HISTOLOGICAL MORPHOLOGY RELATED TO ANGIOGENESIS AND HYPOXIA IN CONGENITAL VASCULAR MALFORMATIONS: EXPLORING AETIO-PATHOLOGY AND FUTURE TARGET THERAPY

A thesis to be submitted to the Imperial College of Medicine and Science for the degree of Doctor of Medicine by

Nonica Maftei

SUPERVISORS

Dr Ann Sandison

Department of Histopathology Charing Cross Hospital

Dr Ewa Paleolog

Kennedy Institute of Rheumatology and Division of Surgery

Faculty of Medicine, Imperial College

Prof. Nigel J Standfield

Department of Surgery and Cancer

Faculty of Medicine, Imperial College

ABSTRACT

Background

Musculoskeletal arteriovenous malformations (AVMs) result from abnormal angiogenesis and belong to congenital vascular malformations (CVMs) group. AVMs persist and have associated morbidity and even mortality. The aetio-pathogenesis of AVMs is unknown. This study analyses histological morphology and the immunohistological expression of specific angiogenesis and hypoxia markers in comparison with control tissues in patients with CVMs. CVM pO_2 was further assessed. This work was performed to gain insight into possible mechanisms involved in aetio-pathogenesis of CVMs.

Methods

Thirty three consecutive CVMs and 10 control specimens were analysed morphologically using routine techniques, histochemical (H&E, Masson Trichrome, Elasic Von Gieson, Toluidine blue) and immunohistochemical (CD68, CD3, CD20, Factor VIII, actin 1-α, ki67 and S100). Further immunohistochemical studies analysed VEGF A, VEGFR-1, Tie-2, αVβ3 and αVβ5 integrins, HIF-1α and HIF-2α, CAIX. The neutral endopeptidase (NEP) was studied by immunostaining for CD10 on 38 CVMs. Intraoperative blood samples from CVMs were analysed for pO_2.

Results

Macrophages ($p<0.0001$), mast cells ($p<0.0001$), vessel number ($p=0.0004$) and diameters ($p=0.0005$) were significantly higher in the CVMs compared to controls. The expression of all angiogenesis markers were significantly higher in CVMs comparing to controls. HIF-1α, HIF-2α and CD10 ($p=0.0002$) were over-expressed in CVMs. There was a positive correlation between HIF-2α expression and VEGF A ($p=0.0001$), VEGFR-1 ($p=0.0043$), Tie-2 ($p=0.03$), αVβ3 ($p=0.029$), αVβ5 (0.045), CD10 ($p=0.0002$) and inflammatory cells in CVMs. Comparisons of CVM and arterial blood pO_2 were not significant ($p=1$).

Discussion

Inflammation and imbalance of angiogenic and hypoxia markers are implicated in CVM pathogenesis. HIF-2α may play a more important role in CVM pathgogenesis than HIF-1α. CVM blood has an arterial pattern in spite of expressing HIF. CD10 may be a potential marker for CVMs in biopsies. Molecular patterns and potential signalling pathways involved in CVM pathogenesis are implicated in this work. This enhanced clinico-pathological correlation should improve therapeutic options and efficacy.

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

STATEMENT OF ORIGINALITY

All the experiments described in this study have been performed by me and are, to the best of my knowledge original research work, which has not been performed elsewhere.

PRESENTATIONS AND PUBLICATIONS

AWARDS

The Friends of Hammersmith Hospital, 'Arthur and Violet Pane and John Spencer Memorial Research Scholarship', Sept 2004 – 'Histological morphology related to angiogenesis and hypoxia in congenital vascular malformations: exploring aetio-pathology and future target therapy' (£40,000)

PRESENTATIONS

1. Maftei N, Sandison A, Farrar M, Forsyth A, Standfield NJ. Morphological and immunohistochemical analysis of arteriovenous malformations suggest an aetiological role of mast cells and mononuclear phagocytes. The Royal Society of Medicine meeting, Norman Tanner Prize, 2005, December 7, London, UK

2. Maftei N, Paleolog E, Sandison A, Farrar, M, Forsyth A, Standfield NJ. Markers of inflammation, hypoxia and angiogenesis in arteriovenous malformations: their role in disease pathogenesis (poster). The XIVth International Vascular Biology Meeting, 2006, June 6-10, Amsterdam, The Netherlands

3. Maftei N, Sandison A, Farrar M, Forsyth A, Standfield NJ. Arteriovenous malformations stromal morphology and clinical classification – a histopathological analysis. The 16th International Workshop on Vascular Anomalies, 2006, June 14-17, Milan, Italy

4. Maftei N, Paleolog E, Sandison A, Farrar M, Forsyth A, Standfield NJ. Markers of angiogenesis in arteriovenous malformations: role in disease pathogenesis? The 16th International Workshop on Vascular Anomalies, 2006, June 14-17, Milan, Italy

5. Maftei N, Sandison A, Paleolog E, Forsyth A, Farrar M, Tran M, Standfield NJ. Neutral endopeptidase (NEP) and hypoxia inducible factors (HIF) expression in arteriovenous malformations (AVMs) and their possible role in modulation of angiogenesis – an immunohistochemical study. The XXIIth World Congress of the International Union of Angiology, 2006, June 24-28, Lisbon, Portugal

6. Maftei N, Sandison A, Paleolog E, Forsyth A, Farrar M, Maxwell PH, Standfield NJ. Neutral endopeptidase (NEP) and hypoxia inducible factors (HIF) expression in arteriovenous malformations (AVMs): possible role in modulation of angiogenesis. The 9th Imperial College Symposium: 'Vascular endothelium: role in disease pathogenesis and as a therapeutic target', 2006, November 23, London, UK

7. N Maftei, NJ Standfield, A Forsyth, M Farrar, LC Brown, A Sandison. Morphological and immunohistochemical analysis of a series of 33 congenital vascular malformations – a single centre experience. The 23rd European Congress of Pathology, 2007, September, Istanbul, Turkey

PUBLICATIONS

1. Maftei N, Paleolog E, Sandison A, Farrar M, Forsyth A, Standfield NJ. Markers of inflammation, hypoxia and angiogenesis in arteriovenous malformations: their role in disease pathogenesis? Vascular Pharmacology, 2006:45(3): el-el67;141-198

2. Maftei N, Sandison A, Paleolog E, Forsyth A, Farrar M, Tran M, Standfield NJ. Neutral endopeptidase (NEP) and hypoxia inducible factors (HIF) expression in arteriovenous malformations (AVMs) and their possible role in modulation of angiogenesis-an immunohistochemical study. International Angiology, 2006; 25(2SupplI):3-204

3. Maftei N, Standfield NJ, Forsyth A, Farrar M, Brown LC, Sandison A. Morphological and immunohistochemical analysis of a series of 33congenital vascular malformations – a single centre experience. Virchows Archiv, 2007; 451(2):113-587

PAPERS IN PROGRESS

1. N Maftei, NJ Standfield, A Forsyth, M Farrar, LC Brown, A Sandison. Morphological and immunohistochemical analysis of a series of 33 congenital vascular malformations – a single centre experience.The Annals of Surgical Pathology – due to be submitted

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABC	Avidin Biotin Complex
Ang	Angiopoietin
APES	3-amino-propyl-triethoxisilane
αVβ3	Integrin beta 3
αVβ5	Integrin beta 5
a-v	Arterio-venous
AVM	Arteriovenous malformations
BCA	Bicinchoninic acid protein assay
CA IX	Carbonic anhydrase IX
CT	Computer tomography
CVM	Congenital vascular malformation
DAB	Diaminobenzidine
DTT	Dithiothreitol
EC	Endothelial cell
ECE	Endothelin converting enzyme
ECL	Chemiluminescent detection of immobilised proteins kit
ECM	Extracellular matrix
EGF	Epithelial Growth Factor
Eph	Ephrin
ER beta	Oestrogen receptor beta
ET	Endothelin
ETA	Endothelin receptor A
ETB	Endothelin receptor B
EVG	Elastic van Gieson
bFGF	Basic fibroblast Growth Factor
FiO_2	Fraction of inspired oxygen
Flt-1	Thyrosine kinase receptor (VEGFR1)
Flt-4	Thyrosine kinase receptor (VEGFR3)
GLUT-1	Erythrocyte glucose transporter protein-1
GM-CSF	Granulocyte macrophage colony stimulating factor
HAF	Human angiogenic factor
HCl	Hydrochloric acid
H&E	Haematoxylin and Eosin
HIER	Heat induced epitope retrieval

HIF	Hypoxia Inducible Factor
HF	High Flow
H_2O_2	Hydrogen peroxide
HRE	Hypoxia responsive elements
HRP	Horse Radish Peroxidase
HSI	Hue, saturation and intensity
HUVEC	Human umbilical vein endothelial cell
IA	Image analysis
IF	Immunofluorescence
IFN	Interferon
IGF	Insulin growth factor
IHC	Immunohistochemistry
IL	Interleukin
IMS	Industrial Methylated Spirits
ISSVA	International Society for the study of Vascular Anomalies
KDR	Thyrosine kinase receptor (VEGFR2)
LF	Low flow
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage colony stimulating factor
MECIF	Monocyte derived EC inhibiting factor
MDECI	Macrophage derived EC inhibitor
MMP	Matrix metalloproteinase
MRA	Magnetic resonanace arteriography
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MRV	Magnetic resonance venography
NICH	Non-involuting congenital haemangioma
NEP	Neutral endopeptidase
NRP	Neuropilin
PAF	Platelet activating factor
pCO_2	Partial carbon dioxide pressure
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule 1
PGE_2	Prostaglandin E_2

PHD	Prolyl hydroxylase
PIER	Proteolytic induced epitope retrieval
PKC	Protein kinase C
pO_2	Partial oxygen pressure
PlGF	Placental Growth Factor
PMSF	Phenylmethylsulphonyl fluoride
PR	Progesteron receptor
RBCs	Red blood cells
RCC	Renal clear cell carcinoma
RGB	Red, green, blue colour model
RICH	Rapidly involuting congenital haemangioma
RNA	Ribonucleic Acid
ROI	Region of Interest
SDS	Sodium Dodecyl sulphate polyacrylamide gel electrophoresis
SMC	Smooth Muscle Cells
STIR	Short T1 inversion recovery
TAM	Tumour associated macrophages
TBS	Tris Buffered Saline
TGF-β	Transforming Growth Factor beta
TIE-2	Angiopoietin receptor
TIMP	Tissue inhibitor of MMP
TLPS	Transarterial lung perfusion scintigraphy
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular Endothelial Growth Factor
VEGFR1	Vascular Endothelial Growth Factor receptor R1
VHL	Von Hippel-Lindau
VM	Venous malformation
VPF	Vascular permeability factor
V/Q	Ventilation perfusion ratio
WBBPS	Whole body blood pool scintigraphy

AKNOWLEDGEMENTS

This MD (Research) thesis is the result of a group effort to which many people contributed with a great deal of time and hard work. Without any doubt, this project would have remained just an interesting project had it not been for the combined efforts and talent of so many people, to whom I would like to offer my appreciation.

First and foremost, I would like to say a special thank you to Professor Nigel Standfield for selecting me for the clinical fellow post at Charing Cross Hospital over eight years ago, for arousing my intrest in congenital vascular malformations (CVMs) and enabling me to take on this research work as well as supervising and guiding me throughout the project. I am very grateful to Professor Standfield for all opportunities he offered me to facilitate my project, for sharing his knowledge about these extremely rare conditions, for his encouragement and stimulation with presentations and publications and for guiding my surgical career and putting me in the position to make good progress.

I reserve the biggest thank you to my dearest friend and supervisor of this thesis Dr Ann Sandison, consultant histopathologist at Charing Cross Hospital. Her trust and agreement to embark on this subject made this thesis happen and I wish to thank her for providing me invaluable help all the way through, starting with me from scratch, being honest with me, guiding me and supporting me with virtually every aspect of this thesis including related publications and presentations and durings the highs and lows of life.

Dr Ann Sandison also introduced me to Dr Ewa Paleolog from the Kennedy Institute of Rheumathology, whose excellent contributions to this project with her ideas and suggestions, gave shape to my thesis. I am particularly grateful to her for encouraging me from the beginning when I was not sure what I was embarking on, for adding insight into this work and supervising me. Most importantly, I cannot acknowledge highly enough her help facilitating my access to equipment in Kennedy Institute of Rheumathology, to the local research meetings of the research group in the department, from whom I have learnt a lot and I express my gratitude to them on this occasion. Thank you especially to Helen Larsen for taking me through the Western blot protocol and to Biju Aravind for teaching me image analysis.

A very big thank you to Amanda Forsyth, who apart from becoming one of my best friends, made this project possible by introducing me to Dr Ann Sandison, by creating the perfect environment for me to work on this thesis at Charing Cross Hospital after an initial failure at Hammersmith Hospital, by collecting and storing the CVM tissue with the greatest care as a tissue bank scientist, obtaining the archived tissue required, by giving me valuble advice and guidance and working with me out of hours and weekends. I cannot say thank you

too many times to Amanda and I felt very sorry when she left for Scotland towards the end of my project.

Special thanks go to Dr Paul Gunning, consultant anaesthetist at Charing Cross Hospital, who has a special interest in CVMs and provided the anaesthesia for the majority of CVM participants subjected to surgery in this study. He kindly helped to obtain crucial data for this project, especially for the blood gas analysis study as well as with interesting discussions we had in relation to this study. Thank you to Dr David Harris, consultant anaesthetist at Hammersmith Hospital for all his help with ethics and setting up the blood gas analysis study.

Many special thanks to Professor Patrick Maxwell and his team, Maxine Tran and Deepa Shukla for their collaborative immunohistochemical work and Western blot analysis, and especially for Professor Maxwell's advice regarding HIF work and oxygen pressure measurements. Thank you to Dyanne Rampling from Great Ormond Street Hospital, London for the collaborative immunohistochemical work for GLUT-1.

I am extremely grateful to all theatre staff at Charing Cross Hospital, and especially to sister Takka and sister Rhona for their administrative and organisational contributions so that my project ran smoothly and without faults. Thank you to all of them for their interest in this work.

Many thanks to Dr Tim Ryder from Histopathology Department at Charing Cross Hospital who facilitated for me the collection and storage of tissues required for this research. Thank you to Michelle Farrar and David Peston for their most valuable technical help and support with the immunohistochemical part of this study.

I would also like to thank everyone in the Department of Vascular Surgery, and especially to Professor Alun Davies whose advice regarding the importance of finalising this thesis was most valuable along with his remarks regarding this work. Thank you to all in Histopathology Department and Kennedy Institute at Charing Cross Hospital for proving a friendly and supportive environment.

My greatest thank you goes to my family and especially to my mother and closest friend (who is one and the same person) who encouraged me and has been a rock of support throughout performing these studies and writing up thesis; without their help this work would have not been possible. Even my little daughter Julia born before my thesis was finalised helped me by tolerating my absence during the time I was writing up the thesis.

Next I wish to thank to all my friends and especially to Ica, who has not only proved to be a close and dear friend in any circumstances I have been through all my life since we have known each other, but she also was very supportive helping me with the statistics of this project and fired my enthusiasm by sharing her own research experiences.

The contributions of Miss Louise C Brown and my friend Lia to the statistical interpretations of results are gratefully acknowledged.

I would like to address special thanks to all patients participating in this study for their willingness to help advancing the knowledge about CVMs, for offering to help in teaching and also, for giving me their time and speaking honestly about their experiences with the hope that their stories would help others in the future.

Thank you to the Medical Illustration Department at Charing Cross Hospital for their help with the photographs.

Finally, I would like to thank the Friends of Hammersmith Hospital, with special thanks to Mrs Frieda Dazeley (who sadly passed away during this work) for offering me the 'Arthur and Violet Pane and John Spencer Memorial Research Scholarship', which provided the financial support for all this research work. Without their trust in my abilities I would have not obtained the grant and this thesis would have not been possible.

I thank you everyone mentioned above from the bottom of my heart and to all those involved and not mentioned, for giving me this great opportunity and honour to perform this study in recognised institutions of excellence under the supervision of great clinicians and scientists.

CHAPTER 1

1. INTRODUCTION

1.1 DEFINITION OF CONGENITAL VASCULAR MALFORMATIONS

Congenital vascular malformations (CVMs), also known as vascular anomalies are common vascular lesions with a heterogeneous clinical, anatomical, histological and patho-physiological presentation. Vascular anomalies occur mainly in the skin, which is the largest organ of the body. They are known to represent proliferations of abnormal blood vessels or morphological defects of the circulatory system that arise during foetal development (Lee, 2004). CVMs are thought to be present at birth but they are not always obvious (Kohout et al, 1997, Vikkula et al, 1998, Lee et al, 2007). The majority of CVMs are sporadic and isolated and occur in healthy subjects. CVMs may also be extensive, affecting multiple anatomical regions, when they may be part of recognised familial syndromes (Fishman et al, 1993, Blei et al, 1998, Rosen et al, 2000, Connors et al, 2005). Their anatomy is variable depending on the type and size of vessel involved as well as on the degree of shunting resulting from the communication of the arterial with the venous side, when this exists. Further details will be given in the following chapters when each sub-category is discussed.

Vascular malformations have often been referred as haemangiomas by various specialists without realising that the two conditions were fundamentally different (Mulliken, 1988). CVMs can occur anywhere in the body. The focus of this research work is on all types of peripheral CVMs with a special emphasis on venous malformations and arteriovenous malformations (AVMs), which pose the most difficult clinical challenges and are the most dangerous vascular anomalies (Lee, 2004). Visceral vascular anomalies or berry aneurysms have been studied within other sub-specialty fields and their aetio-pathogenesis appears to differ from that of peripheral vascular lesions. CVMs are associated with morbidity, high recurrence, and occasional cardiac mortality. Only a small proportion of CVMs can be completely cured (Szilagyi et al, 1976, Enjolras et al, 2000). The lack of scientific knowledge regarding CVMs leads to sub-optimal management of millions of affected individuals. Little research has been conducted on these lesions and data in literature addresses largely haemangiomas, which in many instances are not the most challenging sub-category of CVMs.

