site. Transfemoral access was obtained, and following angiography, balloon-expandable stents (BX Velocity and BX Sonic, Cordis, Miami, Florida) lengths 18-28 mm and diameters 2.5-3.5 mm were deployed bilaterally to bridge the aneurysm neck and lingual

artery. Angiograms were performed immediately following stent deployment, and prior to sacrifice at 10 days (n=2), 10 weeks (n=4), and 20 weeks (n=2) post-stenting.

For the carotid bifurcation model (n=4), through a midline incision, both carotid arteries were exposed and temporarily clipped with aneurysm clips. The left carotid artery was tied off at the origin, mobilized under the trachea, and anastomosed to the contralateral carotid artery. A portion of harvested external jugular vein, either normal or denuded, had two slits cut longitudinally on one side, and was anastomosed to the junction of the two carotid arteries, forming a wide-neck bifurcation aneurysm [20, 21]. Using transfemoral access, angiography was performed, and a single balloon-expandable stent (Herculink Plus, Guidant, Santa Clara, CA), 18 mm in length and ranging in diameter from 4.0-4.5 mm was positioned in the right carotid artery, bridging a portion of the aneurysm neck and the origin of the re-implanted left carotid artery. Angiograms were performed immediately following stent deployment, and prior to sacrifice at 5 (n=2) and 10 weeks (n=2). Animals did not receive any antiplatelet agents or anticoagulants.

The results of treatment were compared by angiography and pathology. Angiographic results were compared using Fisher's exact test, where $P < 0.05$ was required for significance. Aneurysms were scored in a binary fashion, where a score of 0 indicated complete obliteration, and a score of 1 indicated residual or recurrent aneurysm. The number of branches covered by the stent was counted on the initial angiogram and compared to the final angiogram to determine the incidence of branch occlusion. The incidence of hemodynamic alterations, such as sluggish blood flow though a branch vessel was also recorded. The incidence and extent of angiographic in-stent stenosis was determined by measuring the ratio of reference vessel to stent lumen on the same angiogram frame. Significant in-stent stenosis was defined as a reduction of luminal diameter of greater than 50% [23].

Pathological specimens were prepared by cutting the stent/artery complex longitudinally, followed by "en face" microphotography of the aneurysm and branch vessel ostia (Clemex, Quebec, Canada). Pathological findings were studied after formalin fixation, sagittal or coronal sectioning, and staining with hematoxylin-phloxine-saffron (HPS) and Movat's pentachrome stain. Immunohistochemistry was performed on illustrative cases

with markers for endothelial cells (anti-GSL I antibody biotinylated *Griffonia simplicifolia* lectin 1 (GSL-1), Vector Laboratories, Burlingame, CA) and α-smooth muscle actin (1:400, clone 1A4, Sigma, Aldrich).

# Results

***Endothelial denudation procedure:*** Factor VIII staining confirmed the denuding procedure was effective, but 'clusters' of persisting endothelial cells were occasionally observed. Damage to the vessel wall extended to disruption of the internal elastic lamina in a few regions, but no laceration of the media was observed (Figure 1).

***Surgical and Endovascular results:*** All animals tolerated the operative and stenting procedures well. All aneurysms were patent immediately following surgery. Stent placement was judged to be satisfactory in all dogs.

***Angiography:*** Seven of 8 lingual aneurysms treated with stenting and endothelial denudation were completely occluded compared to 2 of 8 aneurysms treated with stenting alone (P=.040). Angiographic results are presented in Table 1, and illustrated in Figure 2.

Of 68 branches covered by the 20 stents, occlusion was noted in 5/68 (7%), 2 of which were on the denuded side (Table 2). Blood flow patterns were altered in 17/68 branches (25%), 8 of which were on the denuded side. Endothelial denudation of the aneurysm did not result in a difference in the incidence of branch occlusion or alteration of blood flow (P=1 and .916, respectively).

Although 7/20 (35%) of stents were noted to have some neointima encroaching on the luminal diameter, in no cases did the degree of stenosis reach 50%.

All denuded (n=2) and non-denuded (n=2) carotid bifurcation aneurysms were still patent at final angiography. However, both denuded aneurysms showed only partial filling, while non-denuded aneurysms remained widely patent. None of the four 'jailed' left carotid arteries became occluded or had altered flow dynamics, and there was no observable neointimal hyperplasia on the parent vessel lumen due to stent implantation.

***Pathology:*** Macroscopic photography of typical results for lingual model aneurysms treated with stenting alone and with stenting and denudation is presented in Figure 3 a-d. The seven aneurysms that were not completely occluded on angiography were grouped into four types; the first were the large saccular recurrences (n=2, both non-denuded) that were associated with very incomplete neointimal coverage at the neck (Figure 3a, c); second, small channels extending from the parent vessel along the vein pouch wall (n=1,

non-denuded); third, small channels opening into larger spaces within the aneurysm fundus (n=3, all non-denuded); these channels were connected to small openings visible through the stent struts (Figure 3b, d); and fourth, a small blunted neck remnant (n=1, denuded).

Lingual model stents were well-incorporated into the arterial wall in 14/16 cases, and 3 of 4 carotid bifurcation stents were well-incorporated in neointimal tissue. Where stents were observed to 'jail' the branch vessel origins, in cases where the ostium was patent, one of two patterns of neointima deposition was observed. Sometimes the stent was devoid of neointima, while in others, the strands of material covered portions of the stent struts, with the ostium patent underneath (Figure 3g, h). In some cases the neointima was seen to have grown over and occluded the branch vessel origin.

Both non-denuded wide-necked carotid bifurcation aneurysms remained widely patent, but smaller aneurysms partially occluded with organized clot were observed in both cases treated with stenting and denudation (Figure 3i).

Staining for α-smooth muscle actin confirmed the presence of numerous myofibroblasts within the organizing thrombus (Figure 4a). Endothelial cell-specific staining with GSL-1 showed lined crevices within the organized thrombus (Figure 4b).

## Discussion

The use of stenting alone was ineffective at totally occluding both lingual and carotid bifurcation aneurysms, at least with the devices we used. Although blood flow patterns were sufficiently modified to provoke thrombosis and clot organization within the lingual aneurysms, the use of the stent alone did not produce a solid organized structure completely obliterating the aneurysm. When the endothelial lining was preserved, small endothelial-lined crevices, which have been associated with recurrences [16, 17], were observed in most cases. However, when stenting was combined with endothelial denudation, occlusions were more often complete and intra-aneurysmal tissue more organized. Removal of the endothelial lining may incite a more vigorous thrombotic reaction within the aneurysm, by exposing a greater collagenous surface. Alternatively, the removal of endothelial signaling may limit or prevent the formation of residual or recurrent crevices. In the future, endothelial denudation could theoretically be accomplished with mechanical, physical, or chemical means. Although denudation removes parietal endothelial cells, circulating endothelial progenitor cells have been described, and likely participate in the repopulation of the maturing thrombus[24].

Attempts to replicate the results of the lingual model in high-flow wide-neck bifurcation aneurysms were unsuccessful. Notably, the stent does not completely cover the aneurysm ostium in this model, and the thrombus is exposed to greater hemodynamic forces. These conditions seem unfavorable for the formation and organization of intra-aneurysmal clot. Aneurysms such as these may require new devices such as bifurcated stents with asymmetrical cells or different therapeutic strategies for definitive treatment.

One difficulty with the use of intracranial stents arises from the conflicting goals of provoking aneurysm thrombosis while sparing adjacent branches. Ideally, the portion of the stent covering the aneurysm would be of low porosity, with minimal blood flow into the aneurysm, with the remainder of the stent comprised of high porosity stent cells. Smaller pore sizes may protect the organizing clot from hemodynamic forces and promote neointimal proliferation up to the boundary created by the stent, permitting greater neointimal formation at the junction of the parent vessel and aneurysm neck.

High-porosity stents such as those currently available for intracranial use are more likely to preserve perforating vessels, but require an additional treatment, usually coiling, to occlude the aneurysm.

The varying observations at the level of the stent struts crossing branch vessel ostia may represent three different pathological outcomes: a) branch vessel thrombosis without recanalization, with dense neointimal coverage; b) branch vessel thrombosis with recanalization, with non-uniform tissue strands covering the stent struts; c) branch vessel remains patent, with bare stent struts crossing a vessel origin. In this series, the use of stents led to branch occlusion in 7.3% of cases, and endothelial denudation of the aneurysm did not worsen this rate. However, stent-related blood flow alterations were observed in 25% of cases, which raises concerns about stent-related tissue ischemia or infarction. The finding of exposed portions of the stent crossing vessel ostia further stresses the importance of compliance with long-term platelet therapy following stenting.

Although in-stent stenosis did not occur with the stents we used, clinical reports of this complication mandate continued surveillance until the natural history of intracranial stenting is better delineated [25].


### Experimental Limitations:

The advantage of the lingual aneurysm model is the multiple branch vessels that resemble many intracranial aneurysms. Aneurysms in this position are exposed to lesser hemodynamic forces, an important factor limiting generalization of our results.

Venous pouches certainly differ from naturally occurring aneurysms, in their cellular composition but perhaps also in their biological reaction. The location of the experimental aneurysm within the soft tissues of the canine neck also represents a different microenvironment compared to the CSF space.

Other limitations include the small number of animals used and the absence of a long-term follow-up group. Finally, the stents used in these experiments differ significantly from the self-expandable stents in current clinical use.

## Conclusion

An additional treatment, such as endothelial denudation, may improve the results of endovascular aneurysm treatment with high porosity stents. More experimental work is needed to determine the optimal stent design to result in durable aneurysm occlusion while preventing ischemic complications. A safe method to remove the endothelium via the endovascular route remains to be developed.

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**Table 1:** Angiographic occlusion rates of aneurysms following endovascular stenting of the parent vessel with or without aneurysm denudation.

## Treatment

| Dog | Time | Stenting Alone | Stenting + Denudation |
|-----|------|----------------|-----------------------|
| 1 | 10 d | Patent | Patent |
| 2 | 10 d | Patent | **Occluded** |
| 3 | 10 w | **Occluded** | **Occluded** |
| 4 | 10 w | Patent | **Occluded** |
| 5 | 10 w | Patent | **Occluded** |
| 6 | 10 w | **Occluded** | **Occluded** |
| 7 | 20 w | Patent | **Occluded** |
| 8 | 20 w | Patent | **Occluded** |

7/8 aneurysms treated with stenting and denudation completely occluded, compared to 2/8 aneurysms treated with stenting alone. (P = .040)

**Table 2:** Stent-related branch occlusion and altered flow dynamic rates with stenting alone and with stenting and denudation.