Although several theories regarding the causes of CVMs have been advanced over the years, their aetiology remains unknown. Amongst the most favoured theories is the concept of abnormal development of the vascular tree, which can take place at different developmental stages. The aim of this work is to study pathways of abnormal angiogenesis in CVMs and to explore the relationship with hypoxia and morphology of the lesion in an attempt to advance knowledge of aetiology of CVMs to facilitate better future therapy.

This introductory chapter describes classification of CVMs, which is the most controversial issue over these lesions. Furthermore, the epidemiology and risk factors for CVMs, the diagnosis, symptoms and treatment of CVMs are discussed. The existing aetio-pathogenetic concepts for CVMs and the background for this study are detailed.

1.2 HISTORICAL ISSUES OVER CLASSIFICATION AND TERMINOLOGY OF CVMs

One of the most complex issues about CVMs is the clinical, aetiological and pathological classification.

The lack of standard, clear and simple classifications added to the confusion over the use of generic terms with respect to these lesions, in spite of extensive published work in the attempt to clarify this matter (Lee, 2004, Hand et al, 2002, Lee et al, 2007, Jackson et al, 1993, etc). This is the result of poor understanding of CVMs and profound lack of knowledge of the intrinsic mechanisms involved in the development and expansion of CVMs and often led and still leads to inappropriate diagnosis and management. (Mueller et al, 1999).

Besides the confusion between CVM and haemangioma, or between the terminologies allocated to either lesion, there are supplementary misunderstandings resulting from the use of name-based eponyms such as Klippel-Trenaunay syndrome, Parkes-Weber syndrome, Proteus syndrome, Maffucci syndrome, Kasabach-Merritt syndrome, Sturge-Weber syndrome, etc, often using various names for same condition. To add even more to the complexity to the terminology, these eponymous syndromes represent in most cases complex combined vascular malformations.

There has been a large spectrum of stages with many various classifications of vascular anomalies and especially of their most commonly encountered sub-category, haemangiomas (or vascular birthmarks or nevi) being elaborated over many decades. These classification systems reflect the understanding of the disease process at the time and were based on a mixture of clinical appearance of the lesions, extent, anatomical location, embryology, dynamics of growth, histopathological features, maturity of endothelium, natural history and other features. Most proposed classification systems proved extensive and too complicated to be widely accepted and used. From failure to initially differentiate between different vascular anomalies, the classification systems gradually developed into accepted classifications of CVMs and improved terminology. Some of the crucial turning points are overviewed below.

1.3 EVOLUTION OF CLASSIFICATION OF CVMs

Details of CVM classification issues over many years are given in the Appendix section.

1.4 CONTEMPORARY ACCEPTED CLASSIFICATIONS OF CVMs

Overwhelmed with many classification systems, it is undoubtedly clear that none of them provide all the information required by the specialist to safely formulate an appropriate diagnosis in every situation and proceed with the best management plan. From all this extensive information it is important to retain that the Hamburg classification (see Appendix) is now generally accepted as the standard classification for CVMs.

In addition, the current classification from the ISSVA (International Society for the study of Vascular Anomalies) (see Appendix) is benefiting from increasing general acceptance. Many clinicians still find the simplest, easiest to use and most useful classifications of vascular anomalies are those dividing them based on proliferative or static features into haemangiomas and vascular malformations and those separating them based on haemodynamics into high and low flow. The distinction of a vascular anomaly as a tumour or a malformation has proven clinically useful. However, the uncertainty in clinical application of CVMs classification systems continues as many authors do not adhere to any of these classifications, so the medical literature is abundant in reports of 'haemagiomas' that are actually vascular malformations or a combination of vascular anomalies.

Vascular malformations and especially venous malformations and AVMs represent the main focus of this study. Over the next chapters reference will be mostly made to these lesions as well as to haemangiomas, from which they could still be difficult to distinguish on occasions.

1.5 EPIDEMIOLOGY OF CVMs

It is common that patients with vascular anomalies have seen a number of physicians before they can have a realistic answer to their problem. Often physicians are puzzled when confronting the magnitude of problems patients with vascular anomalies pose, commonly because of lack of comprehensive experience in this domain. This is because a variety of specialists are usually involved in the study and diagnosis of these lesions and explains why multidisciplinary centres dedicated to the management of these lesions have been established. Although such centres exist all over the world, especially in the United States, there is currently no such centre in the UK as yet.

An inquiry was made to the Health Episode Statistics in the UK with respect to patients with vascular anomalies admitted to hospitals, proportion of patients undergoing surgery, interventional radiology procedures or conservative treatment, treatment of complications such as ulcers within hospitals or community, outpatient visits, health care expenditure and disability costs for patients with vascular anomalies but no clear records were obtained, most likely due to mislabelling of these lesions.

1.5.1 Incidence, prevalence and demographic data of CVMs

There is no current published data regarding the incidence and prevalence of vascular anomalies or any of their sub-categories in the UK. Vascular anomalies are very common skin disorders and they are estimated to occur in up to 5% of newborns (Hohenleutner et al, 2007). It has been reported that in the United States, 40,000 children are born with vascular anomalies every year (Astner, 2005). It is however important to differentiate the proliferating vascular lesions from permanent malformations.

It is estimated that haemangioma, the most common benign soft tissue tumour of childhood occurs in 1-3% of newborns and may be identified in 10-12% of Caucasian infants (Fishman et al, 1993, Takahashi et al, 1994, Esterly, 1995, Drolet et al, 1999, Lee et al, 2007). The prevalence of haemangiomas is estimated at 120 per 1,000 children. Approximately 30-50% are present at birth but become obvious during the first weeks of life, usually between the first and second weeks of life (Finn et al, 1983, Esterly et al, 1996). The median age for the appearance of most cutaneous haemangiomas is two weeks after birth (Mulliken et al, 2000). Female to male infants' ratio is 3.5:1 but the reason for this gender discrepancy has not been established. The sex ratio in preterm infants was 1:1.4 (Amir et al, 1986). Few comments have been made on the gender predominance and suggestions were made that complications and especially ulcerated lesions occur mostly in girls (Vittori at al, 1977, Bowers et al, 1960, Mulliken et al, 1988). The incidence of haemangiomas has been reported to increase to 20% in premature neonates (Amir et al, 1986), in infants whose mothers had chorionic villus sampling (10 times higher) (Powell, West et al, 1987, Burton, Schulz et al 1995) and in twins. They occur in all races but appear less frequently in Africans and Asians (0.8-1.4%). They are usually solitary but multiple lesions have been reported in 20% of affected individuals (Jackson et al, 1993, Blei et al, 1998). Although many authors reported haemangiomas to occur more frequently in prematures (Garzon et al, 2006, Finn et al, 1983, Moroz, 1983), other authors found equal frequency in premature and full-term infants (Holmdahl, 1955, Jacobs, 1957). Between 15% and 30% of infants have more than one haemangioma (Margilath et al, 1965, Moroz, 1983). The majority of haemangiomas occur in the head and neck region (60%), followed by the trunk (25%) and the extremities 15%) (Finn et al, 1983, Wananukul, 2002). In other series, no anatomical preference for site has been noted (Pratt, 1976, Margilth et al, 1965, Simpson, 1959). Superficial haemangiomas have been found to be the most common, representing 50-60% of all haemangiomas. The figure quoted in the literature for lesions with both a superficial and a deep component is 25-35% and 15% for deep haemangiomas. Visceral haemangiomas are known to be uncommon, but the accuracy of this presumption is questionable because asymptomatic lesions may go undetected (Mulliken et al, 2000). They do

not occur in adolescents or adults (Vikkula et al, 1998). Haemangiomas are not an inheritable condition but a family history has been reported in 10% of patients (Margilth, 1965, Moroz, 1983, Finn et al, 1983), possibly because they are common lesions. Although they are considered sporadic, involvement of autosomal dominant mechanisms have been reported (Blei et al, 1998). It has been estimated that 10% of patients have affected family members and is suspected that this figure may be even higher. In contrast, other authors (Cheung et al, 1997) compared the concordance of haemangioma in monozygotic and dizygotic twins and identified no evidence of a strong predisposing inherited component.

Vascular malformations are usually present at birth (Mulliken et al, 1982 and Fishman et al, 1993) but they are not always obvious.

They are less common than haemangiomas but with the variability within this classification it is hard to determine their incidence. The overall incidence of vascular malformations in the general population has been quoted at 1.2-1.5% (Tasnadi, 1993, Eifert et al, 2000).This is higher than other congenital malformations but lower than haemangiomas. Each of their categories could be localised or diffuse (Villavicencio, 2007).

Venous malformations (VMs) are the most common referrals to centres for vascular anomalies, representing up to 50% of patients (Vikkula et al, 1998). It has been reported that venous malformations account for two thirds of all vascular malformations (Simon et al, 2006). Predominantly venous congenital malformations are quoted at 70% (Mulliken, 2000, Eifert, 2000). Other authors indicated that VMs may exist in about 30-50% of the population although they may not be recognised due to their deep location. 15-20% of venous malformations are believed to be mixed lesions (Lee et al, 2002, Lee et al, 2005).

Arterial malformations are usually diagnosed in infancy or childhood (Fishman and Mulliken, 1993). Intra-cranial AVMs are more common than extra-cranial AVMs in the head and neck region, but they do not represent the area of interest of this study. Peripheral AVMs are most frequent in the head and neck, followed by limbs, trunk and viscera. They are usually noted at birth (40%) but often ignored due to their initial harmless appearance. There is no predominance by gender (Enjolras et al, 2000).

Capillary malformations (‘port-wine stain’) are typically noted at birth but they are less common, with reports of 0.3% infants identified (Jacobs et al, 1976).

Approximately 75% of lymphatic malformations occur in the neck and 25% in axilla, but less commonly other areas may be affected. They occur early in childhood and it is estimated that 65% are present at birth, 80% become obvious within the first year of life (Mueller et al, 1999) and 90% will be diagnosed by the age of two years (Brown et al, 1998). Occasionally they appear in adolescence or adulthood.

The combined vascular malformations seem to be sporadic and they are often located on extremities and often only one side is affected. Affected individuals often present bone and/or cartilage abnormalities or overgrowth (Vikkula et al, 1998).

1.5.2 Risk factors, triggers and exacerbating factors for CVMs

Historical perspective

In 1970s, the concepts around congenital malformations, including the vascular anomalies were influenced by superstition and speculation. Kaplan in a review of hamartomas (from Greek, 'hamartia', 'to sin'- defined as a developmental defect) published in 1977 gave an example of a child born with a giant birthmark and concluded that it was dangerous for a woman to look at monkeys during her pregnancy.

Various authors attributed congenital malformations either to teratology, genetic factors, chromosomal changes, maternal risk factors such as viral, metabolic, nutritional, or drugs (Warkany, 1971). These opinions remained hypotheses and stimulated the research in these domains.

As CVMs are not a single disease, but comprise many specific diagnoses, each of them may be distinct in risk factors. There are currently no known risk factors associated with development of either vascular malformations or haemangiomas.

There is a general consensus resulting from observational studies over many years that haemangiomas are associated with female gender, light skin types, head and neck location, premature infants, especially those weighing less than 1500g (Amir et al, 1986, Pratt, 1955, Powell et al, 1987, Chiller et al, 2002). Birthmarks and specifically haemangiomas were reported in a study as having a higher incidence in children with parents exposed to pesticides in the floriculture industry (Munoz et al, 1990).

Few prospective studies (Roganovic et al, 2002, Mertens et al, 1998, Johnson et al, 2006) reported an association between childhood malignancy and birthmarks. In one study (Johnson et al, 2006) children diagnosed with cancer had a three-fold higher risk of presenting a birthmark comparing to those without cancer. Due to the small number of cases with birthmarks within these studies, it was not possible to determine whether any specific vascular anomaly was more commonly associated with specific malignancies or to determine associations of birthmarks with other confounder factors such as gender. It was notable that no genetic syndromes known to be associated with childhood malignancies were recorded in subjects with vascular anomalies (Johnson et al, 2006).

Trauma, surgery, infection, physiological hormonal changes (puberty, pregnancy) or hormonal therapy, have been consistently reported to activate vascular malformations and especially arteriovenous malformations from 'dormancy', representing an asymptomatic status

to symptomatic lesions. The relationship between these potential precipitating factors and vascular malformations is unclear (Rosen et al, 2000) and they remain hypothetical, as vascular malformations may become active in the absence of any obvious precipitating factor.

1.6 DIAGNOSIS, SYMPTOMS, EXAMINATIONS AND NATURAL HISTORY OF CVMs

Only the lesions representing the object of this study, vascular malformations with emphasis on venous malformation and arteriovenous malformations will be presented from now on and no attempt will be made to elaborate on all cutaneous vascular malformations or vascular syndromes as they represent a vast subject. Lymphatic malformation represents a different entity that is not represented in this study. However, reference will be made to haemangiomas as they are the main lesions to differentiate from vascular malformations.

1.6.1 Symptoms, clinical signs, triggers and exacerbating factors

Haemangiomas

Haemangiomas vary considerably in their clinical features depending on their size, depth, stage of evolution or location. Most lesions are asymptomatic but pain may be a feature for some lesions.

Infantile heamangiomas are present at birth and may be represented by a variety of precursor lesions, which gradually develop during the first weeks of life (Hidano, 1972). They typically appear like a barely visible spot, or a small reddish macule that develops thread-like teleangiectases or appearance of ‘cherry’ vascular tufts that may grow quickly to become raised and lobulated lesions (Gampper et al, 2002). The precursor lesions may be a flat, erythematous patch that resembles a bruise or a cluster of small erythematous papules, with or without a pale halo. Those evolving into a deep haemangioma may be blue or purple in colour. Rarely, a superficial ulceration may be the first sign of a haemangioma.

The congenital haemangioma represent a tumour that is fully developed at birth and does not exhibit the natural course of infantile haemangioma. These lesions are bright red, elevated, lobulated nodules or plaques that feel rubbery and fade on applying pressure.

Haemangiomas have sizes varying from few millimetres to greater than 20 centimetres in diameter but most haemangiomas reach a maximum size of 0.5-5cm. Few venous tributaries are found at the periphery of the lesion, commonly. Most haemangiomas remain well circumscribed but rarely, they may be segmental. Deep haemangiomas, located in the deep dermis and subcutaneous tissue may have normal overlying skin or can appear purple-blue, poorly circumscribed and compressible. The overlying skin may present dilated veins or small teleangiectasias as a landmark. Their consistency is firm, rubbery, tense, and expands with increased intravascular pressure such as crying, activity or dependency of the affected part

(Esterly, 1996). These lesions may be warm on palpation. Tenderness on palpation is a variable and uncommon feature.

Many haemangiomas consist of both a superficial and a deep component. Ulceration occurs in 5-10% of haemangiomas, especially the mixed superficial and deep lesions. Secondary infection is common but cellulitis, abscess formation and bacteremia are rarely reported. Intermittent bleeding is common but major haemorrhage is exceptional.

Those lesions resulting in complications such as infection or ulceration may exhibit additional symptoms according to the area involved and extent of the lesion. Visual obstruction, amblyopia and strabismus can occur when a haemangioma involves the eyelids or periorbital tissues. Airway obstruction is a rare complication of haemangiomas affecting upper lips, cervical parapharyngeal or palatal regions.

Diffuse neonatal haemangiomatosis is a potentially life-threatening condition characterized by numerous cutaneous haemangiomas which coexist with visceral haemangiomas.

Cosmetic impairment or disfigurement is an additional complaint of the affected individuals, again depending on the lesion location and extent.

The appearance of haemangiomas may deviate from their classical presentation during various phases in their evolution and their alternative clinical features are presented in the natural history section.

Vascular malformations

Venous anomalies are present at birth but they are not always evident. They are slow-flow (or low flow, or LF – see appendix to introduction) type of vascular malformation and appear like compressible subcutaneous masses with a bluish discoloration of the overlying skin. Most venous malformations are asymptomatic but they may become painful in association with their enlargement and pressure on surrounding structures. Venous malformations of the head and neck are usually unilateral and cause facial asymmetry and progressive distortion of the features. Intra-orbital venous malformations can result in exophthalmia. Pharyngeal, laryngeal or cervical-oropharyngeal venous malformations can cause airway obstruction and sleep apnea. Venous malformations affecting an extremity can involve the skin only or can extend into the muscles, joints and bone and may lead to leg-length discrepancy. Intraosseous venous malformations can cause pathological fractures.

Hyperhydrosis over the lesion is common. Stagnation of blood within venous malformations causes localised coagulopathy and patients may experience recurrent episodes of thrombophlebitis with associated pain.

The majority of venous malformations are isolated but they can occur as part of known clinical syndromes such as Maffucci syndrome or blue bleb nevus syndrome.
VMs present like blue or purple nodules affecting the skin, with prominent surrounding veins and calcified phleboliths (Requena et al, 1997).

Arteriovenous malformations are usually noted at birth but given little importance due to their innocent appearance and may be mis-diagnosed for capillary malformations or haemangiomas. They vary in clinical presentation from asymptomatic spots, a macular rash to erythematous plaques or variable size pulsating nodules or masses with an audible bruit and throbbing pain. Physical examination is usually adequate to separate arteriovenous malformations from venous malformations as the presence of a thrill, bruit, or pulsation indicates the presence of an arterial component within the vascular anomaly. Large AVMs causing an increased cardiac output may demonstrate reflex bradycardia after compressing the lesion (Branham's sign) which is another clinical sign that is not associated with a purely venous malformation (Enzinger, 1988). The increased flow and pressure in the draining veins may result in varicosities. Shunting of blood diminishes nutritive flow and this may result in skin necrosis, ulceration or bleeding. Of all CVMs, they may be associated with bone alteration and may lead to hyperdynamic circulation and congestive heart failure. Lower extremities affected with AVMs may be complicated by pelvic tilting and scoliosis due to leg length discrepancy. Haemangiomas rarely affect bone, whereas vascular malformations are reported to affect bone in 35% of cases (Mulliken et al, 1986).

1.6.2 Natural history

Limited knowledge regarding the natural history and biological behaviour of vascular anomalies is available. The best description is attributed to Mulliken and Glowacki in their biological classification of vascular anaomalies. Rapid growth during the first four weeks is the historical hallmark of haemangiomas. Most haemangiomas undergo a proliferative and an involutional phase with an intermediate phase of inactivity. These are followed by the involuted phase. The length of each phase varies, but generally the proliferative phase lasts for weeks to months. As the lesion grows, it becomes elevated and lobulated with tumoral appearance. Superficial haemangiomas rarely continue to grow beyond the age of 10 months but deep haemangiomas may continu to enlage slowly for few more months. The proliferative phase may be associated with superficial ulceration in about 10% of cases, with larger haemangiomas ulcerating more frequently (Esterly, 1996). Ulceration leads to scarring and there is no evidence that this would result in more rapid regression. After the proliferative phase, haemangiomas may remain quiescent for a period or may begin to involute.

The involuting phase usually begins at the end of the first year or during the second year of life, but variations are reported. Usually the lesion loses its red colour but whilst some lesions may become darker, others lighten and blanch. The lesion becomes softer and flatter, decreasing in size. The haemangioma shrinks from the centre of the lesion but this is less obvious with deep lesions. Some lesions, such as lip lesions are most likely to persist or involute only partially. The time of onset and rapidity of involution of a lesion varies considerably and cannot be predicted. Most published series report 50-65% involution by 5 years of age, 75% by 7 years of age, and up to 90% by 9 years of age (Margilath, 1965, Esterly, 1996). On occasions the involution process takes longer. Nearly normal skin is restored in approximately 50% of children. (Connors et al, 2005). 10-20% of patients reach the involuted phase with residual skin changes such as redundant skin, scars, hypo-pigmentation, dilated veins or teleangiectasia. This is more common for lip, nose, eyelid or ear lesions.

Although haemangiomas cause significant distress for parents and possibly for children, most of them are benign conditions and do not require invasive diagnostic tests or treatment.

The natural history of vascular malformations is distinct from haemangiomas. Most vascular malformations have a slow, steady enlargement. They do not possess the potential for independent growth, but progressive vascular dilatation usually occurs (Meyer et al, 1991, Mulliken et al, 1982, Rao et al, 1992). They get progressively worse with time or persist lifelong, unchanged and sometimes undetected, but never involute spontaneously. They can manifest at any time during life. Venous malformations expand slowly and often enlarge during puberty. They can manifest as pain as a result of vessel dilatation, bleeding, haematoma, haemarthrosis, pathologic fracture, soft tissue over-growth, thrombosis, thrombophlebitis, low-grade disseminated intravascular coagulation. Usually they are non-lethal in comparison with the arteriovenous malformations, which can be limb or life-threatening (Lee et al, 2007). The potential of these lesions to recur represents a significant problem for those diffusely infiltrating malformations categorised as ‘extratruncular’ and this matter should be given serious consideration, balancing risks and benefits, when treating these lesions.

The clinical history of AVMs varies considerable. They may remain dormant for years before becoming symptomatic. Many lesions are silent or have minimal clinical or cosmetic symptoms but others have severe associated conditions such as varicosities, superficial phlebitis, venous stasis ulcers due to venous hypertension, pain, pressure from mass effect, haemorrhage and ischaemic complications. Pelvic AVMs can lead to severe pain, pelvic congestion, sexual dysfunction, high-output cardiac failure and haemorrhage. Extremity lesions in children may cause leg length discrepancy, disfiguring bone or soft tissues overgrowth if left

un-treated (Zhou et al, 2005, Mulliken et al, 1982, Abdool-Carrim et al, 1990). The natural history of progressive lesions is best given by Schobinger staging (appendix to introduction).

Expansion of the arterio-venous channels within AVMs occurs as a result of increased blood flow through collateralisation and recruitment of adjacent vessels and not due to proliferation of cells (Takahashi et al, 1994). Ischaemia may lead to the proliferation of the lesion as it was noted following ligation of the proximal feeding vessels (Coleman, 1973).

Arteriovenous malformations may have aggressive growth patterns resulting in functional or cosmetic deformity or in cardiovascular compromise. Unfortunately, venous malformations and arteriovenous malformations are mostly incurable (Lee, 2002). Szilagyi et al in 1976 also indicated that it would be inaccurate to claim a 'cure' for most vascular malformations. It is estimated that eradication can be performed safely in about 15% of lesions (Riles et al, 2006). There is virtually no data in literature quantifying the natural history of peripheral vascular malformations but there are figures reported for arteriovenous malformations of the brain. This indicates that the natural history of an un-ruptured AVM has a 1-2% bleeding rate and once ruptured a 2-4% annual risk of re-bleeding as well as an associated mortality risk of up to 1% per year (Mattle et al, 2000).

Rapidly involuting congenital haemangioma (RICH) and non-involuting congenital haemangioma (NICH)

Two rare distinctive haemangiomas have been defined separately from the infantile haemangioma. They have similar clinical appearance and some histological similarities but different natural history. Boon et al first described in 1996 a separate entity of haemangioma that can proliferate in utero and manifest as fully developed tumours at birth. These haemangiomas are further described in literature (Berenguer et al, 2003) as rapidly involuting congenital haemangioma (RICH). These are usually large vascular tumours fully developed at birth and unlike the common infantile haemangiomas they fail to further grow postnatal. They instead regress, usually by 1 year.

Another type of congenital haemangioma was described by Enjolras et al in 2001 and named non-involuting congenital haemangioma (NICH). This lesion occurs in utero, has no sex prevalence and it is known to grow with the patient, does not involute, persists or expands slightly. It was noted that biopsy or partial excision of NICH did not lead to recurrence or expansion, as reported to be seen after the incomplete excision of arterio-venous malformations.

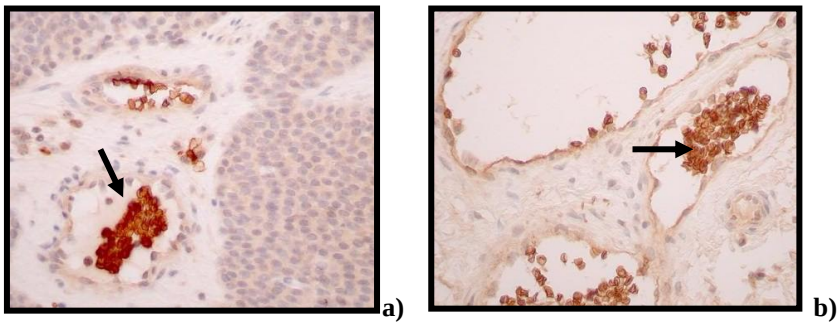
There are very few reports in the literature of spontaneous disappearance of congenital arteriovenous malformations affecting the renal tract (Kubota et al, 2003). Spontaneous disappearance of cerebral and dural arteriovenous malformations has been reported in about 30 cases. The natural history of these lesions is unknown but few hypotheses trying to explain their

regression have been put forward. All had as end-point mechanism thrombosis in the vessels comprising the lesion. This may be caused by compression due to haemorrhage and haematoma formation, vasospasm and oedema causing reduction of blood flow or increased blood turbulence with alteration in flow dynamics. It has also been suggested that the vessels within a vascular malformation may become thrombosed more easily than normal vessels and the flow velocity and direction are not constant. They may easily change according to the pressure balance in the nidus, which in turn may be influenced by various physical or chemical factors.

1.6.3 GLUT-1 – immunohistochemical marker for haemangiomas

Positive endothelial cell (EC) staining with GLUT-1, the erythrocyte-type glucose transporter protein, has been noted during all growth phases of congenital haemangiomas (North et al, 2000). GLUT-1 is a high affinity glucose transporter protein and may facilitate cell growth. Its high expression in proliferating haemangiomas may explain this role. The persistence of high GLUT-1 expression in late-involuting haemangiomas suggests that this is not just an adaptation to the increased demand of the lesion during its proliferative phase. GLUT-1 appears to be a reliable and highly specific immunohistochemical marker to juvenile haemangiomas and previous reports indicate that it does not occur in vascular malformations. GLUT-1 expression was shown to be independent of the mitotic activity of the lesion (North et al, 2000). It is highly expressed by ECs during early foetal development but this feature is lost as the cells differentiate. As the foetus develops, GLUT-1 expression disappears from most micro-vessels but persists in most areas of the brain and in other locations with blood-tissue barrier function.

This marker is a useful diagnostic tool in differentiating late-phase involuting haemangiomas and vascular malformations of similar morphology. Why the ECs of infantile haemangiomas maintain GLUT-1 expression and what is the origin of the ECs comprising these lesions remains unknown. RICH and NICH demonstrate lack of immunoreactivity to GLUT-1, suggesting that these lesions are different from the haemagioma of infancy. The following pictures are examples of GLUT-1 immunopositivity.



a) **b)**

Figure 1. 1. Positive GLUT-1 expression in RBCs in a) glomus tumour; b) haemangioma (stained brown)

1.6.4. Investigations

Details of tests required to confirm diagnosis and plan treatment of CVMs are given in the appendix section.

1.7 TREATMENT OF CVMs

The variety of treatment modalities available for CVMs at present are detailed in the Appendix section.

1.8 THEORIES AROUND AETIO-PATHOGENESIS OF CVMs

1.8.1. Blood vessel development - vasculogenesis and angiogenesis

The vascular system is built by two processes, vasculogenesis and angiogenesis. During vasculogenesis in humans, a primitive vascular plexus is established from endothelial precursors (Breugem, 2001) and the primary circulation is established by the end of the third week of development. The angiogenesis represents the formation of new vessels from these pre-existing vessels by migration and proliferation of ECs (Brugem, 2001, Isner et al, 1999). This is a multistep process involving both the endothelium and the extracellular matrix.

The development of a functioning vascular network requires a perfect degree of coordination between different cell types undergoing complex changes, and is essentially dependent upon signals exchanged between these cell types. Vasculogenesis is largely restricted to early embryogenesis, whereas angiogenesis occurs during embryogenesis and in the postnatal state. The development of new blood vessels is an uncommon physiological event in adults. In the normal adult the turnover of ECs is very low, in humans in the order of years (Denekamp, 1984). The cardiovascular system is the first organ system that forms during embryonic development. The embryonic vascular system develops during the third week of foetal life from mesodermal cells of the 'blood islands' forming the primary capillary network. ECs originate from an embryonic mesoderm during the initial phase of the vascular development. Although initially located in the region of the yolk sac, these cells eventually populate the mesenchyme throughout the foetus; the peripheral cells of the islands give rise to endothelial tubes, and the central cells give rise to the haematopoietic elements. The first step in the formation of blood vessels is the emergence of the inner lining cells, the ECs. It has recently become evident that the primary capillary plexus is made of at least two distinct types of ECs: arterial and venous ECs. An arterial EC type is characterised by its specific expression of Ephrin-B2 and a venous type expresses specifically EphB4 (Gerety et al, 1999, Wang et al, 1998). These cells then associate into tubular structures and other cells types (smooth muscle cells and pericytes), organise themselves around them to make the vessel wall. Structural specialisations such as tight junctions or regionalised adhesion molecules are acquired by differentiating EC. Further changes in size and mural structure lead to the formation of arteries, capillaries, veins and

lymphatics, each with their own characteristics. Vasculogenesis and angiogenesis are the result of a series of genetic events that depend on the orderly expression of at least two sets of tyrosine-kinase receptors, one set largely influencing endothelial differentiation and proliferation and the other governing vascular wall formation and vascular bed morphogenesis (Cines etal, 1998). Initial events in vasculogenesis are dependent on fibroblast growth factor (FGF) and vascular endothelial growth factors A and B (VEGF A and B). Under their influence, ECs differentiate from mesoderm of the blood islands, proliferate, and migrate. The pattern of endothelial differentiation is further determined by VEGF C, the receptor to which (VEGFR-3) is expressed on ECs destined to become veins and lymphatics (Cines et al, 1998). Continued growth of ECs and modelling of vascular walls is dependent on the expression of an additional set of thyrosine-kinase receptors, Tie-1 and Tie-2. The biding of Tie-2 by its ligand, angiopoietin 1, plays a critical role in the induction of components of the vessel wall, as mutations in the genes for either result in defective vessels with poorly formed muscle walls and micro-aneurysms. In contrast to formation of blood vessels in the embryo, new vessel formation in the postnatal state occurs largely as a result of micro-vessel formation from differentiated ECs. Angiogenesis is governed by a complex interaction of pro-angiogenic and anti- angiogenic factors which are under tight control so that there is little, if any, increase in vessels number in the normal state (Cines et al, 1998, Johnson, 1976). However, in response to stimuli such as tissue injury or hypoxia, this balance is perturbed and angiogenesis occurs. These substances therefore serve as ‘signals’ that influence growth, migration, and the permeability of ECs. During angiogenesis ECs acquire protease, which permits them to digest basal lamina and move in the direction of the signal. Foremost among the signalling substances are VEGF and FGF. Once separated from the parent vessel, ECs acquire small vacuoles or primitive lumens, which fuse with lumens of adjacent endothelium to form capillary channels. Although the mechanism by which these signals are processed by the ECs is still the subject of intense inquiry, it appears that endothelial junctions (adherens junctions) play a pivotal role (Dejana, 1997). This specialised apparatus, consisting of a trans-membrane protein VE (vascular endothelial) cadherin bound in its intracellular domain to VE cadherins of adjacent cells, localises or traps extra-cellular signalling molecules. The extra-cellular signals, in turn, alter the cadherin-catenin complex, thereby modifying the confluence and permeability of the EC. The cadherin-catenin complex is also involved in intracellular signalling from the cytoplasm to the nucleus such as gene expression is affected. The primary capillary network is remodelled by regression, sprouting, splitting or fusion of pre-existing vessels and ECs differentiate into arterial and venous types (Yancopoulos et al, 1998). The stabilisation of the vascular network occurs when peri-ECs and pericytes are recruited to the vessel wall. They inhibit ECs proliferation and

migration by stimulating production of extracellular matrix and basement membrane formation (Ramsauer et al, 2002). Stabilisation of a blood vessel is a stage when remodelling has ceased, new branches are not developing and luminal size is constant (Folkman et al, 1996).

To conclude, angiogenesis can be defined as a multi-step complex process, which involves EC activation, increased blood vessel permeability, and local re-arrangement of the basal membrane and extra-cellular matrix. Faults in angiogenesis mechanisms appear to govern the pathology of vascular anomalies but the intimal deficiencies in the signalling pathways are still to be identified. Limited research data investigating angiogenesis pathways in CVMs is available and this is largely restricted to haemangiomas.

1.8.2 The role of angiogenesis in development of CVMs

Laboratory-based research in vascular anomalies lagged behind clinical studies, but the aetiology of CVMs is likely to represent a multifactorial process. With increased understanding of the mechanisms of angiogenesis, vascular anomalies are assumed to be the result of faulty angiogenesis. The hypothesis that tumour growth is angiogenesis-dependent was first proposed in 1971 by Folkman and subsequently proven by a variety of experiments including genetic methods. Little research has addressed this field. Woolard (1922) indicated that CVMs may represent focal persistence of primitive vascular elements but this has never been proven scientifically. Many theories have been advanced to explain pathogenesis of haemangiomas and the most accepted appear to involve increased angiogenesis (Johnson, 1976). These advances are not equalled by similar progress in research work related to vascular malformations (except cerebral AVMs and those associated with hereditary haemorrhagic teleangiectasia, which do not represent the focus of this work). Bastide et al (1990) concluded that the fundamental concepts of embryology and organogenesis allow a better understanding of the pathological characteristics of vascular malformations. He indicated that any cessation or disturbance in the development of the circulatory system will be the source of a vascular anomaly. The stage in which the changes occur will influence the type of the vascular lesion.

Chiller et al in 2003 pointed out that vascular remodelling, which is part of angiogenesis focused on the interactions between pericytes and ECs, was demonstrated to be defective in some experimental models and in some familial cases of vascular malformations.

Haemangiomas

The pathogenesis of haemangioma is still not understood. There is evidence suggesting that pericytes and ECs play a key role (Chang et al, 1999). In this study the majority of mRNA for bFGF and VEGF, which were up-regulated, was localised in pericytes and ECs, the most abundant cell types in the haemangiomas studied.

Another theory is that incomplete maturation of the vessels in the embryo may result in vascular endothelial rests that proliferate after birth more rapidly than normal blood vessels. Although this is just a speculation, it could explain the increased incidence of haemangiomas in premature infants as the likelihood of immature autonomous rests would be greater in these infants than in full-term infants (Finn et al, 1983, Amir et al, 1986).

Other studies have also demonstrated that haemangioma cells cultured in vitro behave more like foetal cells, exhibiting a spindle-shape rather than epitheloid morphology, lower levels of expression of platelet-EC adhesion molecule-1 and von Willebrand factor, and production of interstitial type I collagen rather than epithelium-specific type IV collagen (Dosanjh et al, 2000).

The mechanisms that control the involution of haemangiomas are also poorly understood. Frischer et al, 2004, demonstrated that increased placental growth factor expression in haemangiomas is predictive of involution.

Another hypothesis is that proliferation of ECs is a response to factors secreted by surrounding cells. Smoller and Apfelberg speculated that haemangiomas proliferated from a primitive, uncontrolled stem cell (Smoller et al, 2006).

Another observation is that vessel growth in haemangiomas is associated with infiltration of mast cells, which have been found to stimulate migration of ECs, an important component of angiogenesis. Heparin released from mast cells has been shown to potentate this process (Folkman, 1986). Tan et al, 2004, suggested that mast cells may have a function in both the EC proliferation and the involution of haemangiomas. They also speculated that certain effectors elicited by mast cells may participate in the life cycle of haemangiomas.

The presence of abnormal arteries in some patients with extensive haemangiomas has been attributed to developmental field defects that occur at approximately 8 to 10 weeks of gestational age (Kishnani et al, 1995, Opitz et al, 1992, Pascual-Castroviejo et al, 1996). Haemangiomas have been reported to be the result of an alteration in angiogenesis that allow the uncontrolled proliferation of vascular elements.

A few authors have investigated the complex interplay of pro- and anti-angiogenic factors involved in normal and pathologic processes. (Folkman et al 1987, Klagsbrun et al 1991, Folkman et al 1997). The various stages of haemangiomas can be distinguished by different cellular markers (Takahashi, 1994) but the primary causative defect in haemangiogenesis remains unknown. Nguyen et al (2004) suggest that haemangioma may be a result of vasculogenesis (the formation of primitive blood vessels from in-situ mesenchymal precursor cells) rather than angiogenesis (the sprouting of capillaries from pre-existing vessels). Dadras et al (2004) established strong expression of lymphatic endothelial hyaluronan receptor-1 and

CD34 in haemangioma tissue during the proliferative phase and postulated a maturational arrest in the ECs of proliferating infantile haemangiomas.