## Treatment

|  | Stenting Alone | Stenting + Denudation | p value |
|---|---|---|---|
| **Occluded branches** | 2/32 (9%) | 3/36 (7%) | 1 |
| **Altered blood flow** | 9/32 (26%) | 8/36 (27%) | .917 |

# Figures
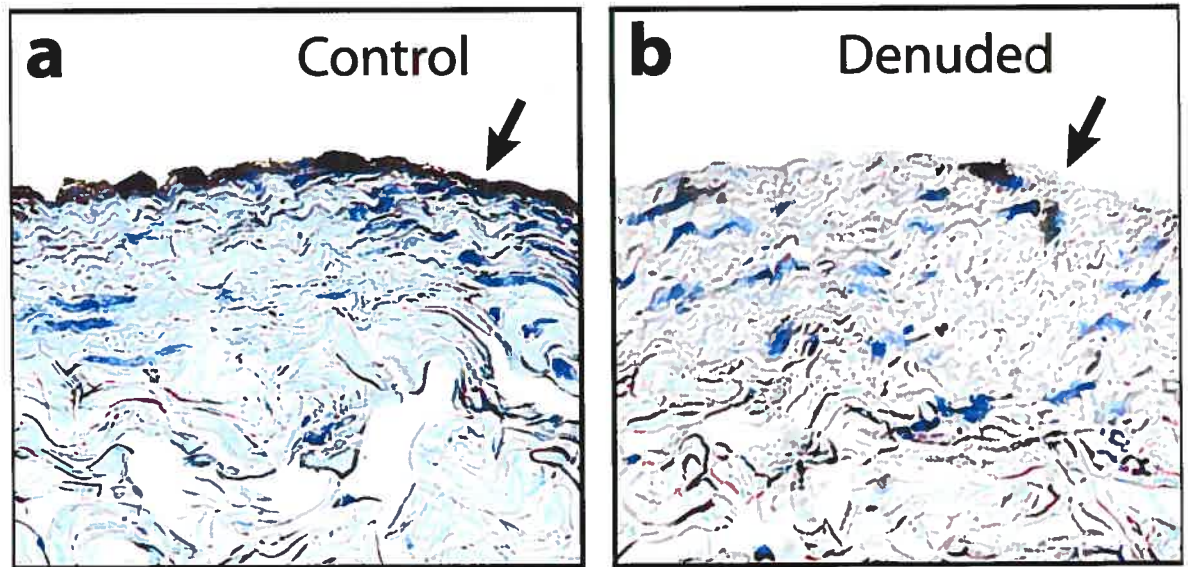


**Figure 1:  Endothelial denudation.**  Vein segments were stained for Factor VIII, showing endothelial cells (arrow in (a)) when intact, but very few endothelial cells when denuded (arrow in (b)).  Original magnification 400x.
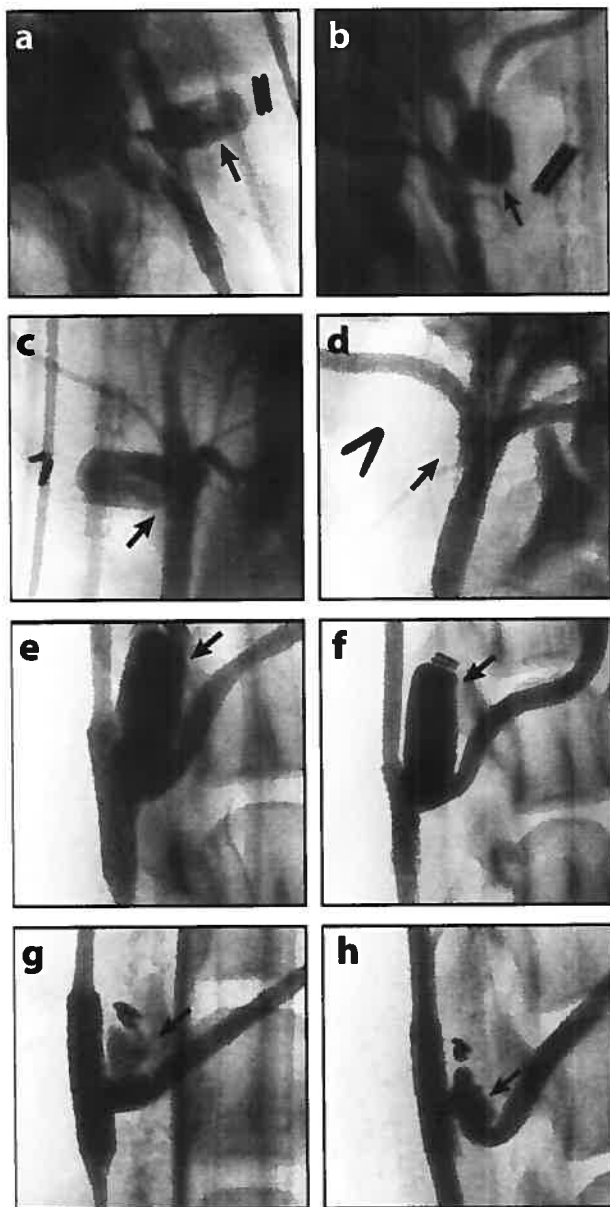
**Figure 2: Angiographic progression of experimental aneurysms.** Initial angiograms at the time of stenting (a,c,e,g) are compared with 10 week follow-up angiograms (b,d,f,h) using the lingual (a-d) or carotid bifurcation aneurysm models (e-h) with (c,d,g,h) or without endothelial denudation (a,b,e,f). Note complete (d) or partial (h) occlusion when the aneurysm is denuded, but large residual lesions when the endothelial lining was intact (b,f).

**Figure 3: Macroscopic photography.** Typical results for aneurysms treated with stenting alone at 10 weeks are shown as 'en-face' views of aneurysm ostia, (a,b), and sagittal sections through aneurysm remnants (c,d). Note the patent lingual artery ostium in c) and the patent channel communicating with an open space within the aneurysm fundus in d). 'En-face' views of aneurysm ostia treated with stenting and denudation are shown in e,f. Neointima formation over branch vessels are shown in (g,h). Partially occluded wide-neck carotid bifurcation aneurysm shown in 3(i) (CC: common carotid, LC and RC: left and right carotid arteries; T: organized thrombus; R: residual aneurysm).
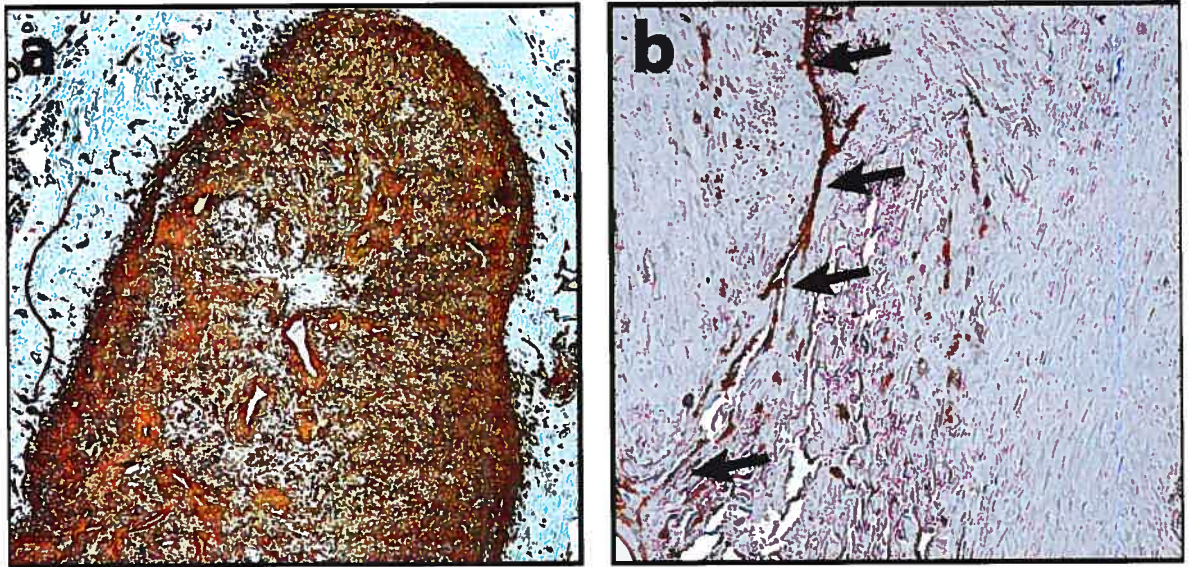
**Figure 4: Immunopathology.** Microphotographs of sectioned aneurysm treated with stenting alone following immunohistochemical staining for smooth muscle actin (a) and with GSL-1 for endothelial cells (b). In b), brown-stained endothelial cells line a channel extending from the neck to aneurysm fundus (arrows). Magnification a: 70X and b: 350X.

## 4.2 Effects of stenting the parent artery on aneurysm filling and gene expression of various potential factors involved in healing of experimental aneurysms

My contribution to the article entitled: Effects of Stenting the Parent Artery on Aneurysm Filling and Gene Expression of Various Potential Factors Involved in Healing of Experimental Aneurysms consisted of planning and executing the experiments, as well as writing the manuscript. During this process, I originally benefited from the assistance of Igor Salazkin for the surgical creation of the carotid artery aneurysms, although I learned to create them independently. Dr. Salazkin also assisted me with the macroscopic and microscopic photography of the aneurysm/stent complexes and histology slides. I was taught to perform angiography and endovascular procedures on the animals, taking over the procedures as the experiments progressed. I also learned to perform necropsies and designed a protocol for rapid tissue harvesting under anaesthesia. I was taught to perform the RNA extraction and RT-PCR procedures as well as the gel separation of the DNA. I organized, prepared, and interpreted the angiographic, pathologic, and mRNA expression data, with the assistance of Christelle Ogoudikpe. I benefited from the assistance of Fatiha Bouzeghrane for the immunohistochemistry of the aneurysm samples. Guylaine Gevry assisted with the formatting of images and text for online submission. Statistical analysis was done by a dedicated biostatistician, Miguel Chagnon. Finally, Jean Raymond provided critical review of the manuscript.

# Effects of stenting the parent artery on aneurysm filling and gene expression of various potential factors involved in healing of experimental aneurysms

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## Acknowledgments

**Effects of stenting the parent artery on aneurysm filling and gene expression of various potential factors involved in healing of experimental aneurysms**

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## Abstract

**Background and Purpose** – Intracranial stents are increasingly used in the endovascular treatment of aneurysms, but very little is known regarding their effect on the cellular and molecular evolution of aneurysms.

**Methods** – Bilateral venous pouch lateral wall carotid aneurysms were created in 20 dogs. All dogs then underwent angiography and balloon-expandable stenting of one aneurysm 4 to 6 weeks later. Fifteen dogs underwent aneurysm harvesting at 1 day (n=3), 4 days (n=4), 7 days (n=3), and 14 days (n=5) for mRNA expression analysis, using axial sections taken from the aneurysm neck and fundus for RT-PCR amplification of four cytokines or growth factors: TNF-α, TGF-β1, MCP-1, and PDGF-BB; two adhesion molecules: VCAM-1 and PECAM-1; five matrix modifying agents; MMP-2, 9, TIMPs 1, 3, 4, and two cellular markers: CD34 and α-SMA. Five other dogs, sacrificed at 12 weeks, were examined for extent of filling of the aneurysm neck with organized tissue and for neointima formation at the aneurysm ostium. Angiography was performed prior to sacrifice in all animals, and compared with initial studies.

**Results** – Eleven of 20 stented aneurysms showed a favorable angiographic evolution, while none of the 20 non-stented aneurysms improved (p=0.001). Pathology showed partially occluded aneurysms, with neointima formation around the stent struts. Observed trends in mRNA expression, that stenting increased expression of genes involved in organization and neointima formation, agreed with experimental hypotheses, but differences between stented and non-stented aneurysms did not reach statistical significance.

**Conclusion** – Parent vessel stenting was associated with angiographic improvement of aneurysm appearance. Modifications in mRNA expression patterns following stenting deserve further study to better establish potential molecular targets to promote aneurysm healing.

## Introduction

Endovascular coiling of intracranial aneurysms is increasing in popularity following the publication of the results of the ISAT[1], but this technique is too often associated with recurrences[2]. Attempts to decrease recurrences have triggered the development of several technical and bioengineering innovations [3-7], including endoluminal stenting. Currently, stents are most often used as an adjunct to coiling, but stents by themselves have been reported to lead to aneurysm occlusion[8-10]. Beyond preventing coil herniation, the beneficial effects of stents are likely realized by modifying flow; they modify intra-aneurysmal hemodynamics, may provoke aneurysm thrombosis, re-establish normal blood flow patterns within the parent vessel, and perhaps buffer the hemodynamic forces implicated in recurrences[11-15]. In addition, the stent may provide a supportive scaffold for neointima deposition at the junction of the parent vessel and aneurysm neck.

The feasibility of treating experimental aneurysms with stents has been established using angiographic and pathologic studies[16, 17], but molecular clues accounting for the variable treatment outcomes remain elusive. Conceptual and molecular parallels likely exist between i) vascular wound healing, organization and neointima formation within the body and neck of the aneurysm[18]; ii) angiogenesis and recanalization at the aneurysm neck[19-21]; and iii) molecular effects of flow modification previously associated with atheroma or stenting[13, 14].