Kleinman et al (2003) demonstrated increased levels of circulating EC precursor cells in children with haemangiomas. Yu et al (2004) demonstrated the presence of EC precursor cells in proliferating haemangiomas and indicated that these cells contribute to the early growth of haemangioma.

Vascular malformations

Arteriovenous malformations have been reported to be the result of errors of vascular development between the 4^{th} and 6^{th} weeks of gestation, clearly distinct from haemangiomas (Takahashi, 1994). Most vascular malformations are sporadic (non-familial) but some may be inherited in an autosomal dominant pattern (e.g. hereditary haemorrhagic teleangiectasia, lymphatic anomalies, etc). Molecular studies indicate that vascular anomalies are caused by dysfunctional signalling processes that regulate proliferation and apoptosis, differentiation, maturation, and adhesion of vascular cells (Vikkula et al, 1998). Chiller et al (2001) indicated they are most likely caused by localised dysfunction in pathways regulating vascular embryogenesis, especially the molecular events responsible for vascular remodelling. Chiller et al (2003) pointed out that vascular remodelling, which is the part of angiogenesis that centres on the interactions between pericytes and ECs, has been shown to be defective in experimental models and in familial cases of vascular malformations.

Vikkula et al (1998) emphasised that TGF-β signalling is important for the proper maintenance of capillaries. When this signalling pathway is altered, the capillary bed is dilated, allowing direct arteriovenous flow and the formation of AVM.

The lack of a suitable animal model for the study of haemangiomas and vascular malformations limits clinically relevant research but in our present study we have undertaken experiments on human tissue obtained with ethical approval from individuals suffering with these conditions.

1.8.3 Angiosomes theory

Taylor et al (1987) introduced important anatomical concepts aiming to better understand vascular anatomy but also, to provide the basis for explaining certain pathological processes. They introduced the term of ‘angiosomes’ and explained that the body is a three-dimensional jigsaw made up of composite blocks of tissue (’angiosomes’) supplied by named source arteries. This concept is important in understanding the management of CVMs and more details are given in the Appendix section.

1.8.4 The genetic theories

Wang (2005) stated that genetic factors play a critical role in the pathogenesis of vascular anomalies. Further details are provided in the Appendix section.

1.9 HISTOLOGICAL MORPHOLOGY OF CONGENITAL VASCULAR MALFORMATIONS

1.9.1 Haemangiomas

There are few recognised histologic features specific to haemangiomas or vascular malformations and thus, these two entities are often difficult to distinguish using routine histology. Some lesions have features applicable to both categories, which imply that present classifications may not reflect the in vivo situation and they are clinically less than satisfactory.

Proliferative haemangiomas represent complex networks of ECs (Mulliken et al, 1982). Chang et al (1999) found that haemangiomas were composed of a heterogenous population of cells with a predominance of vascular elements, ECs and pericytes. Routine histopathology of these lesions varies according to the stage of the haemangioma. In early stages they are characterised by non-encapsulated masses and dense cords of mitotically active, plump ECs in close association with pericytes. The basement membranes are well-developed around primitive vessels. The histological basis of the rapid expansion of haemangiomas is the uncontrolled EC proliferation. As the haemangioma proliferates, the vascular lumen enlarges. As involution progresses, the ECs continue to mature and gain a flatter appearance. The vascular lumen continues to enlarge until few, mature ectatic vessels remain. The proliferating endothelial mass is replaced with fibro-fatty tissue. Various degrees of epidermal atrophy, scar tissue and loss of elastic tissue can be seen in late involuting lesions (Antaya, 2006). In a pathomorphological analysis of 310 CVMs, Leo (1990) found that haemangiomas of small vessels of the arterial and venous type are most of the time accompanied by arterio-venous (a-v) microshunts but haemangiomas involving only capillaries do not contain a-v microshunts. Haemangiomas in connection with vascular malformations are malformations and not tumours. They have no proliferative tendency and usually do not undergo malignant transformation (Leu, 1990).

One of the most interesting findings on histological examination of these lesions was the presence of stromal mast cells and macrophages in some of these lesions. Studies quantitating mast cells in haemangiomas are scanty and not uniform. Increased numbers of mast cells found in haemangiomas have not been clearly linked to their pathogenesis (Glowaki et al, 1982). Details of presence and possible role of these cells in haemangiomas are given in the next sub-chapter.

1.9.2 Vascular malformations

The exact histological morphology of vascular malformations and any derived clinical relevance is unclear. Time, intensity and duration of the aetiologic factors determine the size of the vessels involved and the severity of the alteration (Leu, 1990). AVMs are not recognised as neoplasms but they are locally aggressive. Limited data available in relation to the histopathology of AVMs suggest they are extremely complex containing multiple lacunae septa, diverticula, labyrinths and polypoid structures as well as thickening of the intima. Large vessel malformations are often accompanied by a-v macroshunts. They connect well-differentiated arteries and veins or dysplastic arteries and veins, which have thick walls with enlarged muscular medial layers. The elastic membranes may be absent or rudimentary. Dysplastic veins appear 'arterialised' with a hypertrophic media musculature and hypertrophy of elastic membranes and fibres. In later stage these veins undergo degeneration with atrophy of the wall, loss of elastic fibres, and replacement of the smooth muscles by collagen, ectasia or aneurysm formation. Arteries or veins of less typical structure are directly connected with no intermittent capillary vessels (Leo, 1990). The confluence point of the abnormal vessels in AVMs is clinically/radiologically called a 'nidus'. Details of this are given in the next chapter. Formation of thrombus was frequently observed. The stroma between blood vessels may show fibrosis and calcification. Proliferation and leakage of capillaries as a consequence of raised intra-capillary pressure may be a feature (Pasyk, 1987).

Previous authors indicated that the embryonic vascular system initially consists of interlacing blood spaces in the primitive mesenchyme. Further differentiation involves the development of primitive capillary network, followed by a 'retiform stage', when the primitive capillaries coalesce into large plexiform structures. Blood begins to flow through the retiform plexus from an 'arterial' to a 'venous' side. In the maturation phase, the primitive elements are replaced by mature vascular stems. AVMs are presumed to represent focal persistence of primitive vascular elements. Depending on the stage at which this failure occurs, the abnormal communication may range from just above capillary level to large vessel communications. Leu (1990) stated that the morphology of the vascular malformations appears simple amd monomorphous compared with the clinical issues on classification. He indicated an important difference was between malformations with and without a-v shunts because they were responsible for the haemodynamic consequences of the lesion.

AVMs are still difficult to distinguish histologically from haemangiomas. The evidence for mast cells presence/absence in vascular lesions other than haemangiomas is absent or rather obscure and known data is discussed in the next sub-chapter.

1.9.3 ECs in CVMs

Dysfunction of the EC is central to a number of pathologies, including the two major killers of the Western society (atherosclerosis and cancer) (Bicknell, 1996). Research work over recent years established that there is extensive endothelial heterogeneity. Details of EC and how are these related to CVM pathogenesis can be found in the Appendix section.

1.10 ANGIOGENESIS IN CVMs

Angiogenesis markers

Folkman (1995) suggested that uncontrolled angiogenesis is the cause of haemangioma proliferation and that the increased endothelial proliferation may be the result of abnormal levels of angiogenic stimulators and inhibitors.

Takahashi et al, in 1994 reported elevated levels of bFGF and VEGF in proliferative haemangiomas using immunohistochemistry. Chang et al (1999) also found elevated levels of VEGF and bFGF in proliferative specimens when compared to involuted or involuting specimens and concluded that haemangioma proliferation is the result of aberrant gene up-regulation of these angiogenic growth factors, at least in part.

1.10.1 Inflammatory cells in triggering angiogenesis

The most studied and important inflammatory cells with roles in angiogenesis are mast cells and macrophages.

Mast cells

Mast cells have a variety of activities and their properties make them active in many biological responses. They were originally identified by Ehrlich who named them (Mast = well fed, or fattening in German) because of the specific granules. Their functions include phagocytosis and antigen processing, cytokine production, release of pre-formed (histamine, proteoglycans, proteases) or newly formed (leukotrienes, prostaglandins) mediators (Frangogiannis et al, 1998). Mast cells express an array of adhesion molecules and other surface receptors that allow them to interact with other cells or stimuli (Metcalfe et al, 1997). These characteristics allow mast cells to play an important role in angiogenesis, as well as other processes and conditions such as tissue remodelling, wound healing, chronic inflammatory conditions and allergies (Metcalfe et al, 1997, Gordon et al, 1990, Meininger et al, 1992, Schwartz et al, 1994).

Mast cells develop from haematopoietic stem cells. They become mature in peripheral tissues where they reside and at this stage they are not found within the blood vessels (Wedemeyer et al, 2000). Mast cells are abundant in the skin, gastrointestinal and respiratory tracts. They are often found in close vicinity of blood vessels, nerve fibres, epithelial surfaces or

fibroblasts, all these structures representing possible targets for mast cell-derived mediators (Stead et al, 1987, Blair et al, 1997).

Mast cells are known to produce histamine (White et al, 1987). Histamine exerts its biological activities through histamine-1, -2 and -3 receptors. Heparin and chondroitin sulphates are proteoglycans produced by mast cells. Heparin, which is present in large quantities in mast cells secretory granules, is thought to participate in angiogenesis (Taylor et al, 1982, Folkman eta la, 1983, Fraser et al, 1983) by stimulating the migration and proliferation of ECs. Carboxypeptidase A, chymase and tryptase are neutral proteases found in human mast cells secretory granules, all with important roles in angiogenesis (Serafin et al, 1987) Activated mast cells can produce lipid-derived mediators such as cyclo-oxygenase (prostaglandin D2), lipoxygenase (leukotrienes), arachidonic acid metabolites with important inflammatory activities (Li et al, 2000). Mast cells also secrete tumor necrosis factor (TNF-α), interleukin IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, VEGF, transforming growth factor (TGF-β), basic fibroblast growth factor (bFGF), epithelial growth factor (EGF), platelet-derived growth factor (PDGF), monocyte chemoattractant protein-1 (MCP-1) (Church et al, 1997, Norrby et al, 1997).

Human mast cells are structurally and functionally heterogeneous (Kitamura et al, 1989). Irani et al in 1986 indicated that human mast cells have different neutral protease content, depending on their location. These proteases, which represent the majority of granule protein, are markers of different types of mast cells. Mucosal mast cells contain tryptase without chymase by contrast to connective tissue mast cells that contain both chymase and tryptase. Multiple factors are able to activate mast cells including cytokines (White et al, 1993, Taylor et al, 1995). The degranulation of the mast cells involves fusing of the membrane of the granules containing histamine with the cell membrane such as they become part of the cell membrane and their content dissolves and is secreted. The mast cell left behind is a viable degranulated or partially degranulated cell. Eady et al, 1979 found a significant correlation between the number of mast cells and number of blood vessels in the dermis. Rakusan et al, 1990 noted changes in the mast cell density during capillary proliferation, which may indicate a role of these cells in angiogenesis. The intimal mechanisms of mast cell involvement in angiogenesis are still unclear. Research work has concentrated on the production of angiogenic factors from mast cells (Levi-Schaffer et al, 2001). Activated mast cells can release pre-formed VEGF and they can sustain its release by continuing to secrete the protein (Boesiger et al, 1998). Mast cells also produce and secrete FGF, which has known angiogenic effects. Mast cells can produce IL-8 (Moller et al, 1993, Lin et al, 2001), which has been shown to be a potent pro-angiogenic in studies related to tumour angiogenesis (Lin et al, 2001). TGF-β and TNF-α are involved in angiogenesis and both cytokines can be produced by mast cells (Church et al, 1997, Norrby,

1997). Heparin found in mast cells may be involved in angiogenesis by stimulating the migration and proliferation of ECs (Taylor et al, 1982, Folkman et al, 1983, Fraser et al, 1983, Thornton et al, 1983, Azizkhan et al, 1980). It has been shown that heparin can stimulate the induction of FGF (Thornton et al, 1983). Histamine and mast cell proteases such as tryptase and chymase may also be proangiogenic (Meininger et al, 1995, Muramatsu et al, 2000, Ribatti et al, 2000). Mast cells also secrete type VIII collagen that may be involved in angiogenesis, given the evidence of its implication in the assembly of ECs (Ruger et al, 1994). Angiogenesis mediators such VEGF, FGF-2 and PDGF have been quoted as mast cell chemotactic factors (Gruber et al, 1995). Recent data indicates that human mast cells are a source and target of angiogenic factors and they might play a role in angiogenesis through the expression of several forms of VEGFs and their receptors (Detaroaki et al, 2009). The theories surrounding the presence and roles of mast cells in CVMs are discussed in the next sub-chapter, in relation to the histological morphology of CVMs.

Macrophages

Monocytes and macrophages were originally classified as cells belonging to the reticulo-endothelial system by Aschoff in 1924. Van Furth et al (1972) proposed the term of mononuclear phagocyte system for these cells. Macrophages originate in the bone marrow and they develop in stages, from stem cell, committed stem cell, monoblast, pro-monocyte, monocyte (bone marrow, then peripheral blood) and finally macrophages are reaching tissues. Within the process of migration of blood monocytes into tissues, to become macrophages, monocytes continue to differentiate to become multifunctional tissue macrophages, which do not re-enter the circulation. Monocytes are considered immature macrophages, although they may be fully functional for their location. Macrophages have been classified into normal and inflammatory macrophages. Normal macrophages are those found in connective tissues and the inflammatory macrophages are present in various exudates. It is well-known that macrophages are heterogeneous cells as their isolation from different anatomical sites has a diversity of phenotypes and properties. Because cell function is linked to the signals received from the cell microenvironment, the macrophage heterogeneity is said to be the results of the unique conditions within specific tissues. Macrophages are called ‘activated’ when they exhibit specifically increased functional activity. Within this category there were two classes with distinct phenotypes, identified by Stein et al, as classically activated macrophages (express Th1-like phenotype) and alternatively activated macrophages (express Th2-like phenotype). Both types express a wide range of plasma membrane receptors, suggestive of their interaction with other cells, extracellular ligants from plasma, extracellular matrix and microorganisms. Macrophages play an important role in pathogen recognition and clearance, removal of

senescent cells. They have multiple biological functions, they are involved in the immune response, and they act as killer cells or regulatory cells of the inflammatory response. They also contribute to tissue re-organisation by secreting enzymes such as hyaluronisdase, elastase, collagenase, antiproteases and regulatory growth factors. Macrophages have the potential to potentate the remission of an inflammatory process by releasing TGF-β, PGE_2, IL-10. One of their major roles is the induction and modulation of angiogenesis. They were shown for many years to be key players in the regulation of physiologic and pathologic angiogenesis (Polverini et al, 1977). Macrophages become angiogenic in conditions of low oxygen tension, in presence of lactate, pyruvate or hydrogen ions and they can be activated by cytokines such as IFN, GM-CSF, PAF, or MCP. They respond to hypoxic conditions by up-regulation of hypoxia-inducible factors (HIFs). The cascade of subsequent events may lead to over-expression of VEGF by macrophages located in hypoxic areas, with its inherent pro-angiogenic properties (Burke et al, 2001). Macrophages are found in hypoxic area in tumors (tumor associated macrophages – TAM) but also in ischaemic areas of wounds, atherosclerotic plaques or joints affected with rheumatoid arthritis. What is driving these cells to hypoxic locations is still unknown, although a few theories implicating chemokines and other chemoattractant factors such as monocyte chemotactic protein (MCP-1) have been launched. Macrophages are able to promote all stages of the angiogenic process by means of their own products, when they are specifically activated. Macrophages produce factors able to induce migration and differentiation of ECs. Two of the most important are human angiogenic factor (HAF) and angiotropin. Other factors produced by macrophages and capable of inducing proliferation in capillary endothelium are bFGF, TGF, GM-CSF, M-CSF, VEGF, IL-8, substance P. Whilst many angiogenic factors have been shown to be produced by macrophages, the way they coordinate the angiogenic response is not well understood. Macrophages also release factors that act indirectly by attracting or activating angiogenic cells as well as factors that inhibit migration or mitosis of ECs (monocyte-derived EC inhibitory factor – MECIF-, macrophage-derived EC inhibitor - MD-ECI -, thrombospodin I, IFN. Classically activated macrophages tend to promote inflammation, extracellular matrix (ECM) destruction, apoptosis, while alternatively activated macrophages promote ECM construction, cell proliferation and angiogenesis. Macrophages have the capacity to induce and then, down-regulate the formation of new capillary blood vessels. The persistence of high numbers of macrophages in tumours is associated with on-going growth, neo-vascularisation and poor prognosis (Crowther et al, 2001). By contrast, tumours depleted of macrophages have a reduced capacity to induce neovascularisation. It is suspected that the prolonged or excessive neo-vascularisation in pathological states is the result of a combination of over-production of mediators of angiogenesis associated with a lack of naturally occurring inhibitors of

angiogenesis. It is becoming increasingly clear that genetic control may be responsible for regulating angiogenesis and that expression of macrophages angiogenic activity is under the negative control of a suppressor element (Rastinejad et al, 1989).Better understanding of the angiogenic activity of macrophages is necessary as this may have therapeutic implications in many conditions where angiogenesis is dis-regulated, including possibly CVMs. An account of current data available in respect to macrophages in CVMs is given in the next sub-chapter, where the histological morphology of CVMs is discussed.

1.10.2 Cytokines and growth factors – their role in angiogenesis, classification and regulation

Foremost among the signalling substances with roles in angiogenesis are VEGF and their receptors, FGF, angiopoietin-1 and their receptors.

VEGF A/VEGF-R1 and VEGF family

VEGF A, which is selective for vascular endothelium is one of the best known cytokines involved in vasculogenesis, physiological and pathological angiogenesis, and remodelling of primitive vascular networks. Knockout mice genetically engineered to be deficient in VEGF receptors die of abnormal angiogenesis (Carmeliet et al, 1996, Ferrara et al, 1996).