For neointima and organization to completely fill the defect formed by an aneurysm, the provisional matrix formed by fibrin and platelets must be invaded and replaced by neointimal cells or myofibroblasts and secreted extracellular matrix[18]. In balloon-injury models, the process resulting in neointima formation relies on complex signaling cascades and cellular proliferation, establishment of cell-cell and cell-matrix connections, and matrix degradation and reformation [22]. At the aneurysm neck, another condition for stable occlusion is the absence of recanalizing channels within the thrombus[19-21]. By altering flow with a stent, we aim to observe changes in genetic expression of key factors potentially involved in neointima formation/thrombus organization or recanalization.

The genes that we chose to follow can be classified into growth factors and cytokines (PDGF-BB, TGF-β1, TNF-α, MCP-1), adhesion molecules (integrin αVβIII, VCAM-1, PECAM-1), matrix metalloproteinases and their inhibitors (MMP-2 and 9, and TIMP 1, 3, and 4), and the cellular

marker α-SMA. To study the process of formation of recanalizing channels, we focused on the expression CD34, as well as MCP-1 and the MMPs / TIMPs.

Determination of gene expression changes that are associated with favourable angiographic and pathological results promises to usher in a new era of molecular-based aneurysm treatment, and stenting offers a unique opportunity to study the mechano-sensitive changes in mRNA expression occurring within an aneurysm following endovascular therapy.

## Materials and Methods

Protocols were approved by the Institutional Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. All procedures were performed under general anaesthesia. Twenty beagles weighing 8-12 kg were sedated with acepromazine (0.1 mg/kg), glycopyrrolate (0.01 mg/kg), and butorphanol (0.1 mg/kg), and anaesthesetized with intravenous thiopental (15 mg/kg). Animals were ventilated artificially and maintained under anaesthesia with 2% isoflurane. Post-operative analgesia was provided for three days with a 50 μg Fentanyl patch.

We used the lateral wall carotid aneurysm model of German and Black, as modified by Graves[20, 23]. Briefly, the carotid artery was accessed through a midline incision. A portion of the left external jugular vein was harvested and divided in half to form venous pouches. Temporary aneurysm clips were applied to the proximal and distal carotid, and a 6 mm arteriotomy was created. The vein pouch was attached to the artery via an end-to-side anastomosis with running 7.0 Prolene suture, the apex of the pouches sealed with hemostatic clips, and circulation restored.

After a 4-6 week period to permit post-surgical changes to abate, the endovascular procedure was performed. Following transfemoral angiography, a coronary balloon-expandable stent ranging in length from 13-23 mm and in diameter from 3.0-4.5 mm was positioned to bridge one aneurysm neck and deployed. The contralateral side subsequently underwent balloon angioplasty using the same balloon used to expand the stent. Animals did not receive any antiplatelet agents or anticoagulants. Angiography was repeated immediately following stenting and prior to sacrifice. Fifteen animals underwent a separate harvesting procedure at 1 day (n=3), 4 days (n=4), 7 days (n=3) and 14 days (n=5) post-stenting, where the portions of the carotid arteries bearing the aneurysms were removed under anaesthesia. Animals were sacrificed by barbiturate overdose. An

additional 5 dogs were sacrificed at 12 weeks for pathological analysis; their aneurysms were removed and fixed in formalin.

The evolution of aneurysms following treatment was evaluated by examining the angiographic progression between initial and final angiograms. A binary score of better versus same/worse was given, based on whether the final aneurysm opacified less, equal to or greater than on the initial angiogram following stenting.

Pathological specimens were prepared by cutting the stent/artery complex longitudinally, followed by "en face" photography of the aneurysm ostium (Clemex, Quebec, Canada). Pathological findings were studied after formalin fixation and hematoxylin-phloxine-saffron (HPS), Movat's pentachrome, and immunohistochemical staining.

The fifteen animals analyzed for mRNA expression had each aneurysm cut at the junction with the parent carotid, taking care not to include carotid tissue. 2 mm and 3 mm axial sections of the aneurysm neck and fundus, respectively, were harvested (Figure 1). The tissue samples included the vein pouch aneurysm wall as well as any formed thrombus or tissue adherent to the wall. Samples were frozen with liquid nitrogen. Isolation of total cellular RNA was performed using the phenol method. Aliquots of 1 to 3 μg were used for first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen, Canada) and PCR amplifications were performed with Platinum Taq polymerase (Invitrogen). After determining the linear range for each target gene, amplification of the gene under study was carried out using the primers sequences listed in Table 1. Thermal cycling conditions consisted of enzyme activation at 94°C for 1 minute, then a cycle of denaturation also at 94°C for 1 minute, annealing as described in Table 1 and extension at 72°C, each for 1 minute, repeated 40 times and 35 times for GAPDH. This was followed by a final extension at 72°C for 1 minute. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide and UV transillumination. Quantitative analysis was carried out using a computerized densitometric imager (ImageQuant; Amersham Biosciences, Canada) to obtain gene/GAPDH ratios and thus correct for the variable amounts of tissue within the specimens. For each gene, the mean mRNA expression of the 14 genes at the 4 different time points was quantified for the neck and fundus of each stented aneurysm, and compared to that from the corresponding control aneurysm.

## Statistics:

Angiographic results were divided into two groups: a) decreased opacification b) unchanged or increased size. These groups were compared with the McNemar two-tailed test. mRNA

expression results were treated with ANOVA repeated measurements for three factors: stent, time, and location (neck or fundus), using the Procedure Mixed program of SAS software, version 9. P < 0.05 was considered to be significant for all tests.

## Results

### A) Surgical and Endovascular results:

All animals tolerated the surgical and endovascular procedures well. Stent placement was deemed adequate in all cases, and resulted in an immediate increase in blood transit time through the aneurysm, as qualitatively assessed by the observer.

### 1. Angiographic results:

Of 40 aneurysms created in 20 dogs, 37 were patent at initial angiography at 4-6 weeks, for a spontaneous occlusion rate of 7.5%. Stent deployment had an effect on angiographic progression (Figure 2); 11 of 20 stented aneurysms were angiographically better, while none of the control aneurysms improved (p=0.001). Only one of the stented aneurysms became completely obliterated on angiography at 12 weeks.

### 2. Pathology results:

Macroscopically, stenting seemed to redefine the endoluminal boundary, with neointima formation up to and around the stent struts at least in some cases (11/20) (Figure 3). In other cases (9/20), stented aneurysms remained widely patent. Intermediate cases of incompletely occluded aneurysms showed small channels connecting the parent vessel with dilated openings within the organizing intra-aneurysmal thrombus. Non-stented control aneurysms were usually widely patent, while some had partial filling of the fundus with organized tissue.

### B) mRNA expression results

RT-PCR results in stented and control aneurysms are illustrated in figure 4 and summarized in table 2.

### 1. Growth factors and cytokines:

The kinetic profile of TGF-$\beta$1 expression at the aneurysm neck showed an initial decrease, reaching a nadir at 4 days, with a subsequent slow continuous increase in expression to 14 days, with only slightly greater expression for stented aneurysms compared to non-stented aneurysms.

TGF-β1 expression in the tissue from the fundus was remarkably similar between stented and non-stented aneurysms. Both showed a decrease similar to that of the tissue from the neck, with a more delayed nadir at 7 days.

PDGF-BB expression of neck tissue for stented aneurysms remained fairly constant for 7 days, followed by an increase at 14 days. During this interval, non-stented neck expression of PDGF-BB decreased until the 7 day mark, and then increased to 14 days. At the level of the aneurysm fundus, stented aneurysms showed a small decrease until 7 days, with subsequent increase. Non-stented aneurysms showed a small increase, peaking at 4 days and then tapering off.

The peak expression at of TNF-α at 4 days is compatible with inflammatory cell infiltration and secretion. We observed almost parallel TNF-α expression curves for both stented and non-stented controls at the neck and fundus. Stented fundus showed a slightly higher peak at 4 days and a deeper nadir at 7 days than controls.

MCP-1 expression at the neck was lower for stented aneurysms at all time points compared to controls. At the fundus, the stent led to decreased MCP-1 expression compared to controls over the first 7 days, which slowly increased to become greater than controls at 14 days.

The differences in mRNA expression between stented and non-stented aneurysms were not statistically significant for any growth factors or cytokines assayed at either the neck or the fundus.

## 2. Adhesion molecules :

PECAM-1 expression was variable at the different time-points, showing a biphasic increasing pattern for neck tissue stented aneurysms, with a small peak at 4 days, while non-stented tissue at the neck and stented and non-stented fundus tissue all showed sharply decreasing expression initially that subsequently tapered off at 14 days.

We observed only small increases in VCAM-1 expression for stented aneurysms at the neck and fundus over the first 7 days, which subsequently normalized. Non-stented neck and fundus tissue expressed more VCAM-1 than stented tissue over the first 7 days; this trend was reversed for days 7-14.

We found that integrin αvI expression of neck tissue steadily increased from 1 day to 7 days, followed by a return to low levels of expression at 14 days. During this interval, the expression of integrin αvI of non-stented control necks increased rapidly, peaking at 4 days, and then rapidly dropped to baseline by 7 days, remaining at low levels. For both stented and non-stented fundus

tissue, a very mild increase in expression occurred over the first 7 days, followed by a slow return to baseline by 14 days.

None of the difference in changes in mRNA expression between stented and non-stented controls were statistically significant for any adhesion molecules.

### 3. MMPs / TIMPs:

MMP-2 expression at the neck of stented aneurysms increased to a peak at 4 days and remained elevated, gently decreasing to 14 days. Non-stented neck tissue showed a steady, continuous decrease in MMP-2 expression from day 1 to day 14. The curves for MMP-2 expression at the fundus were very similar between stented and non-stented groups, with a slight predominance in stented MMP-2 expression beginning on day 4 and continuing until day 14.

MMP-9 expression of stented neck tissue increased sharply at 4 days and quickly returned to baseline. Non-stented control neck tissue, as well as fundus tissue from stented and non-stented aneurysms remained at a low level of MMP-9 expression across all time points.

TIMP-1 expression curves were almost parallel for stented and non-stented groups at the neck and fundus. Expression decreased rapidly over the first 7 days, followed by a recovery at 14 days. TIMP-1 expression was slightly less for stented necks compared to all other groups.

TIMP-3 expression increased for all groups to a small peak at 4 days, followed by a decrease to baseline by 7 days, remaining at this low level until 14 days. TIMP-3 expression was slightly less for stented necks compared to all other groups.

TIMP-4 expression profiles were almost parallel for both stented and non-stented necks, with expression levels slightly lower for stented necks. At the fundus, TIMP-4 expression levels of stented and non-stented aneurysms were mirror images of each other; stented tissue decreased at 4 days, followed by a peak at 7 days with a return to baseline by 14 days, while non-stented fundus tissue peaked at 4 days, dropped at 7 days, and then became elevated again at 14 days.

The differences in mRNA expression between stented and non-stented controls were not statistically significant for any of the MMPs or TIMPs.

## 4. Cellular markers:

Neck tissue from stented aneurysms showed a steady increase in α-SMA expression over the first 4 days, which then leveled off. α-SMA expression of non-stented necks was lower than for stented necks for all time points except at one day, a time-point for which we had difficulty with mRNA degradation and sample loss. Tissue at the fundus also showed an overall decrease in α-SMA expression for stented and non-stented controls without clear differences in expression profiles.

Both stented and non-stented aneurysm necks showed increasing CD34 expression, reaching the same peak at 4 days, with subsequent slow return to baseline over 14 days. Non-stented fundus tissue did not show the same peak at 4 days, remaining at a level of expression closer to baseline.

The difference in α-SMA or CD34 mRNA expression between stented and non-stented aneurysms was not statistically significant for tissue from either the aneurysm neck or fundus.