This is why most likely VEGF A is an essential pre-requisite to angiogenesis and is the main focus here, although the other members of family will be discussed. This is a member of a family of dimeric glycoproteins that belong to the platelet-derived growth factor (PDGF) family of growth factors. Other members of the family include VEGF B, VEGF C, VEGF D, VEGF E and placental growth factor (PlGF). VEGF A is the most studied and mediates its effects by interacting with two tyrosine kinase receptors VEGFR-1(FLT-1) and VEGFR-2 (KDR). Recently, a non-kinase recetor, neuropilin (NRP-1), which is less selectively expressed on vascular endothelium was found to potentiate VEGF A binding to VEGFR-2. Disruption of the gene for VEGFR-2 interferes with the differentiation of ECs, leading to the death of embryo at day 8.5-9.5 (Shalaby et al, 1995). Disruption of the gene for VEGFR-1 permits differentiation of ECs but interferes with a later stage of vasculogenesis, resulting in thin walled vessels with larger than normal diameter and death of embryo at day 9 (Fong et al, 1995).

There are three VEGF receptors for the family of VEGF ligands. VEGFR-2 is responsible for mediating microvascular permeability, ECs proliferation and migration but the signalling steps mediated through VEGFR-1 have been less characterised. VEGFR-2 is considered the main receptor involved in the proliferation of ECs after stimulation by VEGF but recent studies attributed significant roles to VEGFR-1, with independent role in cell motility and inhibiting signalling pathways mediated by VEGFR-2 (Dvorak, 2002). VEGFR-1 mRNA is expressed in both proliferating and quiescent ECs, suggesting a role for VEGFR-1 in the maintenance of ECs

(Peters et al, 1993). VEGFR-1 is a potent antagonist of VEGF by binding VEGF with high affinity and reducing its interaction with other receptors (Bicknell et al, 2004). VEGF receptor 3 (VEGFR-3 or FLT-4) becomes specific to lymphatic vessels in adults. The expression of Flt-1 and KDR is largely restricted to vascular endothelium. All receptors are tyrosine kinases; they form dimers and exert their actions via secondary pathways. Each VEGF receptor is able to bind specific VEGF ligands (see diagram below).

VEGF A has been shown to be a critical regulator of EC development and is essential for normal vasculogenesis and angiogenesis. VEGF A seems to have the ability to induce EC proliferation as well as migratory and sprouting activity, and to help promote ECs to form tubule-like structures. These effects seem to be mediated largely by the VEGFR-2 receptor. VEGF A is involved not only in the initial phases of vasculogenesis, but also in the later stages of vasculogenesis, in sprouting, and other aspects of angiogenesis, as well as in maintaining vessel survival (Carmeliet et al, 1996). VEGFR-1 interacts with VEGF B and may be involved in down-regulating VEGF activity to ensure that the right number of ECs is generated (Gale et al, 1999). VEGF is also known as vascular permeability factor (VPF) due to its property of inducing vascular leakage, which is considered an important step in angiogenesis and leading to the formation of extravascular fibrin gel. Induction of vascular permeability appears to be an essential first step in angiogenesis. Roberts et al (1995) suggested that VEGF may be able to induce fenestrations in ECs that may provide additional trans-cellular pathway. Recent studies suggested that VEGF-mediated angiogenesis requires a specific adhesion molecules pathway, mediated by the vascular integrin αVβ5 (Friedlander et al, 1995). Clauss et al (1990) reported VEGF may promote monocyte chemotaxis. The migration of monocytes in response to VEGF was shown to be mediated by Flt-1 (Barleon et al, 1996). VEGF expression is also detectable around microvessels in areas where ECs are usually quiescent (Brown et al, 1992). This would indicate the possibility that VEGF may be required not only to induce active vascular proliferation but also to maintain the differentiated state of blood vessels (Ferrara et al, 1992).

The mechanisms relating VEGF receptor overexpression to that of their ligands are not known but there is evidence that chronic exposure to high levels of VEGF A stimulates the expression of both VEGF A receptors on ECs (Detmar et al, 1998). VEGF A has effects on cells other than ECs, such as lymphocytes, granulocytes, macrophages.

It was suggested that VEGF B may participate in the regulation of angiogenesis, particularly in muscle (Olofsson et al, 1996).

VEGF A is over-expressed in the majority of solid human cancers and is increasingly found in lymphomas and haematologic malignancies (Dvorak, 2002) Elevation in VEGF levels have been detected in the serum of some cancer patients and expression of VEGF receptors is known

to be up-regulated in tumours compared to normal vessels. VEGF expression was correlated with vessel involvement and metastases and patients with VEGF-positive tumours had a worse prognosis than those with VEGF-negative tumours (Maeda et al, 1996). VEGF up-regulation has been reported in other diseases such as rheumatoid arthritis, psoriasis, bullous disorders, endometriosis, Graves' disease. Non-EC populations may express one or more VEGF receptors (Wang et al, 1998). Whether this is required for recruitment of other cells, specifically smooth muscle cells or pericytes or occurs as a consequence of cytokine synthesis by activated ECs, it is not known.

VEGF A expression is subject to a number of control mechanisms. Oxygen tension is the major regulator of VEGF gene expression (Minchenko et al, 1994, Schweiki et al, 1992, Shima et al, 1995, Brogi et al, 1994). Hypoxia has been proposed to play an important role in the regulation of VEGF receptor gene expression (Tuder et al, 1995). Hypoxia up-regulates expression of VEGF A by increasing m RNA transcription and stabilisation (Claffey et al 1996, Shih et al, 1998, Levy et al, 1995, Forsythe et al, 1996). Its up-regulation is associated with up-regulation of both VEGF A receptors. Hypoxic up-regulation of VEGF A is mediated by hypoxia-inducible factor 1 (HIF-1) and the details of this pathway are discussed in the hypoxia sub-chapter. Hypoxia has been reported to up-regulate VEGFR expression independent of VEGF A (Gerber et al, 1997).

The von Hippel-Lindau (VHL) tumour suppressor gene has been implicated in the regulation of VEGF gene expression (Siemeister et al, 1996).

Cell differentiation has been shown to be important in the regulation of VEGF gene expression (Claffey et al, 1992).

Several cytokines or growth factors up-regulate VEGF mRNA expression and induce release of VEGF protein in various tissues. Amongst those quoted in literature are: epidermal growth factor (EGF), TGF-β (Frank et al, 1995), TGF-α (Detmar et al, 1995), IL-1β (Li et al, 1995), IL-1α, PGE_2 (Ben-Av et al, 1995), IL-6 (Cohen et al, 1996), IGF-I, tumour necrosis factor (TNF), FGF-2 (Dvorak, 2002). Dexamethasone appears to down-regulate or to prevent cytokine-induced up-regulation of VEGF A expression (Nauck et al, 1998). Placental growth factor (PlGF), strongly expressed in placenta, has been suggested to play a vital role especially in pathological angiogenesis potentate the activity of VEGF A, and was shown to reconstitute haematopoietic stem cells via VEGFR-1 (Hattori et al, 2002).

Tie-2/Angiopoietins

Both VEGF/VEGF receptor system and the angiopoietin/Tie-2 system were identified as central regulators of embryonic angiogenesis (Breier, 2000).

Tie-1 and Tie-2/Tek represent the Tie family. Angiopoietin-1 and Angiopoietin-2 have been reported to be antagonistic and stimulatory ligands for Tie-2 Tyrosine kinase receptor (Maisonpierre et al, 1997, Suri et al, 1997). The four known Angiopoietins all bind to Tie-2, but it is not clear whether they utilise the closely related Tie-1, for which no ligands have been identified as yet. Both Tie receptors are required during embryonic development for the integrity and survival of ECs, especially in areas undergoing angiogenic sprouting of capillaries and later for EC maintenance (Dumont et al, 1994, Puri et al, 1995). Angiopoietins appear to influence how a simple EC-lined tube develops into a mature vessel (Folkman et al, 1996, Korpelainen et al, 1999). Unlike other well-known angiogenic factors, the angiopoietins are not mitogenic for ECs and their function is not well understood (Carlson, Feng et al, 2001). Defects in mice lacking Ang-1 or Tie-2 suggest that this system is critical for normal remodelling, maturation and stabilisation of the developing vasculature. Unlike VEGF, Ang-1 cannot promote mitogenic responses or tubule formation, but promotes vessel branching and remodelling, maturation and stabilisation of vessels. It has been suggested that Angiopoietins act in a 'permissive' way allowing for proper interactions between ECs and supporting cells, resulting in a system that can respond to other stimuli (Suri et al, 1996). Maisonpierre et al, 1997 indicated that Ang-2 facilitates vascular remodelling in adults by blocking a constitutive stabilising action on Ang-1, allowing vessels to revert to a more plastic and instable state. Existing data suggest that VEGF A and angiopoietins not only have quite different roles during vascular development, but also very complementary and coordinating roles (Gale et al, 1999). All members of the VEGF family and their specific receptors are shown in the Figure 1.2.

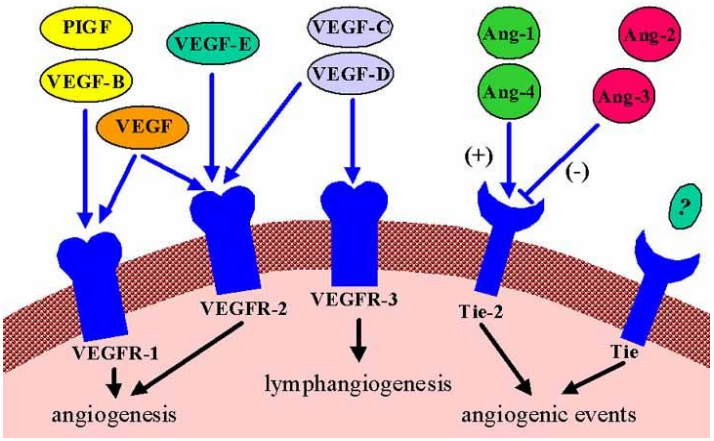


Figure 1. 2. VEGF family and their specific receptors
Diagram adapted from www.bioscience.org

1.10.3 Other molecules with implications in CVM related angiogenesis

Integrins

Adhesion of EC to one another and to the matrix is mediated by a variety of surface receptors that belong to several families of cell adhesion molecules such as cadherins, integrins, immunoglobulins and proteoglycans. There is evidence that EC adhesive structures might play a morphogenic role during blood vessel formation in both embryonic and postnatal life (Bazzoni et al, 2000). Targeted null mutation studies have supported the concept that EC-matrix and cell-cell adhesionis involved in the formation and maintenance of the vascular tree (Bazzoni et al, 1999).

EC adhesion molecules of the integrin family have emerged as critical mediators and regulators of angiogenesis and vascular homeostasis (Ruegg et al, 2003).

The integrins are a family of adhesion receptors involved in many physiological functions. They are adhesion molecules with well-established roles in angiogenesis as they were found to facilitate cell migration, proliferation and survival and the regulation of matrix degradation. They can also mediate cell-cell adhesion and influence the expression of differentiation related genes (Danen et al, 2003). They co-operate with growth-factor receptors to enhance signalling (Miyamoto et al, 1998). Integrins have both common and specific pathways for signalling into the cell (Ruoslahti, 1996). The family of human integrins comprises 24 members, each of which is a heterodimer consisting of 1 of 18 α- and 1 of 8 β-subunits (Carlson et al, 2001).

Endothelial Cell Adhesion Molecules

Integrin families and their ligands.

Integrins	*Ligands*	*Integrins*	*Ligands*
β1		β3	
α1β1*	Coll, Lm	αIIbβ3	Fb, vWF, Fn, TSP
α2β1*	Coll, Lm	αvβ3*	Vn, Fn, TSP, OP, PECAM-1
α3β1*	Lm-5		
α4β1	VCAM-1, Fn	**β4**	
α5β1*	Fn	α6β4*	Lm-5
α6β1*	Lm		
α7β1	Lm, Fn	**β5**	
α8β1	Fn, Lm, TN	αvβ5*	Vn, Fn
α9β1	TN		
αvβ1*	Vn, Fn	**β6**	
		αvβ6	Fn, TN
β2		**β7**	
αLβ2	ICAM-1, -2, -3	α4β7	MAdCAM-1, VCAM-1, Fn
αMβ2	iC3b, ICAM-1, Fb, FX	αEβ7	E-cadherin
αXβ2	iC3b, Fb		
αDβ2	?	**β8**	
		αvβ8	Fn, Lm, Coll

Integrins expressed on vascular EC are marked with an asterisk (see Text). *Abbreviations*: Coll, collagen; Lm, laminin-1; Lm-5, laminin-5; Fn, fibronectin; Vn, vitronectin; Fb, fibrinogen; vWF, vonWillebrand factor; TSP, thrombospondin; OP, osteopontin; TN, tenascin-C; VCAM-1, vascular cell adhesion molecule-1; PECAM-1, platelet endothelial cell adhesion molecule-1; ICAM, intercellular adhesion molecule; iC3b, inactivated complement factor 3b; FX, coagulation factor X; MadCAM-1, mucosal addressin cell adhesion molecule-1.

Figure 1. 3. Integrin families and their ligands

The picture above is adapted from Morphogenesis of Endothelium, edited by Werner Risau and Gabor M. Rubanyi, 2000, p 49.

Most integrin mediating cell adhesion to ligands is present in basement membranes, extracellular matrix, or plasma proteins. A subset of integrins binds receptors expressed on the surface of other cells, being involved in heterotypic cell-cell adhesion. Members of the β1, β3, β4 and β5 integrin families are expressed on EC, with significant heterogeneity in their expression dependant on the origin of ECs, species, etc.

There is strong evidence to suggest that endothelial integrins might influence vasculogenesis and angiogenesis, as follows (Bazzoni et al, 2000):

1. The integrin expression and function in ECs showed that integrins are not involved in providing attachment only, they mediate pro-angiogenic functions, such as cell migration, proliferation, survival and regulation of matrix degradation.
2. Blocking integrin-mediated EC adhesion affects blood vessel formation.
3. Gene targeting experiments in mouse indicated a role for integrins in vascular morphogenesis.

Activation is key to integrin function in different processes and is termed dynamic regulation of integrin adhesive function or 'inside-out' signalling (Hughes et al, 1998).

Integrins have permissive roles for angiogenic programme of ECs. Some integrins are known to be regulated by angiogenic growth factors and studies with inhibitors of integrin functions implicate some of these molecules in vasculogenesis and angiogenesis. So far it is unclear how angiogenic growth factors and integrin-mediated adhesive events cooperate in the process of angiogenesis.

There is a large and growing body of evidence implicating various integrins and their ligands in vascular development but it is not clear which integrins are the most important or what exactly each of them does. From the vast family of integrins of which some have been studied on animal tissues, αVβ3 and αVβ5 merit attention as potential important regulators of angiogenesis. αVβ3 is a widely expressed integrin with a broad range of ligands and it is often called vitronectin receptor, although it can also bind fibronectin, von Willebrand factor, thrombospondin and fibrinogen (Dejana et al, 1990). αVβ3 is expressed on both abluminal and luminal surfaces of EC and it can bind both matrix components and plasma proteins (Conforti et al, 1992). Endothelial αVβ3 also binds circulating leukocytes through the receptor PECAM-1 (Piali et al, 1995). αVβ3 is constitutively expressed on EC but it can be up-regulated by TGF-β (Defilippi et al, 1995) and bFGF (Boudreau et al, 1997). ECs can express at least αVβ3 and αVβ5 and perhaps αVβ1, although resting ECs express little or no αVβ3.

αVβ3 has several functional properties which indicate that this integrin might have a permissive role for the angiogenic program of EC. αVβ3 co-localises with vitronectin at the basal surface of newly formed vessels (Drake et al, 1995) and promotes EC migration on vitronectin (Leavesley et al, 1993). αVβ3 regulates matrix degradation by binding MMP-2 and co-localising it at the surface of invading EC in an active form (Brooks et al, 1996). αVβ3 mediated adhesion also promotes EC survival and maturation and induces apoptosis (Brooks et al, 1994). αVβ3 was found to be markedly up-regulated on vessels undergoing angiogenesis. In the process of building up focal adhesions, it was shown that a necessary cross talk should occur between αVβ3 and VEGFR2, in order to transduce the necessary VEGF signals. (Masson-Gadais et al, 2003).

Angiogenesis requires cell-extracellular matrix interactions, which are mediated in part via integrins αVβ3 and αVβ5 (Nisato et al, 2003). They were found to be involved in the EC invasion and capillary-like tube formation. αVβ5 was also found to bind vitronectin and to promote angiogenesis. The two integrins identify different angiogenic pathways: αVβ3 is essential for PKC-independent angiogenesis induced by bFGF and TNF-α, whilst αVβ5 mediates PKC-dependent angiogenesis induced by VEGF and TGF-α (Friedlander et al, 1995). It was noted that in spite of different mechanisms of action, αVβ5 might compensate for αVβ3 and explain the normal vascular development in Glanzmann's thromboasthenia, a disease characterised by mutations of the integrin β3 gene and defective αVβ3 expression. Cheresh et al have shown that different angiogenic stimuli rely on either αVβ3 (FGF-2, TNFα) or αVβ5 (VEGF) and have shown that αVβ3 can bind the MMP-2 in a fashion that contributes to an invasive response and to angiogenesis.

Previous research has tried to establish if αV integrins are as essential for developmental angiogenesis as they are for adult angiogenesis. Preliminary reports indicate that if null mutation of the αV gene abolishes the expression of nearly all vitronectin receptors, the vasculature develops normally, and around one fifth of the αV-null progeny is born alive (Bader et al, 1998). It was concluded that if not essential for blood vessel formation, αV integrins might become relevant at later stages for maintaining vascular integrity, as the αV-negative mice that develop to term die after birth with severe haemorrhages in brain and intestine.

Brooks et al (1994) reported expression of αVβ3 in blood vessels of human wound granulation tissue but not in normal skin. A monoclonal antibody to αVβ3 blocked angiogenesis induced by bFGF and TNF-α, but had no effect on pre-existing vessels. The authors concluded that αVβ3 may be a useful therapeutic target for diseases characterised by neo-vascularisation. Antagonists of αVβ3 and αVβ5 disrupt angiogenesis in response to bFGF and VEGF (Berman et al, 2003). Antagonists of αVβ3 suppress angiogenesis in many experimental models and are

currently tested in clinical trials for their therapeutic efficacy against angiogenesis-dependent diseases, including cancer (Ruegg et al, 2003).