## Discussion

The salient findings from this work are that stents improve the angiographic and pathologic appearance of lateral wall aneurysms in 55% of cases, and that this improvement correlated with several mRNA expression trends of genes implicated in neointima formation and thrombus organization: i) increased expression of the growth factors TGF-β1 and PDGF-BB for aneurysm neck tissue starting at 4 days; ii) decreased expression of the pro-inflammatory and chemotactic agents TNF-α and MCP-1, and iii) increased expression of MMP-2 and MMP-9 at the aneurysm fundus, as well as decreased TIMP-1, 3, and 4 at the aneurysm neck. The improved neointima formation was further reflected in a trend towards increased α-SMA expression of tissue from the neck of stented aneurysms. There were no observed trends in mRNA expression of PECAM-1, VCAM-1, or integrin αv to suggest a role for adhesion molecules to account for the improved appearance of stented aneurysms.

The contribution of stenting to aneurysm healing is difficult to assess when stents are used as adjuncts to coiling. The observed angiographic improvement following stenting suggests that their role in aneurysm healing may extend beyond prevention of coil herniation; we hypothesized that this effect was at least in part mediated by flow disruption with subsequent changes in genetic

expression. Hypothetical stent-related modifications of gene expression are summarized in Table 2.

TGF-β1 is a multifunctional regulator; its actions are dependent on species, cell phenotype, growth conditions, and interaction with other growth factors[24, 25]. In atherosclerosis, with disrupted blood flow patterns, TGF-β1 promotes lesion formation and vascular smooth muscle cell (VSMC) proliferation[26, 27]. Alterations in shear stress can upregulate TGF-β1 expression[28], and we were able to detect a non-significant increase in TGF-β1 expression at the aneurysm neck following stenting, which may provide a molecular explanation for the increased neointima formation observed at pathology.

PDGF-BB is a potent chemoattractant and mitogen produced by activated platelets and macrophages that stimulates VSMC proliferation and migration[22]. Experiments that surgically decreased blood flow in an animal model increased PDGF-BB expression [29]. Cultured endothelial cells increased PDGF-BB expression in response to increased shear stress and turbulent blood flow [30]. The marginal increase in PDGF-BB expression in stented aneurysm neck and fundus tissue beginning after 7 days may have contributed to increased neointimal formation.

The pro-inflammatory effects of TNF-α are well-characterized[31-33]. This cytokine is produced by macrophages, neutrophils, endothelial cells and VSMCs, and exerts multiple biological effects on a multitude of cell types [31, 33]. TNF-α locally augments the expression of adhesion molecules and MCP-1 and attracts leukocytes. TNF-α also modulates growth factor expression, stimulates MMP activity, and can trigger endothelial cell apoptosis [33, 34]. TNF-α has been implicated in the generation of atherosclerotic lesions at site of turbulent blood flow; laminar blood flow inhibits TNF-α expression[32], whereas atheromatous areas characterized by turbulent blood flow have high TNF-α levels[35]. Because stents disturb blood flow, we had expected stenting to increase TNF-α expression, and for this to increase expression of adhesion molecules. We did not however observe expression changes of TNF-α or adhesion molecules.

MCP-1 is implicated in the formation of atherosclerotic lesions [36], neointimal hyperplasia following balloon injury[37, 38], and may also play a role in the recanalization of organized intravascular thrombi [21]. Laminar flow inhibits MCP-1 expression [39], and we had anticipated that flow disruption due to stenting would increase MCP-1 expression. As with TNF-α, we observed a trend towards lower expression levels of MCP-1 for stented aneurysms at all time points compared to controls. Changes in MCP-1 expression are known to occur ultra-early (within 5 hours) in cultured endothelial cells[40], and so it is possible that the time-points we chose

may have missed important early events. Nonetheless, because MCP-1 is also implicated in promoting the recanalization of organized thrombus (and thus potentially, recurrences), a relative decrease in expression of this chemokine may prove beneficial.

The balance of MMP and TIMP activity is essential to maintain the proper 'fluidity' of the extracellular matrix[41]. The activity of the collagenases MMP-2 and MMP-9 is thought to be essential to permit cellular migration [25] of neointimal cells into the provisional matrix of platelets and fibrin within a treated aneurysm. MMPs are also essential to the process of angiogenesis[42], and contribute to the formation of neovessels within organizing thrombi. A strategy to decrease the incidence of aneurysm recurrences due to the formation of recanalizing channels or neovessels connected to the parent vessel could aim to inhibit MMP or promote TIMP activity. Proper timing of intervention would be necessary to ensure that sufficient neointimal cells had migrated into the provisional matrix prior to form a robust neointima prior to altering the balance of MMP/TIMP activity to minimize the formation of recanalizing neovessels.

MMPs can also modulate signal transduction pathways by acting on inflammatory mediators, growth factors, and growth factor receptors[43]. MMP-2 and MMP-9 production and activation are increased following arterial balloon injury and subsequent remodeling [44, 45], and MMP-9 has been localized to atherosclerotic plaques in humans[46]. In an in vitro model, elevated shear stress induces changes in MMP-2 and MMP-9 expression that follow different temporal patterns: two days after shear onset, MMP-2 expression levels increase and remain elevated, while MMP-9 peaks quickly and decreases[25]. MMP-9 expression is also responsive to shear pattern, with oscillatory or turbulent blood flow resulting in greater, more sustained expression compared to unidirectional shear [47]. We anticipated that the flow disturbances caused by stenting would increase expression of both MMPs, with a more sustained elevation for MMP-2 than MMP-9. We further anticipated a decrease in TIMP activity to result in a net decrease in the MMP/TIMP balance.

We observed only small increases in MMP-2 activity in the necks and fundi of stented aneurysm compared to controls, which started after 4 days. Because MMPs are potent enzymes, perhaps only small changes in MMP expression, in conjunction with the observed decreased TIMP expression may be sufficient to lead to matrix degradation and allow greater neointimal cell migration and promote aneurysm healing. The sharp, transient increase in MMP-9 expression at 4 days for stented neck tissue was in agreement with published findings, and may reflect the increased exposure of cells of the aneurysm neck to the mechanical force of blood flow.

The α-SMA cells were anticipated to follow the pattern of migration observed in arterial balloon injury models, where cells begin to proliferate in the media after approximately 24 hours [48], then

migrate into the intima at 4 days, continue to proliferate and form extracellular matrix for at least 3 months[49]. In our experiment, we hypothesized that the stent would provoke thrombosis within the aneurysm, and the expression of α-SMA would increase as the cells that contribute to neointima formation populate the provisional matrix formed within the thrombosed aneurysm. With the exception of the time-point at 1 day, the kinetic profile of α-SMA expression for stented tissue at the neck was in accordance with expectations for progressive thrombus invasion with cells destined to populate and from the neointima. The presence of these cells is likely of particular importance at the neck of endovascularly treated aneurysms, to promote robust neointima formation at the junction of the parent vessel and aneurysm neck.

As observed with the resolution of deep venous thrombi, the process of recanalization within and around the formed intra-aneurysmal thrombus may contribute to aneurysm recurrences. To investigate the molecular aspects of this process, we followed expression levels of MCP-1, MMPs/TIMPs, and CD34. The chemokine MCP-1 has recently been implicated to stimulate the monocytic drilling of 'tunnels' within thrombi [50], and the uniformly lower expression of MCP-1 of stented aneurysm neck tissue may partially account for the better angiographic and pathologic outcomes observed at 12 weeks.

There was no reliable means to differentiate MMP or TIMP activity needed for neointimal cell migration from that of neovessel formation. By observing the kinetics of TIMP expression following aneurysm treatment, the initial decrease in expression lasted 7 days before increasing again; perhaps this period of time is sufficient to allow for neointimal cell migration, and future pharmacologic interventions to alter the MMP/TIMP balance to prevent recurrences might be timed for 7 days post-stenting.

CD34 expression was assayed to search for a possible increase in cells of endothelial lineage within the aneurysmal thrombus, particularly at the neck. Neovessels propagating through the thrombus could originate from the tissues surrounding the aneurysm aiming to provide blood supply to the neointimal cells; alternatively, they could represent nefarious recanalizing channels originating from the parent vessel. Circulating endothelial progenitor cells may also contribute to the observed CD34 expression. The CD34 expression profiles of stented and non-stented aneurysms did not clarify the role of recanalization in aneurysm recurrences in this experimental model.

The greatest limitation of this experiment was the small number of dogs we used. As with all animal experimentation, we are limited by ethical and practical considerations; given the size of the effects we observed here, perhaps 50 or more animals would have been needed to attain statistical significance, a sacrifice we could not justify given the lack of indications of where potentially

findings were. Other sources of variation of our model include the variability of positioning of the stent struts relative to the aneurysm ostium, which may have resulted in inconsistent modulation of the fluid dynamics and relatively large changes in mRNA expression. Although we corrected for cellularity by using GAPDH:gene ratios, variability in the formation of thrombus within the aneurysmal pouch may have affected the mRNA expression results. More refined molecular tools also remain to be developed for use in the canine model. Alternatively, we may have omitted some key molecular factors that change dramatically in response to mechanical forces, or our window of sampling may have missed important ultra-early or late events. The cellular composition of a venous pouch aneurysm is different from that of a naturally occurring aneurysm, and the local environment of the experimental aneurysms certainly differed from subarachnoid aneurysms. Finally, balloon-expendable coronary stents are not alike currently available self-expendable intracranial stents.

## Conclusions

Parent vessel stenting is insufficient to cause complete occlusion of lateral wall aneurysms when used alone. However, stenting does contribute to the healing process, as evidenced by the improvement in angiographic and pathologic appearance in 55% of cases. We were unable to detect statistically significant stent-related differences in mRNA expression, although we observed trends in expression of growth factors, cytokines, and matrix agents that may prove useful to the future design of a molecular-based therapy for aneurysms.

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## Table 1. Sequences of primers of selected genes chosen for RT-PCR.

| | Gene | Tm | NT(bp) | Foward primer | Reverse primer | Type |
|---|---|---|---|---|---|---|
| markers | CD-31 | 52 | 687 | 5'-CCCAGGGTGACACTGGACAA-3' | 5'-CCTTCTGGATGGTGAAGTTGG-3' | Consensus human/mice |
| | CD-34 | 64 | 434 | 5'-GCCCAGTCTGAGGTGAGGCCTCA-3' | 5'-CAGGTGTTGTCTTGCTGAATGG-3' | Dog (NM 001003341.1) |
| | αᵥ | 64 | 622 | 5'-GTCAAGGAGGATTCAGCATTGAT-3' | 5'-AGCAGCAATTGCAATATCATTGA-3' | Consensus human/mice |
| | VCAM | 58 | 694 | 5'-CTCTTGGAGAACCCAGATAGACAGTC-3' | 5'-ATGTTCCAGAATCTTCCAGCCTCATAGCAATTA-3' | Dog (NM 001003298.1) |
| Reporter | GAPDH | 64 | 500 | 5'-GCCAAAAGGGTCATCATCTC-3' | 5'-GCCCATCCACAGTCTTCT-3' | Consensus human/mice |
| MMPs | MMP-2 | 58 | 580 | 5'-ATGCAGAAGTTCTTTGGGCTGC-3' | 5'-TTGCCATCCTTCTCAAAGTTGTA-3' | Dog (AF 177217) |
| | MMP-9 | 64 | 476 | 5'-TATGACACCGACCGTCGGTTC-3' | 5'-GTACATGAGCGCTTCTGGCAC-3' | Dog (AF 169244) |
| TIMPs | TIMP-1 | 52 | 341 | 5'-GCGTTATGAGATCAAGATGAC-3' | 5'-CTGGTCCGTCCACAAGCA-3' | Dog (NM 001003182.1) |
| | TIMP-3 | 52 | 260 | 5'-TACCAGTACCTGCTGACAGG-3' | 5'-GCCCATCCTCGGTACCAGCT-3' | Dog (XM 53840.2) |
| | TIMP-4 | 58 | 331 | 5'-ATCTCCAGTGAGAAGGTAGT-3' | 5'-GTGGTGATTTGGCAGCCACAGTTC-3' | Consensus human/mice |
| Growth Factor | TGFβ1 | 64 | 366 | 5'-TTCCTGCTCCTCATGGCCAC-3' | 5'-GCAGGAGCGCACGATCATGT-3' | Dog (NM 001003309) |
| | PDGFbb | 64 | 393 | 5'-ATGAATCGCTGCTGGGCGCTCTTCCT-3' | 5'-GGAGCAGCGCTGCACCTCCAC-3' | Dog (NM-001003383) |
| | TNFα | 64 | 436 | 5'-CCTCCAACTAATCAGCCCTCT-3' | 5'-TCTCAGCGGCTGAGTCGATCA-3' | Dog (NM 001003244.1) |
| | MCP1 | 64 | 305 | 5'-ATGAAGGTCTCCGCAGCGCTC-3' | 5'-TCATGGCTTTGCAGTTTGGGTTTG-3' | Consensus human/mice |

**Table 2.** Genes potentially involved in the molecular mechanisms of aneurysm healing, with the hypothesized expression changes obtained from a survey of the literature of other vascular processes, and our experimental observations.

| GENE | Expression Changes due to Stent | |
| --- | --- | --- |
| | Hypothesized | Observed |
| TGF-β | ↑ | ↑ |
| PDGF-BB | ↑ | ↑ |
| MCP-1 | ↑ | ↓ |
| TNF-α | ↑ | ↓ |
| VCAM-1 | ↑ | - |
| MMP-2 | ↑ | ↑ |
| MMP-9 | ↑ | ↑ |
| TIMP-1 | ↓ | ↓ |
| TIMP-3 | ↓ | ↓ |
| TIMP-4 | ↓ | ↓ |

**Figures**
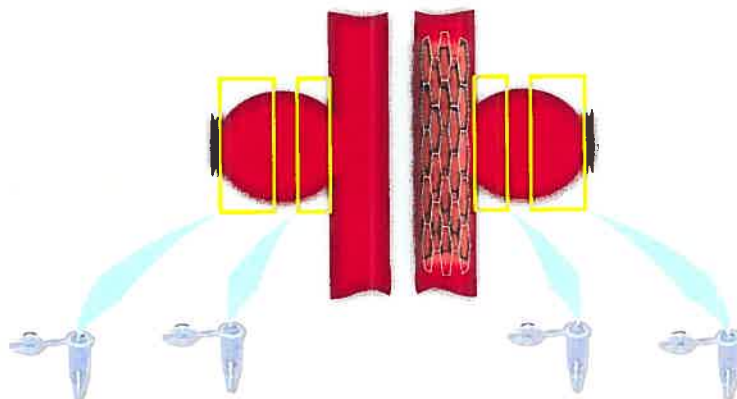


**Figure 1.** Schematic of tissue chosen for mRNA expression studies: 2 and 3mm axial sections of aneurysm tissue, from the neck and fundus, respectively, were harvested.
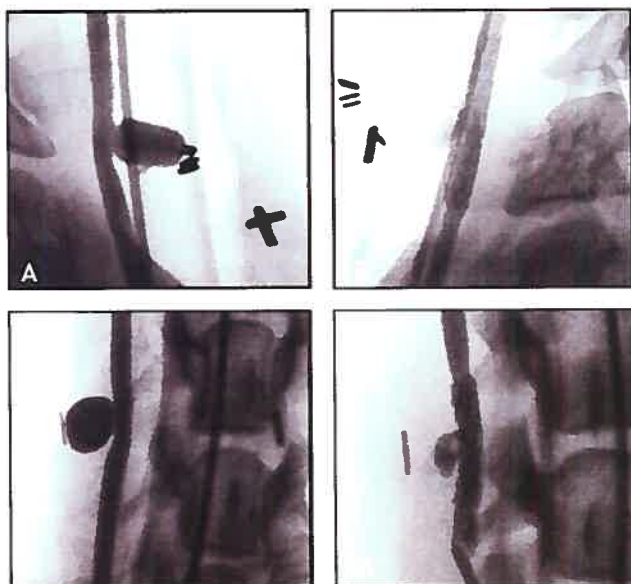


**Figure 2.** Angiographic progression of stented lateral wall aneurysms with time. A, C) represent initial images at 4-6 weeks following surgical creation. B, D) show improved angiographic appearance following stenting, but not complete obliteration, at 4 days and 12 weeks respectively.
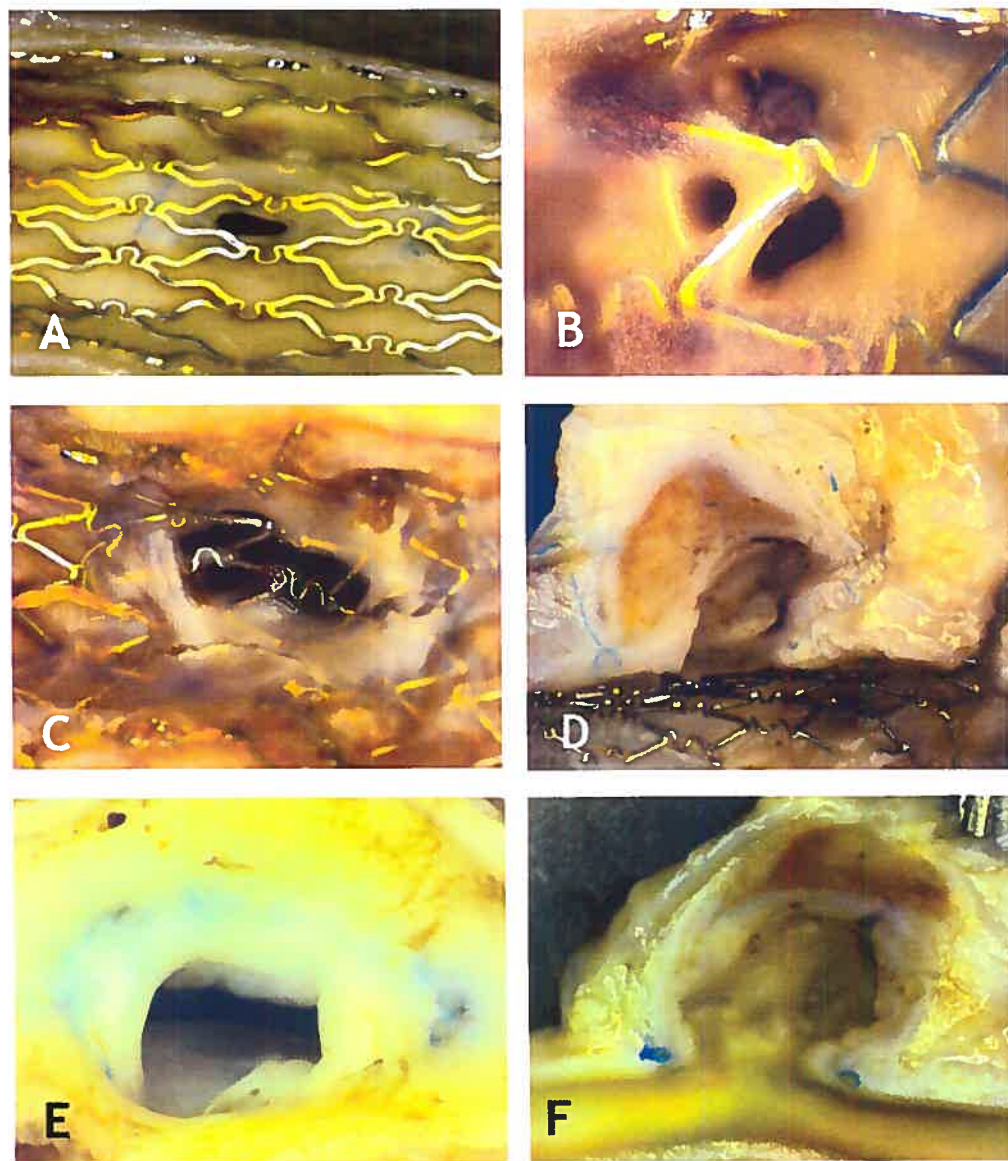
**Figure 3.** Photographs of stented (A-D) and non-stented (E-F) control aneurysms. Good neointima formation in (A, B), filling aneurysm body and neck and incorporating stent struts. C, D) reveal incomplete neointimal filling, with the aneurismal defect evident on en-face as well as sagittal views. E, F) demonstration the widely patent ostia and minimal neointimal filling typical of non-stented control aneurysms.

**Figure 4.** Gene expression associated with stenting. Results of RT-PCR analyses, expressed as specimen/arterial controls ratio, according to time. A) Gene expression at the fundus B) Gene expression at the neck of aneurysms. (● = Stented; ○ = Not stented).

A)
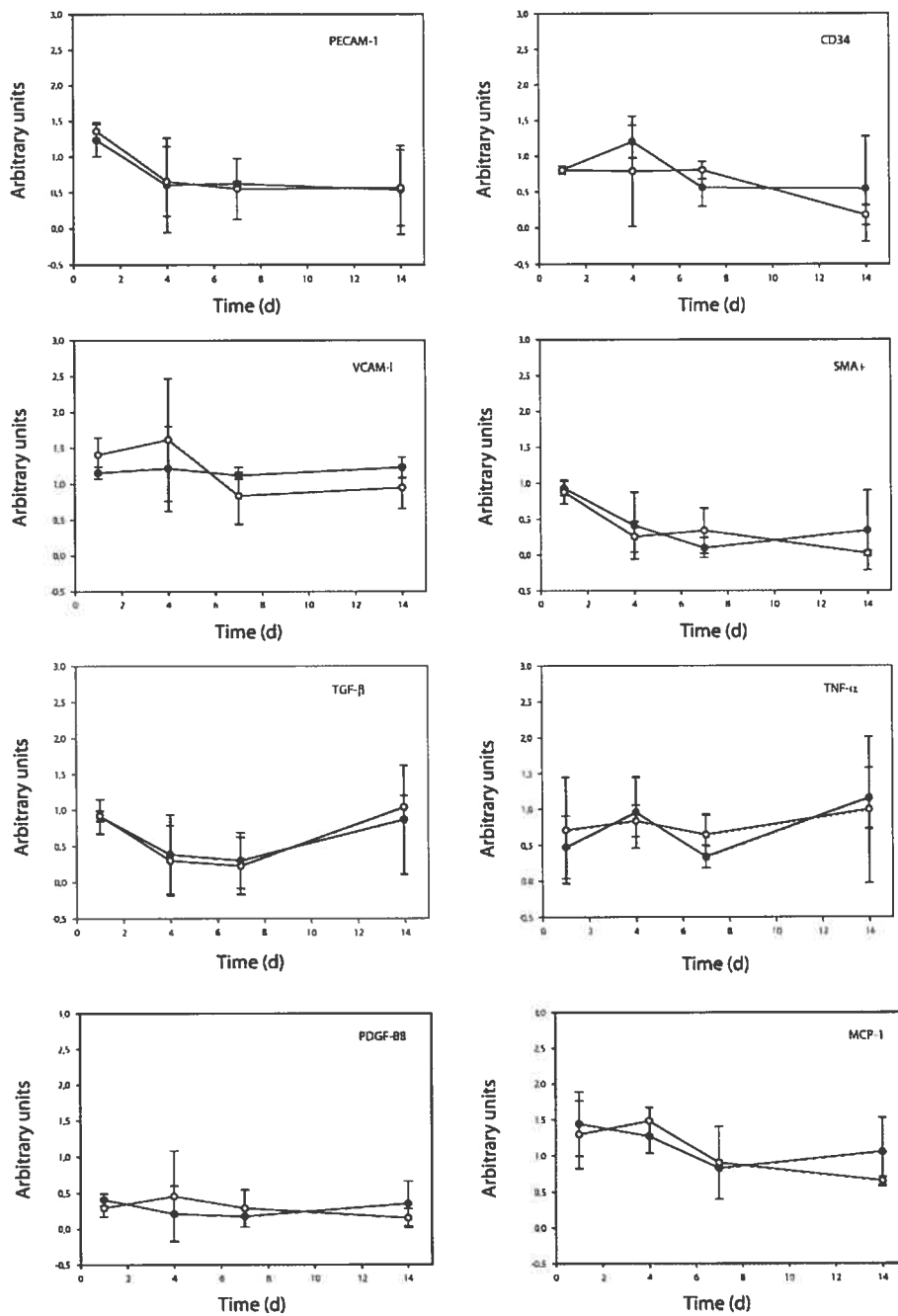
B)
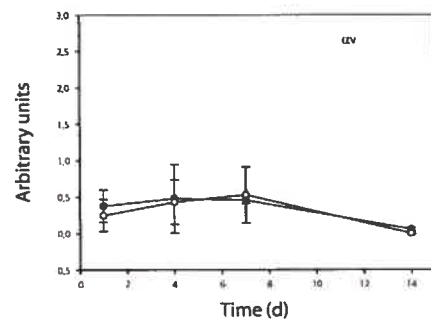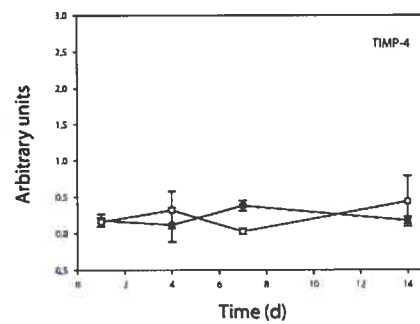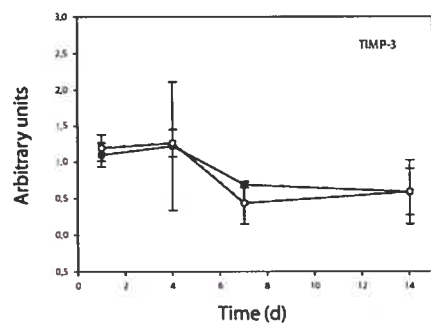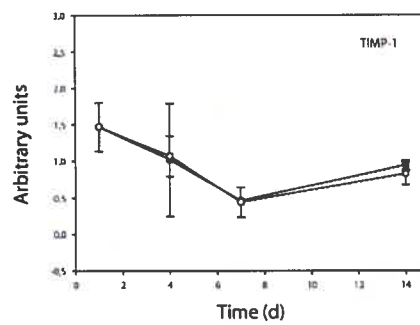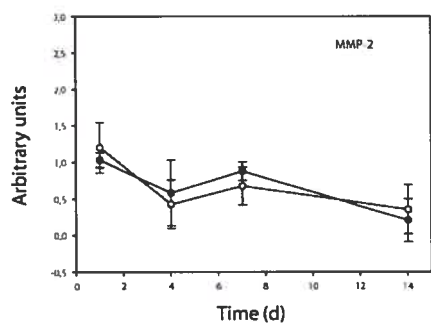