Integrin activation provides a mechanism for VEGF to induce a broad spectrum of cellular responses (Hood et al, 2003). Byzova et al (2000) indicated that VEGF activates integrins. Shattil (1995) found that beta 3 integrins, including αVβ3, are prominently expressed on the surfaces of platelets and ECs. These locations place them in strategic positions to perform adhesive functions required in haemostasis, wound healing and angiogenesis.

ET-1/ET_A/ET_B

Endothelin (ET) is a potent vaso-constrictive peptide, initially isolated from cultured ECs medium, in 1988 (Masaki, 2004) and comprises 21 amino acid residues. Subsequently three endogenous isoforms were discovered: ET-1, ET-2 and ET-3. The predominant ET produced in ECs is ET-1.

Endothelins which exert their effects by binding to two distinct cell surface receptors, ETA and ETB, are produced by a variety of normal cells, including ECs, vascular smooth muscle cells and act as autocrine or paracrine growth factors (Bagnato et al, 1998). Both receptors are transmembrane proteins with different molecular and pharmacological characteristics and functions based on their location. ET-1 promotes the growth of various kinds of cells, including ECs, smooth muscle cells, mostly via ETA receptors (Simonson). ET-1 modulates various stages of neovascularisation, including EC proliferation, migration, invasion, protease production, tube formation (Salani et al, 2000). ETA interaction with ET-1 results in vasoconstriction and cell proliferation. By contrast, ETB receptors are suggested to be involved in apoptotic mechanisms of cells (Masaki, 2004). Its activation results in nitric oxide induced vasodilatation. ETB plays a determinant role in the clearance of circulating ET-1(Attina et al, 2005). The ETA receptor binds ET-1 and ET-2 with greater affinity than it does ET-3, whilst the ETB receptor binds all three isoforms with equal affinity (Masaki, 2004).

In physiological states, ET-1 actions result in a complex modulation of vasomotor tone, tissue differentiation and cell proliferation driven by the interplay between effects on ETA and ETB receptors. In pathological states, ET receptors are differently regulated and there is a tendency towards vasoconstriction and ET alteration may be involved in diseases affecting both micro and macro-vasculature.

The increased expression of ET-1 is related to increased micro-vessel density and VEGF expression in some tumours (Salani et al, 2000) suggesting that ET-1 and VEGF might have complementary and coordinating roles during neovascularisation in such tumours. In these tumours ET-1 increases VEGF mRNA, expression and induces VEGF levels increase, especially in hypoxic conditions. ET-1 is up-regulated by hypoxia and could promote

angiogenesis by increasing VEGF production through an HIF-1α dependent mechanism. In hypoxic conditions, ET-1 potentiates hypoxia by amplifying HIF-1α stability and VEGF production, data derived from research on tumours (Gupta et al, 2003). In the presence of oxygen, HIF-1α binds to the VHL tumour suppressor protein leading to its ubiquitination and rapid degradation. In tumours HIF-1α stability was analysed and results indicated that the increase in HIF-1α protein levels induced by ET-1 results from enhanced HIF-1α stability (Bagnato et al, 2003).

In other tumours, ET increases αVβ3 integrin expression (Bagnato et al, 2004). ET-1 was reported to be expressed by macrophages (Mencarelli et al, 2009) and clearly both macrophages and mast cells of human hyperplastic tonsils expressed ET-1(Niwa et al, 1999). ET-1 expression by these cells is postulated to play a pathogenic role with a possible pro-inflammatory action on macrophages. ET-1 induced vasoconstriction was suggested to be increased by macrophages via stimulation of macrophage ET receptors (Magazine et al, 1994).

The gene encoding ET-1 is on chromososme 6 (Treiber et al, 2003) and stimuli such as shear stress, thrombin, hypoxia, growth factors, cathecolamines, angiotensin II can induce transcription of ET-1 mRNA. ET is not stored and released but instead generated in response to stimuli which vary between different tissues. ET is produced from a precursor named pre-proendothelin. This is selectively processed by an enzyme to the biologically inactive intermediate named big-endothelin. Big-ET is converted into active ET by the endothelin-converting enzyme (ECE). Two pathways have been described for clearance of ET: ET_B receptor mediated uptake followed by lisosomal degradation (Burkhardt et al, 2000, Bremnes et al, 2000) and catabolism by extracellular neutral endopeptidase (NEP) (Vijayaraghavan et al, 1990, Battistini et al, 1993). ET-1 production is stimulated by a variety of cytokines and growth factors, including IL-1β, TNF-α, TGF-β, PDGF, vasopressin, hypoxia and shear stress. Nitric oxide, prostacyclin and atrial natriuretic peptides are inhibiting ET-1 (Remuzzi et al, 2002).

Soon after the ET discovery in 1988, researchers became interested in ET receptor antagonists, because of the unique pharmacological response initiated by ET (sustained contraction) and the possible involvement in the cardiovascular disease (Masaki, 2004). Many selective and non-selective ET antagonists are currently available and tested in trials in a series of cardiovascular diseases, with variable success.

1.11 HYPOXIA AND HYPOXIA INDUCIBLE FACTORS (HIFs)

In the context of CVM development, there is no hypothesis as to how initial insult of hypoxia could be brought about or whether hypoxia is a leading signalling pathway in these lesions or if this is just a pre-requisite of a leading process of exacerbated angiogenesis. It is possible that abnormal micro-circulation comprising abnormal blood vessels of CVMs may

cause blood stasis with subsequent ischemia and injury to the ECs of the vessel intima as well as tissue hypoxia. It is also possible that the blood within CVM vessels, supplying CVM tissue is well oxygenated as a result of arterio-venous shunting and its delivery to the tissue is altered or normal with normal oxygenation of CVM tissue. However, nothing is known about HIF expression in normoxia. Several stimuli have been reported to induce HIF stabilisation and transcriptional activity, including mechanical stress, hormones, cytokines and growth factors or alteration of HIF degradation pathways are present, such as loss of function of the von Hippel-Lindau ubiquitin ligase complex (Gaber et al, 2005).This occurrence represents 'pathological hypoxia' and it has been described in relation to abnormal angiogenesis found in some tumours.

The effects of hypoxia have been sub-divided by time span. Acute hypoxia activates ECs to release inflammatory mediators and growth factors which encourage neutrophil adherence to the endothelium (Wood et al, 2000), as opposed to chronic hypoxia, which is thought to increase the expression of specific genes that encode for growth factors such as vascular endothelial growth factor (VEGF) and other various cytokines (Tipoe et al, 2006, Semenza et al, 2006). These genes are modified by transcription factors, the most important being hypoxia inducible factors (HIFs).

HIF is a 'key regulator' of the adaptive response to alterations of oxygen tension and contains two (α and β) sub-units. HIF molecules bind to hypoxia-responsive elements (HRE) in the promoter or enhancer regions of various genes, including VEGF. Under normoxic conditions, HIF-α sub-units have a short half-life, due to hydroxylation of proline residues by prolyl 4-hydroxylases, which require oxygen as a co-substrate. This allows binding of the von Hippel-Lindau ubiquitin ligase complex, which targets HIF-α for proteasomal destruction. Additional oxygen-dependant hydroxylation of asparaginase within HIF-α regulates recruitment of transcriptional co- activators. The dependence of prolyl and aspariginyl hydroxylation on oxygen means that, under conditions of hypoxia, HIF-α accumulates within the nucleus. Here, upon binding to constitutively expressed HIF-1β and recruitment of co-activators, it recognises HRE within target genes, leading to their transcriptional activation.

In parallel to the oxygen-dependant pathway, HIF-1α is also regulated by receptor-mediated signals, although this pathway is less well understood. Hypoxia induces expression of numerous pro-angiogenic mediators as a result of stabilisation of hypoxia inducible factor-1alpha. The specific involvement of the HIF transcription factors 1 and 2 in the angiogenic response of ECs is not clear. These more subtle changes in HIF-1α levels and/or transcriptional activation are stimulated by growth factors and cytokines.

1.11.1 Carbonic anhydrase IX (CA IX)

Carbonic anhydrase is a transmembrane glycoprotein, which belongs to a family of at least 13 different carbonic anhydrases and also acts as an enzyme. CA IX is strongly induced by hypoxia and therefore can be considered an endogenous marker of hypoxia. CA IX catalyses the reversible inter-conversion between CO_2 and HCO_3^-, one of the most fundamental reactions in cells. This reaction is essential for organisms and influences respiration, pH regulation, exchange of electrolytes, and many metabolic pathways.

A HIF binding site was found on CA IX and previous authors (Beasley et al, 2001) showed that a hypoxia responsive elements and HIF-1α were essential for CA IX transcription under hypoxia. CA IX expression is induced as early as two hours after HIF activation and persists for several days, meaning that CA IX reflects both previous and present hypoxia in cells.

CA IX it is not present in most normal tissues but is known to be overexpressed in VHL-defective tumours and under hypoxic conditions (Wykoff et al, 2000, Loncaster et al, 2001, Mandriota et al, 2002). In tumours, CA IX was noted to be involved in maintaining extracellular acidic pH, which resulted in tumour growth and spread (Ivanov et al, 2001). These facts prompted the investigation of expression of CA IX in CVMs, since one of the aims of this study is to investigate hypoxia in CVMs.

1.11.2 Interaction of HIFs with NEP and ET-1 signalling pathway

Activation of the hypoxia inducible pathway is a well-established potentiator of angiogenesis. Stabilised HIF-1α leads to the transcription of target genes including ET-1, which binds to its receptors enhancing HIF-1α stability (details in Chapters 6 and 7).

1.11.3 Neutral endopeptidase (NEP)/CD10

NEP (neprilysin) is a metallopeptidase known to degrade ET-1 and it has been shown to modulate the bioavailability of peptide mediators and growth factors. NEP exerts an important role in controlling inflammatory responses. Details are given in Chapter 6.

1.11.4 Measurement of blood gases

Measurement of blood gases or blood gas analysis or arterial blood gas analysis is a test which measures the amounts of oxygen and carbon dioxide in the blood, as well as the pH (acidity) of the blood. Further details are provided in the Appendix section.

1.11.5 Blood-tissue gas exchange

This term represents the movement of oxygen from the blood stream into the tissues and additional information is given in the Appendix section.

1.11.6 Account of ventilation-perfusion inequality

The rate at which oxygen reaches an alveolus is determined by its ventilation and the inspired pO_2. The rate at which O_2 leaves the alveolus is determined by its perfusion. pCO_2 in the alveolus will depend on the delivery of CO_2 to the alveolus by the venous blood and the ventilation. All these factors could influence the results of this study attempting to assess CVM oxygenation. More details are available in the Appendix section.

1.11.7 Oxygen delivery to tissues and oxygen consumption

The quantity of the oxygen made available to the body in one minute is known as the oxygen delivery. The pO_2 results from the balance between oxygen delivery and its consumption. Additional explanations related to this matter can be found in the Appendix section.

1.11.8 Hypoxia as a cause for CVMs – relationship to angiogenesis

It is well established that the angiogenic mechanisms are stimulated in response to a hypoxic environment. It is not known whether CVMs are hypoxic lesions or not and this is part of the aim of this research work (see Chapter 6). Should CVM prove to be hypoxic lesions and express HIFs, then the question is whether CVMs are primarily hypoxic or the hypoxia is secondary. Theoretically it is possible for CVMs to represent hypoxic tissues because of potentially decreased oxygen delivery to the tissues as a result of blood shunting. On the other hand dis-regulated angiogenesis could be the original culprit and hypoxia may accompany the angiogenic process. Further details as to the possible hypoxia and angiogenesis signalling pathways involved in the CVM pathogenesis are given in Chapter 7.

1.12 HYPOTHESIS

CVMs result from aberrant blood vessel development due to altered pro-angiogenic markers and their interaction with abnormal stromal cellular constituents and/or stimulation by hypoxia.

1.13 OBJECTIVES OF STUDY

1. To investigate the histological morphology of CVMs by histochemistry and immunohistochemistry.
2. To determine the immunohistochemical expression of the following pro-angiogenic markers in CVMs: VEGF A, VEGF-R1, Tie2, αVβ3, αVβ5, and ET-1.
3. To identify hypoxia-mediated responses relevant to CVMs by establishing whether hypoxia is a feature of CVMs measuring oxygen pressures within abnormal blood vessels of these lesions and by correlating expression of HIFs with hypoxia and with the expression of the selected pro-angiogenic markers in CVMs.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 SELECTION OF PATIENTS

Ethical approval has been granted for the collection of vascular malformations and control tissue as well as blood samples, for all studies comprising this project by the Hammersmith Research Ethics Committee: 04/Q0406/37 – 'Immunocytochemical studies of congenital vascular malformations', and 064/Q04064/29 – 'Study of partial oxygen pressures within blood vessels in congenital vascular malformations'. The study 04/Q0406/37 was subsequently amended to add additional immunohistochemical work (histochemistry, new antibodies to be tested and children'specimens to be included) and this was approved in January 2006. Data collected from recruited subjects were anonymised and protected according to the rules set out in the Human Tissue Bill (2004).

Patient selection, inclusion and exclusion criteria

Part of this proposed study involved tissue analysis and comprised two elements: one was prospective and one was retrospective. Both elements concern non-CVM control tissue as well as diseased tissue from patients with CVMs.

Only the prospective element of this study involved research participants. These participants were assessed, investigated and recruited in two ways: from a consultant led vascular clinic at Hammersmith Hospital and from the hospital (Charing Cross, Hammersmith or, on rare occasions from Chelsea and Westminster Hospitals) prior to their surgical intervention.

Prospectively, diseased tissue was obtained from participants with planned interventions, independent of this study. For collection of vascular malformation tissue, patients were seen by a Consultant Surgeon in the vascular outpatient clinics before appropriate surgery was decided. CVMs were operated for symptomatic reasons only. Those patients with lesions causing bleeding, ulceration/gangrene, mass pressure effect, functional impairment, cardiomegaly, severe pain or a combination of these problems, were offered surgery when location of the lesion made surgical intervention feasible. Patients undergoing operation to excise or debulk (serial removal without complete excision) CVMs were chosen for the study.

Inclusion and exclusion criteria for patients undergoing vascular malformation surgery were established to avoid faulty results. Those patients suffering from any form of malignancy or those diagnosed with or suspected to have atherosclerotic disease, connective tissue diseases or any other conditions known to alter angiogenesis were excluded from this study. Also patients with respiratory diseases, acute or chronic, musculoskeletal conditions or conditions causing depression of the brain's respiratory centre were also excluded from the blood gases analysis study.

Clinical details were obtained for all retrospective participants in the study, from the archived medical notes and all clinical data was recorded. The purpose of investigation was to exclude from the study those retrospective participants suffering with malignancy or inflammatory conditions such as rheumatoid arthritis, connective tissue disorders, atherosclerosis and systemic diseases that could result in stromal infiltration by inflammatory cells.

Arteries and veins for comparison with vascular malformation vessels were harvested from control participants. Other control tissue for this study consisted of normal skin taken from patients undergoing other surgical resections; abdominoplasty, limb amputation, reconstruction using flaps and breast reduction. A clinical history was taken from all control participants and care was taken that none of the control patients had known malignancy, advanced atherosclerosis or any other disease process that could alter angiogenesis within the peripheral vessels.

Informed consent for participation in the study was obtained usually when patient was seen in clinic, prior to operation, or in some cases, on the day of the operation. Details of the study, the type of tissue collected and the experiments planned were provided in an information sheet that was given to all participants along with an invitation letter. Separate consent forms were obtained prospectively from agreeing participants to have photographs taken by the Medical Illustration Department at Charing Cross Hospital or by authors, with the purpose of using them for publications, presentations and other teaching opportunities. Reassurance was provided regarding protection of data and tissue disposal after experiments.

Patients, from whom control tissue for this study was obtained, were approached for consent during their hospital stay before the operation. They were invited to participate in this present study and they were provided with an information sheet. Details of the study, tissue collection and disposal were provided as patients were given an information sheet and an invitation letter and informed consent was obtained.

A very small amount of tissue was necessary for the purpose of this study and this was delivered to us in the form of redundant tissue after a preliminary assessment of the whole specimen sent to histology as a routine, to confirm the diagnosis (see collection of tissue section).

CVMs were identified from a retrospective surgical cohort (1993 – 2003) consisting of 20 patients but 3 patients were represented by blocks of tissue for more than one specimen. All samples were formalin fixed and stored as paraffin blocks within the Histopathology archive, Hammersmith Hospitals NHS Trust (using local standard protocols).

Prospective cases were collected from theatre (2004 – 2009) and tissues were both snap frozen (when enough tissue was available) and formalin fixed. The prospective tissue samples

from patients recruited in this study consisted of 16 new patients and 2 patients initially identified from the retrospective cohort; further surgical debulking of their CVM allowed fresh tissue accrual. All tissues were collected and stored through the Human Biomaterials Resource Centre (Tissue Bank). All of these 33 samples had fixed tissues to allow immunohistochemical studies, but frozen tissue was limited to a variable number (7 - 20) of patients due to surgical limitations. In four patients normal skin away from the CVM was sampled. During this study the process of recruiting patients continued throughout its duration, averaging 7 new patients per annum. By the end of this research work, there were a total number of 20 new CVM patients agreeing to participate in this study but it was possible to consider 16 patients. All patients were initially adults but ethics has been altered to include specimens from children increasing recruitment to 11 CVMs per annum (see ethics approval section).

Ten control participants were recruited for most analyses in this study although by the end of this research work control tissue was obtained from 55 patients who agreed to participate in this study. The control samples represented a variety of specimens including skin from healthy areas (following operations like breast reductions, abdominoplasties, and amputations) and sometimes macroscopically normal skin adjacent to the excised CVMs, arteries and veins from pedicles, redundant at surgery involving flaps and varicose veins. After the initial morphological, histochemical and immunohistochemical analysis of the ten control specimens considered, given the uniformity of results, it was considered unnecessary to include more control participants.

Pre-operative MRI Scans and angiography

All the patients who had CVMs included in this study had either MRI scans or angiography when dealing with HF CVMs, or both investigations. Duplex scans, although feasible and generally used to define the type, location and extent of the lesions, were not used routinely in the institution where this research was performed. They were not considered as useful and accurate as MRI scans, due to their limitations associated with the small field of view, and restricted depth of penetration.