*Arbitrary units = mRNA levels of gene of interest/mRNA levels of GAPDH

# 5. DISCUSSION

The primary goal of therapy for intracranial aneurysms is usually to prevent future aneurysm rupture. Clinically, digital subtraction angiography is considered the best surrogate outcome to assess for risk of future rupture and thus evaluate aneurysm treatments. While angiography remains the best clinically available measure of aneurysm treatment, it cannot reliably distinguish those aneurysms destined to remain occluded from aneurysms likely to recur. However, studies of experimental aneurysms demonstrate associations between type of aneurysm treatment and pathological outcomes, and have identified features associated with durable aneurysm occlusion[93]. Autopsy specimens of healed human aneurysms after embolization are few, but findings suggest that stable angiographic results are associated with neointima formation at the neck, without recanalizing crescents[196]. Molecular biology may hold the key to understanding the causal links leading to these variable pathological outcomes. Insight into the molecular mechanisms of thrombus formation, cellular invasion, thrombus organization and neointima formation may lead to therapies to closely control the amount of tissue formed within treated aneurysms; complete filling of the aneurysm body and neck may promote more durable results. Further characterization of the role of endothelial cells in the treatment response, as well as the kinetics and mechanics of re-endothelialization and thrombus recanalization may reveal new targets for molecular-based aneurysm treatment strategies.

The molecular processes occurring in the area of the aneurysm following endovascular treatment are very complex, and remain poorly understood. In the absence of established knowledge regarding the molecular mechanisms of aneurysm healing, research into this nascent field relies on established literature from other domains. The arrival and formation of cells and extracellular matrix within the body of the aneurysm and the formation of a mural structure at the neck may resemble the events occurring following balloon-angioplasty injury with neointimal hyperplasia, while the flow-related genetic modifications due to stenting may mimic those known to occur in atherosclerosis. The invasion of the matrix within the aneurysm with cells seeking to vascularize this newly

formed tissue may share conceptual similarities with angiogenesis. Finally, the DVT recanalization process known to restore blood flow past occluded veins may also act on iatrogenically formed intra-aneurysmal thrombi. The molecular biology portion of this work relies on the principle of parsimony of nature; we only stand to make discoveries if considerable molecular similarities exist between aneurysm healing and other known vascular phenomena.

Research using experimental aneurysms permits us to seek the biochemical or molecular mechanisms that lie behind observed pathological phenomena. Virchow's triad inspired our search for modifiable factors possibly involved in aneurysm healing; endothelial denudation as a form of controlled trauma to the blood vessel and endoluminal stenting as a means to modify flow and promote intra-aneurysmal stasis were the manifestations of this inspiration. We also aimed to capitalize on the fact that stent placement within the parent artery allows us to harvest aneurysm tissue without artifacts caused by metal coils. Another advantage of using stents to study genetic effects of flow modification is that stents likely cause a more reproducible disruption in flow compared to endosaccular coils.

In the spirit of Virchow, I will begin by focusing on the pathophysiological processes of intra-aneurysmal neointima formation/thrombus organization and recanalization, followed by a discussion of the potential role of endothelial cells. I will conclude with the potential role of stents and flow disruption as a means to treat aneurysms.

## 5.1  NEOINTIMA FORMATION / THROMBUS ORGANIZATION

In an effort to maintain clarity of language regarding these processes, some preamble is necessary. The cellular events occurring within an endovascularly treated aneurysm involve the formation and maturation of thrombus, spanning from the depths of the aneurysm fundus to the thrombus nearest the aneurysm neck that is exposed to blood flow. Traditionally, a distinction has been made between the processes of thrombus organization and neointima formation, with the latter term reserved exclusively for a vessel wall reaction to some type of injury. However, at the level of the aneurysm ostium

there is no vessel wall and the 'neointima' is synonymous with 'thrombus organization' occurring at the interface of the blood vessel and circulating blood flow. The largely artificial distinction between what goes on inside the sac of the aneurysm and the ostium, which is a continuous process without clear-cut boundary in pathological specimens (Figure 5.1), although important to classical pathological vascular paradigms, is hereafter disregarded; the process of cellular proliferation, migration and extracellular matrix production that occurs at blood-flow exposed regions is considered to represent the same process that occurs to fill the substance of the aneurysm. Morphological differences remain, perceived as a specific orientation of cells as we come closer to the circulating blood at the residual neck, but the distinction is here conceived as a nuance of the same phenomenon.



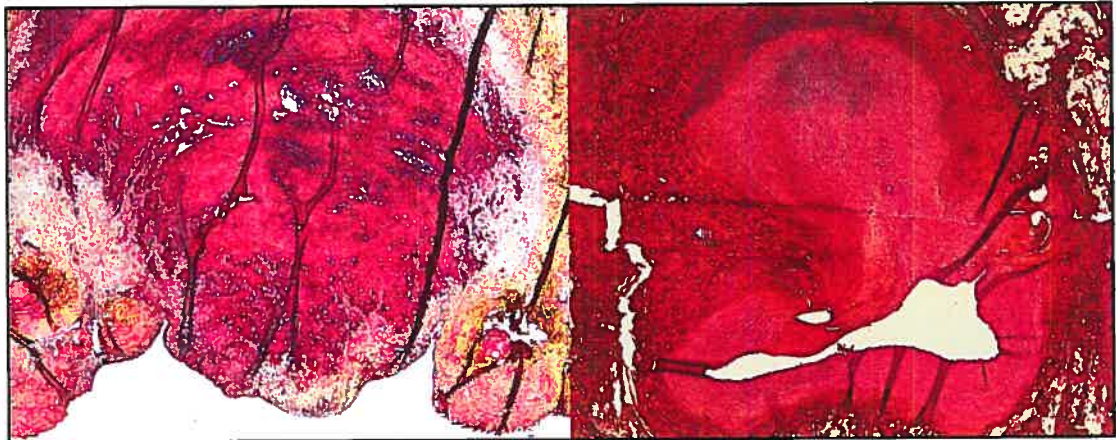**FIGURE 5.1** Photomicrographs of axial sections through an aneurysm.

The evolution of the thrombus formed within the aneurysm seems to be variable. In some cases, following treatment, a robust provisional matrix is formed within aneurysm, followed by invasion of cells destined to organize the thrombus and form neointima. Sufficient extracellular matrix is deposited, and neovessels (or extensions from pre-existing

vessels) develop to supply the newly formed intra-aneurysmal tissue. The parent vessel then undergoes a period of remodeling, resulting in a blood flow conduit that recreates normal flow patterns and stably resumes a form that does not include an aneurysmal dilation. In other cases, the aneurysm does not become completely obliterated. This outcome results from either incomplete treatment, where the residual neck or saccular remnant is immediately angiographically apparent, or from aneurysm recurrence following treatment initially deemed angiographically complete. Compared to completely treated aneurysms, incompletely treated aneurysms are more likely to recur[197, 198]; this determination has stimulated the use of packing density as a measure of the extent of treatment, a well as the development of modified coils which swell following deployment and increase the volume of space filled by the coils[26]. However, structural-based attempts to increase the volume of the aneurysm filled by coils do not address the biological phenomena that lead to recurrence of both incomplete and completely treated aneurysms.

The formation of intra-aneurysmal thrombus is the sine qua non of aneurysm healing, as the platelet and fibrin thrombus forms an essential provisional matrix for subsequent cellular invasion. The strength of adhesion of the formed thrombus to the deposited coils and wall of the aneurysm may impact the evolution of treatment. Although antiplatelet or anticoagulation use following stenting is common in clinical practice, the more active canine fibrinolytic system permits the use of stents without the same risk of thrombotic sequelae seen in humans.

The dissecting effects of blood flow may form channels into the thrombus/coil mass, an effect which seems particularly evident at the inflow zone of experimental lateral wall aneurysms. A layer of thrombus immediately apposed to the aneurysm wall may also facilitate the invasion of cells from adjacent tissues into the provisional matrix filling the body of the aneurysm. (The presence of an intact endothelial layer may interfere with the close apposition of these layers; the endothelium is discussed later on). Another potential reason why firm adhesions between the thrombus and aneurysm wall may be important is that the contractile forces of the $\alpha$-smooth muscle actin positive cells within the maturing thrombus tends to create a centripetal force collapsing the aneurysm (see Figure 1.6). This

natural wound healing process helps minimize the dead-space that must be filled by healing cells, but in the case of endovascularly treated aneurysms, the contractile force may pull the thrombus away from the aneurysm wall and form fissures (Figure 5.2) that subsequently develop into recurrences. The balance of matrix metalloproteinase activity at the margins of the thrombus/aneurysm wall interface may also play a role in the strength of adherence and the resistance to fissure formation.
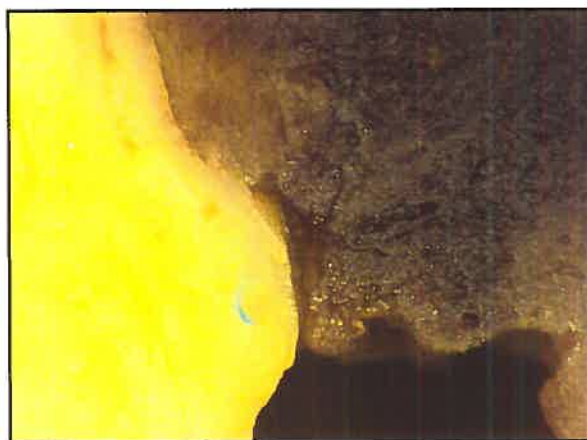


**FIGURE 5.2**
Photograph of coronal section through a lateral wall aneurysm.

The formation of neointima depends on adequate proliferation and migration of cells into the aneurysm body, replacing the provisional matrix with more permanent ECM proteins. Cardiologists are greatly interested to discover ways to limit the formation of neointima and prevent re-stenosis following balloon angioplasty and stenting, and the literature is full of examples of new drugs targeting the cell cycle or nuclear signaling pathways to limit the proliferation and migration of neointimal cells. Endovascular treatments directed towards aneurysms are interested in modulating neointima formation differently at two different sites. First, we aim to *promote* neointima formation at the aneurysm neck and organization within the aneurysm body, to recreate an arterial blood flow conduit without a pathological dilation. By augmenting neointima formation, we hope to decrease the rate of recurrences from incompletely treated aneurysms. Increased neointimal thickness, at least in animal models, has been correlated with better healing[25, 146, 199]. The second site where neointima formation is of interest to us applies to endovascular strategies using stents, where good neointima formation around the stent is required to incorporate the stent into the wall and cover the potentially thrombogenic surface. Of course, like the

cardiologists, we are also concerned about over-exuberant neointima formation leading to parent vessel stenosis or branch occlusion.