MRI scanning included T1- and T2- weighted imaging in multiple planes, fat-saturated T1-weighted imaging with intravenous administration of a gadolinium-based contrast agent. Dynamic contrast-enhanced MRI scan was used to distinguish between HF and LF vascular malformations, as well as to establish the extent of the CVM and any potential involvement of vital/important structures. The main feature in characterising vascular malformations on conventional MRI is represented by a mass/soft tissue abnormality and the presence or absence of flow-voids (HF vessels).

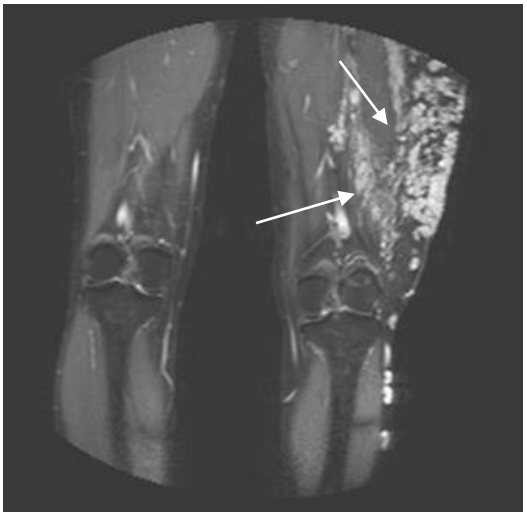


Figure 2. 1. MRI scan of a left thigh LF AVM

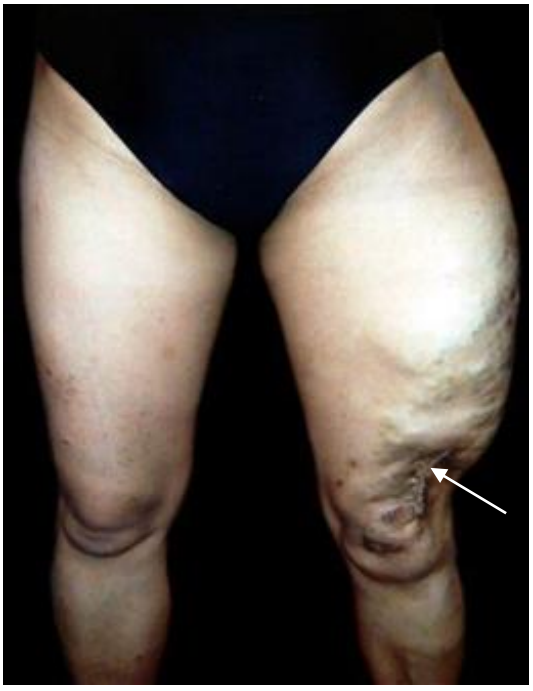


Figure 2. 2. Left thigh LF AVM following a first debulking operation

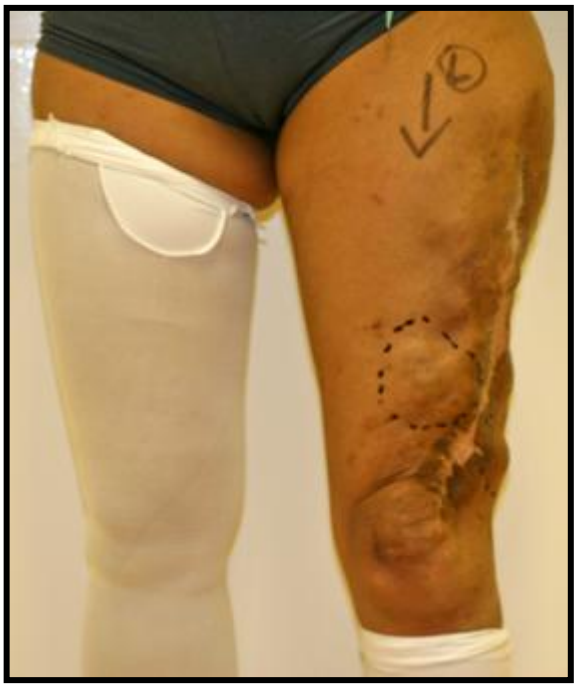


Figure 2. 3. Left thigh LF AVM following more debulking surgery

The Figure 2.1 is the MRI scan of a LF vascular malformation included in this study. This represents a young woman with a large left thigh AVM. Coronal images (Philips 1.5T) through the lower extremity showed an extensive left thigh LF vascular malformation, which is involving the vastus lateralis and the superficial fat overlying the lateral and posterior aspect of the thigh. Vastus medialis appeared un-involved; Figure 2.2 is a clinical picture of the vascular malformation described in Figure 2.1 following a limited debulking operation at the lower part of the lesion (arrow). Figure 2.3 represents the clinical picture of the same lesion, following a third debulking operation (original photographs).

Angiography, which includes arteriography, venography and direct intralesional contrast agent injection, was used as standard criteria to evaluate HF CVMs pre-operatively. In addition, those lesions suitable for embolisation were embolised 24 -72 hours prior to the surgical intervention.

Figure 2.4 is an example of angiography performed in an extensive HF vascular malformation involving left medial thigh in a young female with recurrent ulcerations and bleeding from overlying skin, participating in this study. The associated clinical pictures are also provided. The AVM nidus (arrows) is deriving its supply mostly from branches of the profunda femoris and anterior division of the left internal iliac artery. The radiologist reported extremely rapid arteriovenous shunting into dilated veins, which communicate with the superficial femoral vein. This patient had multiple femoral embolisations for a period of 4 years before she had the first debulking operation. The figure 2.5 is a clinical picture of the HF AVM

with intramuscular and soft tissue components as described in figure 2.4, obtained following the first debulking operation. Figure 2.6 is a clinical picture of the same patient, obtained after the second debulking operation showing that most of the lesion was removed (original photographs).

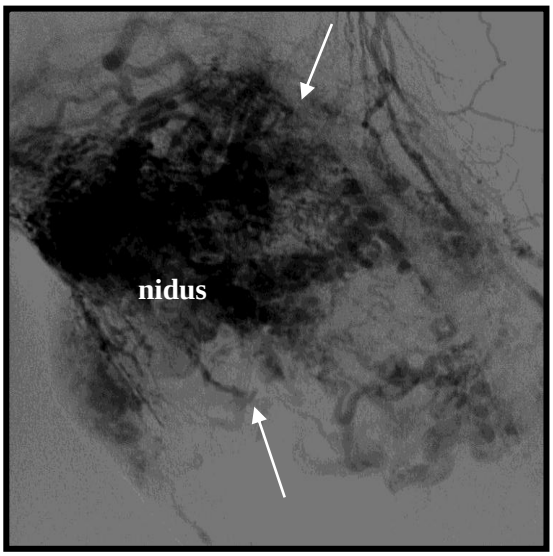


Figure 2. 4. Angiography of a thigh HF AVM

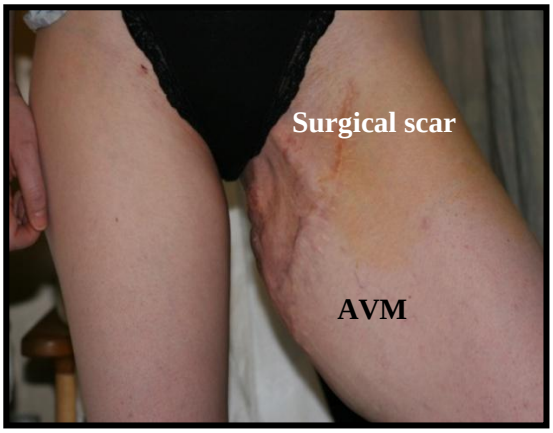


Figure 2. 5. HF AVM following first operation

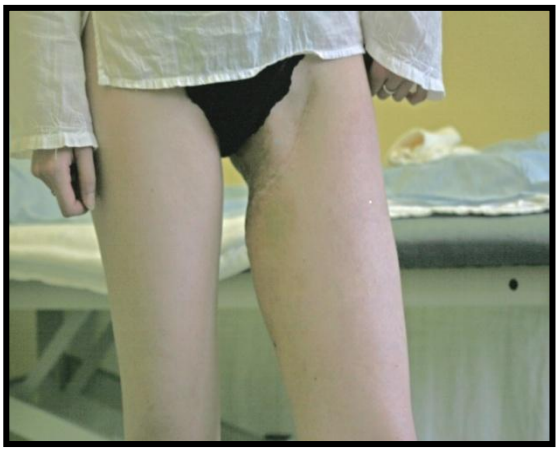


Figure 2. 6. Same HF AVM as in fig 2.5 after more surgery

2.2 COLLECTION OF TISSUE

2.2.1 Operations for CVM and collection of CVM tissue

The principle behind the operation for CVMs is to excised the lesion completely or, if this is not possible, to remove as much as possible from the lesion ('debulking'), process which is often limited by the amount of the blood loss or by the involvement of vital adjacent structures by the CVM. Further debulking operations can be performed, usually spaced at minimum six months interval, to give patient the chance to fully recover and to assess the result of previous operation. The extent and location of incision for these lesions varies extensively with the location and extent of the lesion itself.

Information about the risks of anaesthesia and surgery, the success and the failure rates of treatment, complications, recurrence rates and the follow up management were discussed with each prospective participant before the procedure.

Patients were given general anaesthesia and their position on the operating table varied with the location of the lesion. The exposed operating area was cleaned using antiseptic solution and draped. The length of the incision varied from about 4-5 cm in limited lesion to more than 30 cm in extensive CVMs. As the skin incision was already made, in most studied CVMs, the lesions could immediately be seen or they would announce they presence through extensive bleeding. Careful control of haemostasis is taken during all operation using diathermy forceps or suture-ligation of the bleeding vessels. The aim of the operating surgeon is to get around the whole lesion, or at least a good bulk of it, getting into a plane of normal, bloodless tissue and avoiding other structures such as nerves, important feeding vessels or vital organs. The length of operation varied considerably from under one hour to more than six hours, depending on the technical difficulties. Usually the surgical specimen will represent one main bulk of tissue, which is removed from the patient once all dissection around the CVM mass is performed.

When this was about to happen, the tissue biologist (AF) working in close relation to the consultant pathologist with a special interest in CVMs (AS), was called to the operating theatre to collect the specimen in a sterile recipient. This fresh tissue was immediately analysed by the consultant histopathologist, who confirmed the macroscopic appearance and supported the suspected clinical diagnosis of vascular malformation. Part of the fresh tissue collected, which was redundant to that necessary for the confirmation of histological diagnosis, was snap frozen and kept for research.

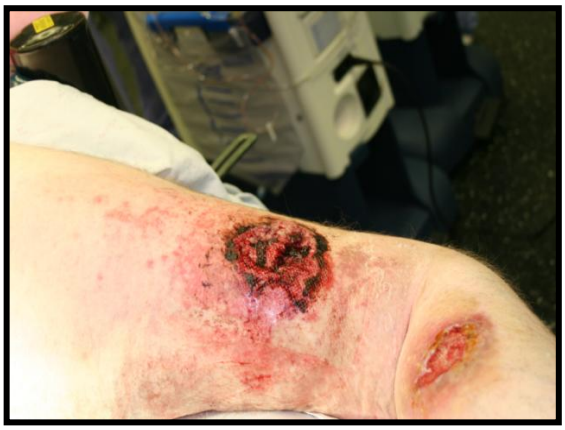


Figure 2. 7. Patient with HF AVM on operating table prior to surgery

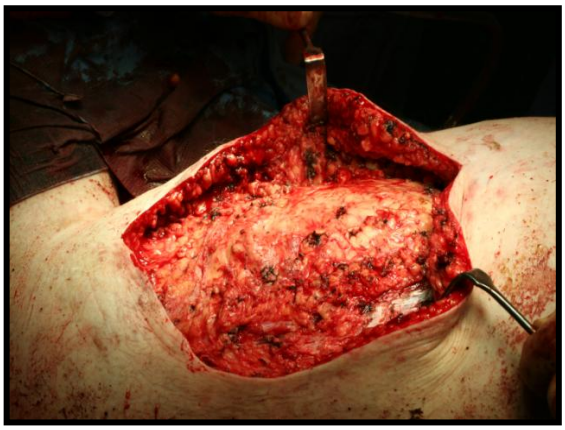


Figure 2. 8. HF AVM exposed at surgery

The CVM tissue retained for research purpose was divided in two parts (when enough tissue was available) using a sterile surgical blade under sterile precautions. One part of tissue was placed in formalin (4%), while the other part was snap frozen in liquid nitrogen and placed in -80°C freezer for biochemical analysis, after coding the tissue. At this stage, the tissue segments were assigned code numbers to protect participant identity and to blind the investigator. When research tissue was scanty, the specimen available was formalin fixed and subsequently embedded in paraffin.

The average recorded blood loss varied significantly from 50mls to 25L. In spite of this, due to the fact that majority of participants were young, their hospital stay was an average of nine days and this was mainly related to the required length of time to keep drains within the wound.

2.2.2 Collection of control tissue

Normal arteries and veins from location with similar provenience as CVM tissue were sampled for this research work in order to compare the morphological and immunohistochemical properties of CVMs with standard vessels. Control vessels were carefully chosen after detailed discussion between all involved in this study, and peripheral vessels were selected, as all CVMs we considered in this study were peripheral CVMs. The vessels in CVM include a mix of arteries and veins embedded in stromal tissue. Therefore, skin vessels were considered appropriate as control tissue for CVMs. Skin controls used in this study were obtained from patients undergoing operations such as: breast reduction surgery, abdominoplasty or leg amputations, where the tissue removed was redundant. None of the control patients had any other known disease process; tumour malignancy, advanced atherosclerosis or any other condition that could alter angiogenesis. All types of control tissue were treated as a homogenous, singular reference control for the CVM tissue. The tissue bank scientist was informed when an appropriate operation took place and once the tissue was about to be removed from the participant, this was collected in sterile containers. As described above for the CVM tissue, the control tissue was also divided in two pieces, one piece was preserved in formalin (and subsequently embedded in paraffin) and the other was snap frozen in liquid nitrogen.

2.3 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

2.3.1 Histological analysis of CVMs

Paraffin embedded CVMs tissue was used for histological analysis with H&E (haematoxylin and eosin), Masson Trichrome, Elastic Van Gieson and Toluidine blue. Histological morphological analysis of CVM vessels and comparison to control vessels were undertaken using these special histological stains. H&E stained tissue was used for general assessment and screening of sections, to assess quality of tissue and staining, presence of artefacts, to study the distribution of inflammatory cells of interest and as a reference for immunostained tissue. Masson's Trichrome stained connective tissue green and muscle in red. This stain helped studying the assessment of architecture of CVMs in comparison with control tissue and was used to calculate percentage of collagen tissue present in average sections in CVMs in comparison to controls. EVG stained tissue was used to assess disruption of elastic lamina in comparison to control vessels. The EVG stain colour the elastin in black and the muscles in red. Toluidine blue showed mast cells in blue and this stain was used to count

average number of mast cell present per/field (1 mm^2 section of CVM tissue) in comparison to control tissues.

All staining techniques used in this research work were standardised and performed according to the protocols established by the Pathology Department at Charing Cross Hospital, London (described in a later section). The CVM and control tissues preserved in formalin and set in paraffin blocks were routinely cut into 3μm sections with a microtome. The special stainings were obtained following the stages described in the Appendix section.

2.3.2 Immunohistochemistry (IHC)

This method was used to localise specific antigens in the CVM sections and compare it with their presence in control sections. The methods IHC are linked to Alfred H Coons and his colleagues (Coons and Kaplan, 1950) who were the first to label an antibody with a fluorescent dye and use it to identify an antigen in tissue sections with a fluorescence microscope. This technique has been expanded by introducing enzyme labelling with peroxidase (Nakane and Pierce 1967) and alkaline phosphatase (Mason and Sammons 1978), as well as other enzymes. IHC was chosen in this project to study specific antigens in the CVM and control tissue in preference to immunofluorescence (IF), because it better allows the visualisation of cytological details and tissue architecture.

2.3.3 Computer assisted microscopy and IA of CVMs and control vessels

Microscopy and part of the quantitative IA was performed using a computerised IA system. This was represented by an Olympus Optical BH2 microscope (Olympus Optical; Tokyo, Japan). The microscope was connected with a JVC KY-F55BE video camera (Victor Company of Japan, Tokyo, Japan) linked to a Gateway 2000 computer (Gateway 2000, North Sioux City; USA). The digital images were acquired at low and high power as required and were processed using the AnalySIS IA software (Soft Imaging Software GmbH, Munster, Germany). This software was used for: 1) Blood vessel count, 2) Blood vessel diameter, 3) Cell count, 4) Nerve fibre count, 5) Collagen percentage, 6) Quantification of immunopositivity to antibodies, 7) General description of CVM morphology. The detailed protocols for all these measurements and assessments are given in the Appendix section.

2.4 WESTERN BLOT FOR HIFs PROTEIN ANALYSIS

This technique was used in this study in the attempt to analyse the HIFs pathways and confirm the presence of HIF downstream proteins in CVM tissue, as HIFs were immunohistochemically expressed in CVMs (see Chapter 6). Details of the protocol are provided in the Appendix section.

2.5 BLOOD GASES ANALYSIS

This component of the research aim to determine if hypoxia is an element of CVMs, by measuring partial oxygen pressures (pO_2) in the abnormal blood vessels comprising CVMs (during their operative management) and comparing it with the arterial oxygen pressure, as well as venous oxygen pressures from ipsi-lateral and contra-lateral veins to the CVM. Subsequently, markers of hypoxia such as HIFs were quantified immunohistochemically and analysed in respect to oxygen pressures in the CVM tissue. The protocol of this part of the study is provided in the appendix section.

2.6 STATISTICAL ANALYSIS

The morphological analysis of CVMs was performed in two stages. Initially, a pilot study was carried out, to determine the optimal method of assessment, followed by a more comprehensive study, in order to support initial findings. The aim of both studies was to compare numbers of vessels, nerve fibres, vessel diameters, collagen percentage, disruption of elastic lamina/fibres and stromal cells in CVMs to those in control tissue.

The demographic data of the population in the pilot group of the morphological study was analysed using Mann-Whitney U test. The same test was used to analyse the difference in the number of cells, blood vessels, nerve fibres, vessel diameters, collagen percentage, degree of elastic fibres disruption and cells number between the groups studied as data could not be assumed to be parametric.