Our attempts to study the effects of flow modification on the genetic expression of key molecules involved in aneurysm healing focused primarily on growth factors, cytokines, adhesion molecules, and cell surface markers potentially important to the process of neointima formation, as well as the enzymes involved in regulating cell migration through the matrix. (We also looked at the expression of one cell surface marker, CD34, to study the progression of endothelialized channels within the treated aneurysm, which will be discussed later.) The discovery of a growth factor able to locally stimulate neointima formation within the aneurysm might permit modulation of aneurysm healing using biologically coated coils. We were also seeking a molecular explanation for the observed improved angiographic and pathologic results following stenting. Many of the factors we studied had been shown to be responsive to flow in other models of vascular disease, and we did notice some interesting trends in mRNA expression changes following stenting, even though these differences did not reach statistical significance (see section 5.5.1 for experimental limitations). Nonetheless, the trends suggest two things: First, the growth factors and adhesion molecules we chose seem to play a role in the formation of neointima and thrombus organization; second, that there is an increase in endothelialization over time *within the substance of the aneurysm*. These findings shed some light on the molecular mechanisms of aneurysm healing, and also support a role for using stents to improve the results of aneurysm treatment, even though stenting did not lead to the dramatic mRNA expression changes we originally expected.

Strategies aiming to improve aneurysm healing by targeting the action of MMPs or their inhibitors present new exciting possibilities. These enzymes are potentially important to two separate processes; the migration of cells destined to form neointima, as well as the formation of new vascular channels within the organizing tissue. Strategies that promote matrix degradation to promote cell migration must be viewed with extreme caution; MMPs are potentially very destructive enzymes, and the potential consequences of thinning the matrix around recently treated aneurysms should not be underestimated. The more interesting therapeutic goal of preventing MMP activity and inhibiting the formation

of new vascular channels within the thrombus is further discussed in the following section on recanalization.

## 5.2 RECANALIZATION

Recanalization is known to occur at the margins of deep venous thromboses[200, 201]. In linear vessels occluded by thrombus, small neovessels attempt to form channels around the thrombus, to re-connect the patent portions of the vessel on each side and re-establish blood flow. Recanalization is also likely at work in the setting of endovascularly treated aneurysms, originating from the aneurysm ostium and attempting to form channels towards the fundus. Unlike the situation of a thrombus separating linear blood vessels, however, the aneurysm remains a blind-ended pouch, and the process of recanalization serves only to recreate another blind-ended channel which may develop into another aneurysm. There are at least three factors which likely promote recanalization of intra-aneurysmal thrombus: monocytic 'tunnels', increased matrix metalloproteinase activity, and hemodynamic forces.

The recent discovery of MCP-1-dependent tunnel drilling by monocytes occurs within intravascular thrombus, in conjunction with the discovery of circulating progenitor cells, suggests a new mechanism for thrombus recanalization[79, 94]. In this scheme, the monocytes that coat the luminal surface of an intravascular clot form tiny crevices or 'tunnels' into the thrombus, into which circulating cells may subsequently settle and differentiate. This possibly represents a mechanism for the creation of the observed neovessels implicated in aneurysm recanalization. By implicating monocytes and MCP-1 in the process, it also presents a possible therapeutic target to decrease recurrence rates.

Another potential means to decrease the formation of neovessels through the thrombus involves modulating the ratio of MMP/TIMP activity. Specific inhibition of MMPs has been shown in models of angiogenesis to decrease the formation of capillary tubes[60], a finding which has important implications for many vasculo-proliferative processes, including aneurysm recurrences. Selective inhibition of MMPs involved in recanalization (neovessel formation from the parent vessel) while permitting matrix degradation for cellular migration and thrombus organization and preserving the formation of vessel

supplying neointima cells is the goal of this treatment strategy. This work focused on the more modest goal of identifying MMP or TIMP family members potentially important to these processes in aneurysms. Our observation of a transient sharp increase in MMP-9 expression with stenting, although not statistically significant, may merit further investigation.

Hemodynamic forces are implicated in the formation and rupture of aneurysms, and are also known to affect the evolution of treated aneurysms[194, 202, 203]. Pulsatile blood flow orients and directs the formation of neovessels in models of angiogenesis[60], and may stimulate the expansion and growth of small channels into the maturing thrombus, both by mechanical separation and spreading of the organizing thrombus, as well as by altering cellular signaling cascades of mechano-responsive cells. Figure 5.3 depicts the decreased blood flow into a lateral wall aneurysm following stenting. One of the possible beneficial effects of endovascular stenting may be to buffer the hemodynamic forces implicated in recurrences by absorbing and dispersing the pulsations of blood flow through the stent structure. This buffering effect would be more pronounced with more rigid stents, potentially necessitating a trade-off for the increased flexibility and intracranial navigation of newer generation stents. The ability of a stent to absorb and disperse hemodynamic forces also certainly depends on the magnitude of force acting on the formed thrombus.
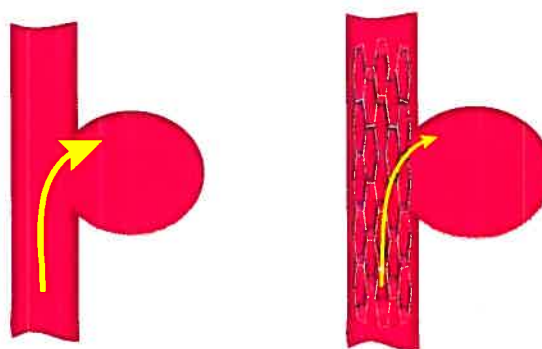


**FIGURE 5.3** Schematic showing the direction and attenuation of blood flow into a lateral wall aneurysm.

## 5.3 STENTS AND BLOOD FLOW MODIFICATION

The clinical use of intracranial stents for the treatment of aneurysms is still in its infancy. There remain many unanswered questions, including: i) the optimal stent design to provoke aneurysm thrombosis yet minimize thrombotic risks, both to the parent vessel and perforating branches covered by the stent; ii) the nature of the response of the parent vessel and aneurysm to stenting, and how to control it; and iii) how flow alteration impacts aneurysm healing. Finally, and most importantly, given the higher recurrence rates currently associated with endovascular treatment, justifying the choice of endovascular treatment over surgical clipping requires more thorough knowledge of the risks associated with the various intravascular devices.

The optimal stent design that attains the therapeutic goals of provoking aneurysm thrombosis while sparing adjacent branches depends on the local microanatomy of the targeted aneurysm. More basic research is required to understand the effect of different stent cell patterns on blood flow disruption. In the future, stents designed and oriented to conform perfectly to the parent artery with a high density mesh over the aneurysm ostium and openings to permit unobstructed blood flow into branch vessels may be manufactured according to specifications and measurements taken from an angiogram, personalizing the structural component of the stent to meet the therapeutic goals. As we progress into a new era of DNA mapping, the biological agents used to coat endovascular devices may also take into account the patient's genome and proteome to promote stable aneurysm occlusion and minimize in-stent stenosis. Advances in molecular biology may one day allow us to engage in preventive medicine directed against aneurysm formation and reduce the prevalence of aneurysms and incidence of SAH. In the meantime, we will continue to attempt to characterize the molecular and cellular processes occurring inside aneurysms treated endovascularly, including the effects of flow alteration on genetic expression of cells within an aneurysm.

Mechanotransduction is a relatively new area in vascular biology research, especially the effect of mechanotransduction on the molecular mechanisms of aneurysm healing. Endoluminal stents likely contribute to the treatment outcome by promoting thrombosis

within the aneurysm, redirecting blood flow within the parent vessel, forming a scaffold for neointima formation, and by buffering hemodynamic forces implicated in recurrences. This work provides further evidence that stents alone are not a reliable means to treat aneurysms, only occasionally resulting in aneurysm thrombosis. For this reason, an additional endosaccular thrombotic agent (most often coils) is often used to promote thrombosis. Coils properly placed beyond the margin of the aneurysm neck do not modify flow in the parent vessel, and remain passive recipients of the hemodynamic effects of pulsatile blood flow. Stents at least partly serve as a guidance tube through the parent vessel, decreasing blood flow into potential space outside the confines of the stent. This reduces the dissecting force of radial-directed blood entering the aneurysm inflow zone.

In lateral wall aneurysm models, recurrences are typically characterized by a larger recurrence at the inflow (distal lip of the ostium) compared to the outflow zone (Figure 5.4). The force of blood flow striking the freshly-formed aneurysm thrombus at the inflow zone may promote the formation of an endoluminal wall defect at the site of maximal impulse.
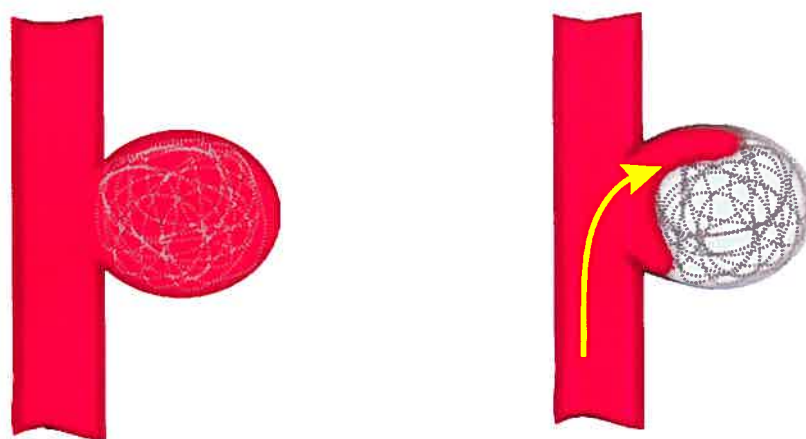


**FIGURE 5.4** Schematic illustrating the larger aneurysm recurrence at the distal lip compared to the proximal aneurysm lip.

For blood that continues to enter the aneurysm, passing through the stent struts likely increases flow turbulence and decreases shear stress. Studies of blood flow in atherosclerosis have shown that turbulence increases neointima formation; cellular proliferation is stimulated, while laminar blood flow patterns exerts a suppressive effect [64]. Neointima formation is greater in the lee of the main thrust of blood flow, such as on the proximal (non-flow divider) side of a branch vessel[67]. The observed increased neointima with turbulent flow suggests that akin to atherosclerosis, the disrupted flow characteristics blood entering a stented aneurysm may initiate changes in gene expression that translate into biological effects including formation of more robust neointima.

The location of the stent at the junction of parent vessel and aneurysm neck, as compared to the often more-recessed platinum coils, may have promoted neointima formation up to and around the stent struts. The scaffolding properties of the stent may therefore serve to help re-create a new endoluminal boundary and maximize neointima formation at the site crucial to prevent recurrences - the junction of the parent vessel and aneurysm neck.

Finally, the ability of the stent to reduce the impulse of blood entering the aneurysm by dispersing the force over a greater surface area may have served to buffer nefarious hemodynamic signals transmitted to buried endothelial cells. As further discussed in the following section, parietal or seeded circulating endothelial cells covered by thrombus may rely on mechanical guidance cues transmitted through the provisional matrix to form endothelialized channels through or around an intravascular thrombus. These small channels may be a necessary precursor to aneurysm recurrences, and the ability of the stent to disperse and partially absorb these mechanical signals may promote stable aneurysm occlusion by limiting the success of formation of channels back to the parent vessel from within the aneurysmal thrombus.

## 5.4 THE ROLE OF THE ENDOTHELIUM

The endothelial lining is known to be associated with residual necks and aneurysm recurrences after embolization in animal models[93, 98]. Initially assumed to be a

quiescent layer of cells simply defining the boundary of the endoluminal space, the ability of this dynamic cellular layer to dramatically alter disease processes is increasingly recognized.

Removal of the endothelial layer likely influences aneurysm healing through a combination of at least three mechanisms. First, physical removal of endothelial cells eliminates the thrombolytic and anti-thrombotic EC surface, exposing a large surface area of thrombogenic collagen, which may promote a more vigorous thrombotic and healing reaction. Classical experiments in vascular pathology have associated the extent of endothelial denudation with subsequent neointima formation, and implicated the role of re-endothelialization in limiting re-stenosis[204, 205]. The effects of exposing subendothelial tissue on circulating or local factors involved in the vascular healing response is incompletely known, but the activity of some growth factors is known to be modified by binding to collagen[206].

Denudation also removes the endothelial contribution to cellular signaling cascades. In aneurysms with intact endothelium, thrombus formation within the aneurysm sac coats the endothelial cells and deprives them of their blood supply. The ensuing hypoxia and acidosis likely triggers changes in genetic expression that may impact vascular healing mechanisms. Although these molecular pathways remain obscure, a negative impact of endothelial cell signaling on durable experimental aneurysm occlusion seems biologically plausible. The endothelium is also important in recruiting cells of monocytic lineage, possibly in an MCP-1 dependent fashion, and monocytes may in turn play an important role in recanalization[207, 208].

Finally, the layer of endothelial cells buried under the intra-aneurysmal clot may participate in the formation of neovessels within the thrombus. (Recent evidence supporting the seeding of thrombus with circulating endothelial progenitor cells may also place endothelial cells within the substance of the thrombus as well as at the thrombus/wall interface.) In angiogenesis, isolated clusters of endothelial cells have been shown to burrow in multiple directions. In experimental models, spatially separate endothelial cells line up along force lines and proliferate in defined directions in response to mechanical

signals sensed through the extracellular matrix [60, 180]. The clusters of proliferating endothelial cells form contacts with other burrowing endothelial cells and form cords, which are the precursors to blood vessels.

Should buried endothelial cells survive the low oxygen tension within the thrombus, they may participate in the formation of two types of neovessels: those proliferating to supply the neointimal cells of the organized thrombus, or those participating in the process of recanalization. Centripetally-directed migration of neovessels (likely originating from vasa vasorum or adventitial tissue layer) into the substance of an organizing aneurysm may encounter and incorporate buried endothelial cells, restoring their blood supply. The endothelial cells thus form lined channels within the substance of the aneurysm to reach buried neointimal cells. For this reason, the tissues surrounding an aneurysm may be important to aneurysm healing processes; an insufficient blood supply may lead to insufficient organization of the aneurysmal sac. With respect to our canine model, it is important to note that the soft tissues of the canine neck surrounding the experimental aneurysms certainly represent a different environment compared to the cerebrospinal fluid of the subarachnoid space. The second type of 'neovessel-like' structure to which buried endothelial cells may join up with are channels or crescents involved in recanalization (see also section 5.2). Endothelial cells buried within the organizing thrombus may be incorporated into and contribute to the formation of recanalizing channels. Because these channels originate from the parent vessel and extend into the substance of the treated aneurysm, it seems plausible that they could enlarge and dilate secondary to contraction of the organizing clot and the presence of appropriate hemodynamic factors, forming a recurrence.

Because of the implication of the role of the endothelium in aneurysm recurrence, removal of this cell layer seems a desirable treatment goal. However, there is considerable concern about the safety of denuding aneurysms via the endovascular route. Because aneurysm rupture ultimately occurs due to an abnormal thinning of the blood vessel wall, any treatment that further removes cell layers threatens to promote aneurysm rupture. One potential solution to this problem would make use of the fact that most aneurysms rupture at the dome; a staged approach with coil deposition to protect the friable dome

could be followed by a subsequent treatment, where the remainder of the aneurysm neck could be denuded to promote good neointimal formation where it is most needed, at the junction of the parent vessel and aneurysm neck. A potential means to accomplish this would be to inflate a balloon across the neck of the aneurysm with a microcatheter inside the aneurysm, which could release a de-endothelializing liquid into the aneurysm, followed by further embolization with coils and possibly a stent.

One final note regarding endothelial denudation bears mentioning. The published benefit of endovascular coiling over surgical clipping was demonstrated with old-generation coils, which were stiffer and more difficult to deploy, likely resulting in at least some damage to the endothelium. As newer generation softer coils designed to decrease the risk of aneurysm perforation are introduced to the market, inadvertent denudation may be decreasing. Any treatment benefits accruing from this inadvertent damage to the endothelium may disappear with softer coils, further reinforcing the need for rigorous pre-clinical evaluation of all new endovascular devices.

## 5.5 EXPERIMENTAL LIMITATIONS

The limitations of this work can be divided into general limitations of animal models of aneurysms and those related to the use of stents, as well as concerns specific to the individual projects.

### 5.5.1 General Limitations

As with all studies using animals, costs and ethical concerns limits the number of animals used. The small number of dogs affected our statistics, and by using such a small n, we may have missed a significant result. Other general concerns include surgical and procedural variability, as aneurysm creation was a responsibility shared by two different surgeons. Surgical decision-making at time of operation included how much vein to harvest to form the vein pouch, which in turn determined the height of the aneurysm; the

size of the arteriotomy, which dictated aneurysm neck size, aneurysm hemodynamics, and the amount of stretch of the vein segment required to form the anastomosis. Mechanical manipulation of the artery bearing the aneurysm at surgery occasionally led to vasospasm, which may have transiently affected the hemodynamics of blood entering the aneurysm. The endovascular procedures, although primarily performed by one operator, were subject to considerable variability, in terms of contrast injection volumes and rates, variable catheter positioning, and inflation pressures, which may have affected angiographic and mRNA expression results. Variable positioning of the stent struts with respect to the aneurysm ostium may have led to variability in the manner and degree to which flow was disrupted. Furthermore, the limited number of available stents led to the use of more than one size and type of stent design, which may have variably affected hemodynamics and tissue reaction. Finally, for pathological analysis the presence of the metallic stent led to difficulties in tissue processing, introducing artifacts into the pathological specimens. Notably, it made the study of the endothelium covering the luminal portion of the stent very difficult. Nonetheless, the uniform tubular stent handled more predictably than endovascular coils.

Venous pouches also certainly differ from naturally occurring aneurysms, in their cellular composition but perhaps also in their biological reaction. The location of the experimental vein pouch aneurysms within the soft tissues of the canine neck differs from that of the subarachnoid space where most aneurysms are found; the surrounding environment may influence the cellular and molecular response to aneurysm treatment.

Finally, because the fibrinolytic systems of dogs is more active compared to humans, the withholding of antiplatelet agents in experiments using this canine model may alter the process of thrombus maturation or neointima formation within aneurysms.

## 5.5.2   Specific Limitations

### 5.5.2.1 Lingual Lateral Wall and Carotid Bifurcation Project

We chose the lingual lateral wall aneurysm model because the presence of multiple peri-aneurysmal branch vessels mimics most human intracranial aneurysms. The smaller

arterial caliber and aneurysm size at the lingual position, with lesser hemodynamic forces, may be an important factor limiting generalization of our results. Attempts to replicate the results of stenting and endothelial denudation of the lingual model in the bifurcation model (with greater hemodynamics) failed, suggesting that aneurysms subjected to greater forces may require a different or additional treatment strategy.

### 5.5.2.2 mRNA expression Project

The small number of dogs used per time-point was our greatest experimental limitation. We experienced considerable difficulty with mRNA degradation at the outset of our experiments, leading to the use of Trizol to preserve the aneurysm tissue prior to freezing in liquid nitrogen. In spite of this precaution, often the expression of the ubiquitous enzyme GAPDH was nonexistent, leading to the loss of one specimen. Alternatively, after running the gel, the expected bands were found to be destroyed, with nucleic acid fragments settling out into the bottom of the gel. Part of the difficulty with attempting to amplify and quantitate the genetic expression of tissue forming within an aneurysm using RT-PCR is that in some cases, the aneurysm fundus would be devoid of tissue, while in others it would contain variable amounts of cells and extracellular matrix. Because RT-PCR amplifies the mRNA present and compares it to the expression of a known standard, in some cases the aneurysm was an empty space, while in others there was found a variable amount of thrombus formation, leading to difficulties in comparing tissue samples. To address these problems, molecular tools appropriate to canines need to be developed. Alternatively, the development of an adequate aneurysm model in a smaller species, such as mouse or rat would greatly facilitate biochemical and molecular studies and enable the use of a greater number of animals. It is worth mentioning that the PCR data generated with this method was only semi-quantitative; real-time PCR would likely yield more precise results. This technique also does not permit quantitation of protein levels.

Another problem related to the biochemistry project included the incidence of crushed or migrated stents. We discovered that some dogs wore collars and leashes while being cared

for by the animal technicians, and rambunctious activity may have led to the collar crushing the stents or triggering migration. This problem would not affect humans, where the skull would protect stents from external compressive forces, although crushed stents have been reported in more accessible locations, such as the carotid bifurcation[209].

## 5.6 FUTURE DIRECTIONS

As in many areas in medicine, more work needs to be done in the domain of public health to reduce the prevalence of smoking and tobacco-related disease in our population. Although the indications for treatment of ruptured aneurysms are becoming more clear, the best treatment, if any, of unruptured intracranial aneurysms remains a contentious issue. The multicenter randomized TEAM study, initiated by the clinical arm of our laboratory, promises to shed light on the natural history of unruptured aneurysms, which will guide clinical decision making regarding aneurysms. More work remains to be done to characterize the ideal stent design for the treatment of intracranial aneurysms, taking into account aneurysm morphology, location, and hemodynamics, and the adjunctive treatments being used. In our laboratory, we will persist in our search to uncover the molecular mechanisms of aneurysm healing. In light of the major difficulties secondary to the unavailability of biomolecular tools for dogs, there is considerable interest in our laboratory in developing a murine aneurysm model for molecular biology work. Although attempts thus far have led to unacceptable experimental mortality rates, we will continue to devise new and safer ways to create experimental aneurysms in a smaller model. One future project that promises to yield insight into the role of the endothelial lining in aneurysm recurrences would explore the differences in mRNA expression between denuded and non-denuded aneurysms.

Finally, a safe method to remove the endothelial lining through an endovascular approach remains to be found.

## 5.7 CONCLUSIONS

The process of aneurysm healing following endovascular therapy remains a complex, incompletely understood process. Nonetheless, several conclusions emerge from this body of work: First, stents alone are usually insufficient to treat aneurysms, but they can significantly improve angiographic and pathologic results. Second, the beneficial result of stenting can be augmented by combining it with endothelial denudation, which is sufficient for aneurysm occlusion in the low-hemodynamic lingual model. Third, the use of stents is not without risk to perforating vessels originating from under the stented segment.

Trends in mRNA expression changes of cells of the aneurysm wall in response to stenting supported a role for flow modification in the process of neointima formation and thrombus organization. We also found a trend to support the formation of endothelialized channels within the aneurysm thrombus, implying that recanalization affects endovascularly treated aneurysms. Unfortunately, the mRNA expression differences due to stenting did not reach statistical significance.

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