In the pilot study, macrophages and mast cells were counted by two independent assessors (NM and AF) using a microscopic grid under light microscopy, as well as image analysis. Therefore one of the objectives was to examine the difference between microscopic grid method and image analysis when compared with controls and between themselves. Wilcoxon Signed Ranks was used to investigate the difference between microscopy and the image analysis method for both macrophages and mast cells.

The other objective of the morphological study was to assess if there was any significant correlation between morphological findings in CVMs and control tissue and in different clinical patterns of CVMs (based on flow characteristics, i.e. HF and LF). Spearman's rho correlation coefficient was used to establish the relationship between clinical categories and any of variables that will show significant difference between the CVM group and the control group. SPSS for windows was used as statistical package to analyse the pilot data, two-sided test was used with 5% and 1% level of significance.

Furthermore, analyses were performed on data obtained from 33 vascular malformation specimens obtained from 33 patients and ten controls using the Stata 8 statistical software (Stata Corporation, Texas, USA). Data were analysed according to a pre-defined statistical analysis

plan. Mann-Whitney test was used to examine the difference between the CVM group and the control group for all variables as data could not be assumed to be approximately normally distributed. In this case, as ten outcomes have been analysed on the same dataset, a Bonferroni correction was applied such that $p<0.005$ indicates significance. Data are quoted as median [interquartile range] as the group sizes are small. Data assessed within the CVM group was given as median without being statistically analysed, due to small numbers.

In the angiogenesis markers immunohistochemical expression study, all immunostaining was quantified by two independent assessors using a scoring system from 0 to 3, depending on distribution and intensity of stain. Data obtained was analysed for agreement using kappa coefficient analysis. The demographic data of CVM participants and controls in this study was given as means±SD.

The immunostaining scores for CVM tissue were compared for each antibody with the immunostaining scores of control tissue using the Mann-Whitney U test. Correlations were made between expression of each angiogenesis mediator by CVM tissue and the stromal infiltrate of macrophages and mast cells present using Spearman's rho correlation coefficient for non-parametric data.

Similar methods of interpreting results were used for the analysis of hypoxia markers in Chapter 6. HIFs and CD 10 were also quantified by two independent observers using the scoring system from 0 to 3 and results were analysed for agreement with kappa coefficient analysis. HIFs and CD10 demographic data was similarly reported as means±SD. HIF-1α, HIF-2α and CD10 staining scores for CVM tissues were compared to the staining scores in control tissue using Mann-Whiney U test. HIFs staining scores in CVMs were correlated with those of each angiogenic marker studied as well as with the staining score of CD10 and the numbers of macrophages and mast cells per field, in the corresponding tissues, using Spearman's rho correlation coefficient.

For the blood gases analysis study (Chapter 6, sub-chapter 6.3), it was noted that for 95% power and a confidence interval of 27, 13 subjects were required for analysis. Two main comparisons were made in patients with CVMs: 1) paired comparison of arterial pO_2 in the radial artery and the CVM blood vessel in patients undergoing surgical correction of their CVM; 2) paired comparison of venous pO_2 in the venous systemic blood (on the same side as well as on the opposite side to the CVM) and the CVM blood vessel in patients scheduled for surgical correction of their CVM.

Unpaired t-test and Wilcoxon matched-pairs signed-rank test (assuming non-parametric data) was used to compare the difference in pO_2 between the radial/venous systemic and CVM vessels.

All comparisons and correlations in the angiogenesis, hypoxia and blood gases analysis studies were performed using GraphPad Prism version 4.03 statistics package.

CHAPTER 3

3. HISTOLOGICAL ANALYSIS OF MORPHOLOGICAL CHANGES IN CVMs

3.1 INTRODUCTION

There are few recognised histologic features specific to haemagiomas or vascular malformations and these two entities are often difficult to distinguish using routine histology. Some lesions have features applicable to both categories, which imply that present classifications may not reflect the in vivo situation and are clinically less than satisfactory. The exact histological morphology of vascular malformations and the clinical relevance is unclear. They appear as an abnormal proliferation of blood vessels of variable calibre and wall thickness with variable density. Complete destruction of the confluence point of the abnormal vessels in CVMs (‘nidus’) is considered by some authors to be the only cure for CVMs. Specific histology of the ‘nidus’ is not described. The stroma between blood vessels may show fibrosis and calcification and Mulliken and Glowacki noted normal endothelium and normal mast cell count within vascular malformations. Proliferation and leakage of capillaries interpreted as a consequence of raised intra-capillary pressure, was sometimes noted by histopathologists. All features described are largely non-specific. Percutaneous sclerotherapy and trans-catheter embolisation are often used to treat these lesions and both involve the injection of a foreign material that can further alter the histological morphology. Published data has described hyperplasia of elastic fibrils in the arterial wall and inflammation and necrosis in the venous wall at 2-3 weeks after the injection of sclerosant material.

3.2 OBJECTIVES

There is limited description in the literature of the morphological changes in CVMs and the main problem is recognising whether the lesions described are truly CVM, given the still existent controversies regarding their nomenclature and classification. On the other hand, different clinical categories of CVMs are not distinguishable histologically and features described in the literature are non-specific. Even more, there is no correlation described between the clinical behaviour of CVMs and their histological appearance and immunohistochemical profile.

The purpose of this part of the study was to compare the structural features of CVMs with those seen in control tissue (i.e. normal vessels, which were skin vessels from breast tissue, abdomen or lower limbs). An understanding of the potential morphological changes specific to CVMs, for example increased number of blood vessels per power field, increased vessel diameter, was essential for the subsequent studies of cellular infiltration (Chapter 4) and alteration in angiogenesis markers (Chapter 5).

Counting the blood vessel number per power field and measuring the blood vessels diameter was performed in this analysis, they were easily recordable, reproducible and

comparable characteristics. In addition, descriptive morphology was provided for the specimens of CVMs, analysed as part of the initial pilot study. This study was performed to elucidate histology of these uncommon but often aggressive lesions and to gain insight into possible mechanisms involved in their aetio-pathogenesis.

3.3 MATERIALS AND METHODS

3.3.1 Selection of patients and tissue collection

This part of our proposed study was both retrospective and prospective. A pilot study was initially performed and this was followed by a larger study, incorporating more participants and validating the data obtained in the pilot study. Both studies involved CVM and control participants. A total number of 33 CVM and 10 control participants were included. Whilst CVM specimens were obtained both retrospectively from archive and prospectively, control specimens were all obtained prospectively. All participants included in the pilot study had oestrogen and progesterone receptors (ER beta and PR) immunostaining to assess if these results would permit further morphological stratification. This initiative was supported by the clinical finding that CVM become clinically active during times of hormonal variation such as pregnancy or puberty. Therefore, differences in hormonal immune-stain may give indications of the CVM activity.

Details of patient selection criteria, consent forms and tissue collection are given in Chapter 2.

3.3.2 Assessment of blood vessel numbers and diameter in CVMs and controls

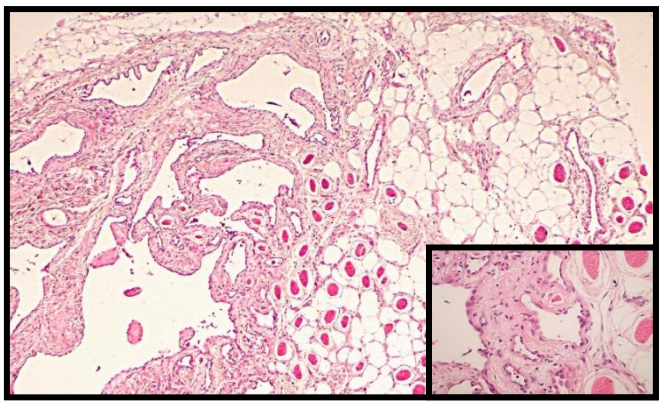
Factor VIII stained CVM and control tissue were used to delineate blood vessels for the purpose of both counting the blood vessels and measuring their diameters in CVMs and control tissue. These techniques were thoroughly described in the Appendix.

3.3.3 Histological and immunohistochemical studies

CVM and control non-diseased tissue, were stained with Haematoxylin and Eosin (H&E), Elastic von Gieson (EVG), Masson's Trichrome. In addition to the above histochemical stains, immunohistochemical stains for S100 and SMA 1-α were used to further characterise the morphological changes in CVMs. All histochemical and immunohistochemical techniques used in this part of the study were performed on formalin fixed and paraffin embedded tissues and they are described in the Appendix.

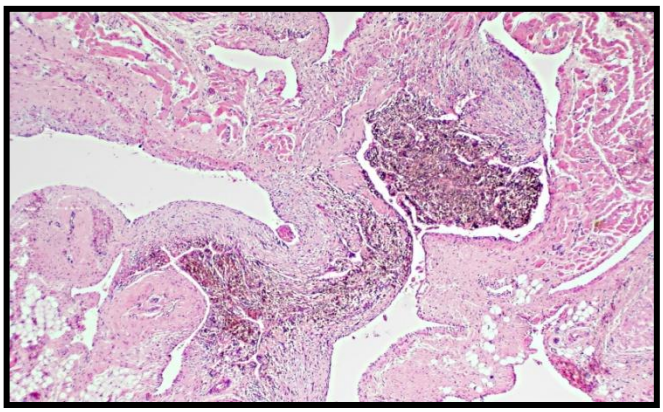
The aim of this study was to analyse the morphological changes within the vascular malformation in comparison with the normal/non-diseased vessels.

The H&E stained slides were used to identify the vascular malformation and ensure the tissue was representative and screen for various tissue artefacts, which may have affected the interpretation of proposed stains.



x25/x200

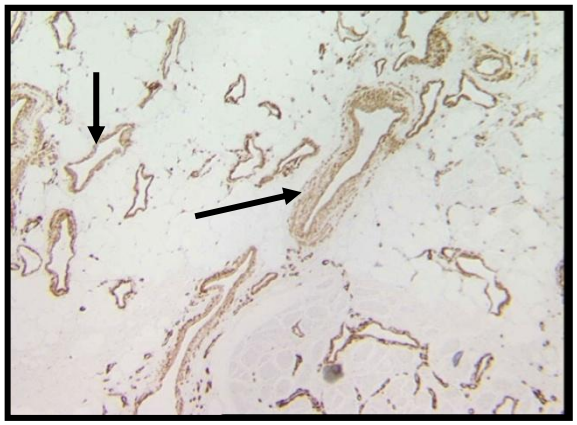
Figure 3. 1. Typical appearance of H&E slide of a HF AVM



x25

Figure 3. 2. H&E slide of a CVM showing thrombus formation

The following figures demonstate smooth muscle actin 1-α in CVMs.



x25

Figure 3. 3. Positive expression of SMA 1-α in an AVM

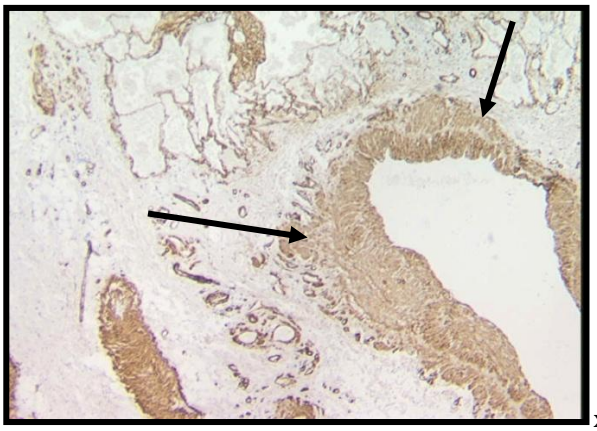


Figure 3. 4. Positive expression of SMA 1-α in a VM

There is a similar appearance of SMA 1-α stain in both CVMs, demonstrating thick and thin vessels of variable calibre.

S100

In the analysis of the morphological changes in CVMs, account was taken of presence of nerve fibres within CVM tissue and they were visualised using S100 immunostain.

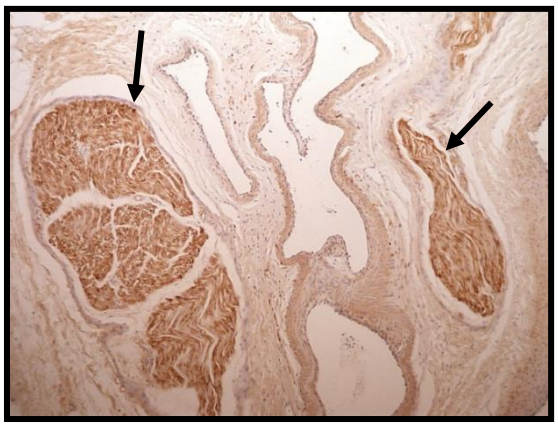


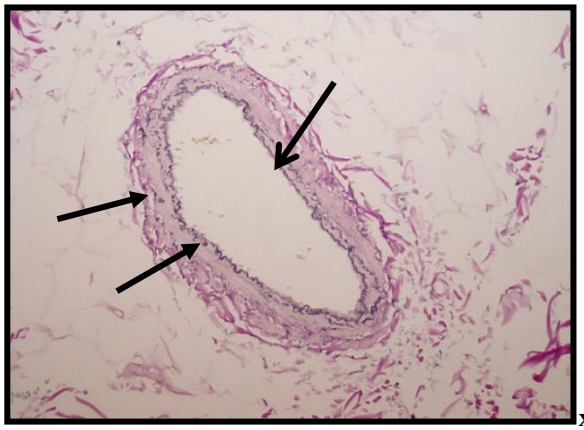
Figure 3. 5. Positive expression of S100 stain showing nerve fibres in an AVM

3.4 RESULTS

All CVM lesions analysed in this section (33 lesions) were negative for GLUT-1, therefore they were vascular malformations, not haemangiomas. All vascular malformations (17 lesions) analysed in the pilot part of this study for ERβ and PR receptors were negative for both receptors. Therefore, no comments can be further made regarding any association of ERβ or PR with the CVMs morphology or level of clinical activity. Most likely more specific hormonal stains need to be performed but this issue was not pursued further in this work.

The H&E stains of vascular malformations consistently showed large and small, thick and thin vessels lined by ECs, as well as a significant cellular infiltrate within the perivascular and extravascular space. Based on this stain, different clinical categories of CVMs cannot be distinguished, they appear similar.

In the EVG stained sections, both internal and external lamina of blood vessels were clearly visualised as black fibres, within control tissue (Figure 3.6). The external elastic lamina formed a well demarcated layer around the tunica media of the vessel wall, with no features of disruption. These features were similar in all control specimens studied and represented normal variant for the types of vessels analysed.



x40

Figure 3. 6. Elastic von Gieson stain of a skin control

Elastic Van Gieson stain, demonstrated disruption of the elastic fibres within a large proportion of AVMs. This finding was difficult to quantify, but EVG stain was used to assess the degree of disruption of the elastic fibres within the vascular wall, using a grading system from 0 to 3 (0 = no disruption, 1 = mild, 2 = moderate, 3 = severe). Beside this, it was noted that some CVM specimens contained a considerable amount of elastic tissue, sometimes in multiple layers, which was difficult to localise due to the nature of the specimen, containing really large blood vessels and at the same time giving the impression that the elastic fibres were localised outside the vessels, within the stromal tissue. This was thought to be due to the fusion of external elastic laminae of a number of adjacent vessels. The observation of dis-organised elastic fibres in some of the CVMs studied here could be associated an imbalance of pro-angiogenic mediators such as metalloproteinases (Marler, Fishman, et al, Nikkari, Hoyhtya et al, 1996).

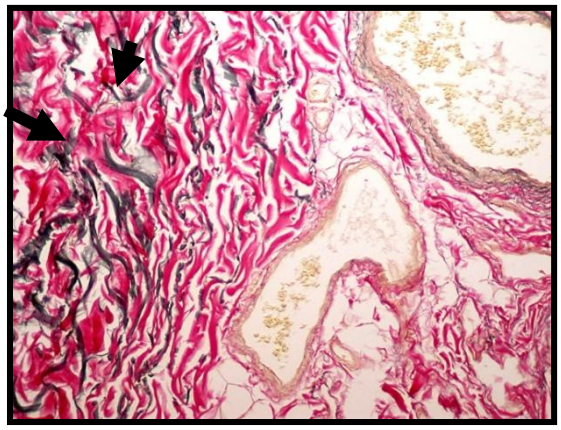


Figure 3. 7. Elastic von Gieson stain of an AVM

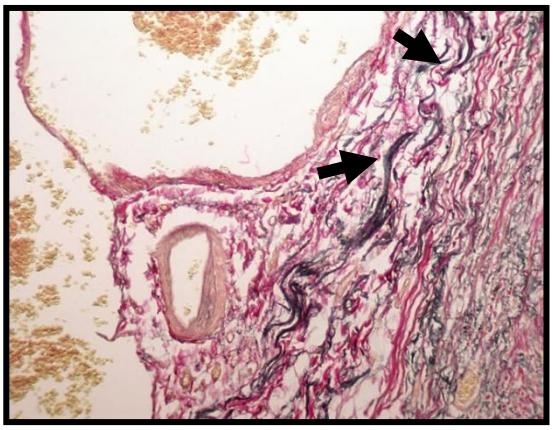
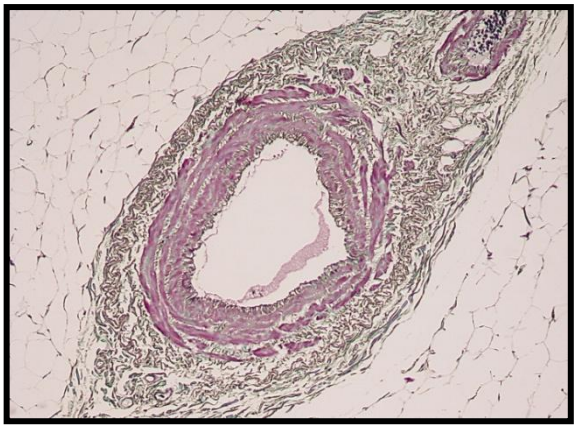


Figure 3. 8. Elastic von Gieson stain of a VM

The figures 3.7 and 3.8 show dis-organisation of the elastic laminae (arrows), with no much difference between AVMs and VMs.

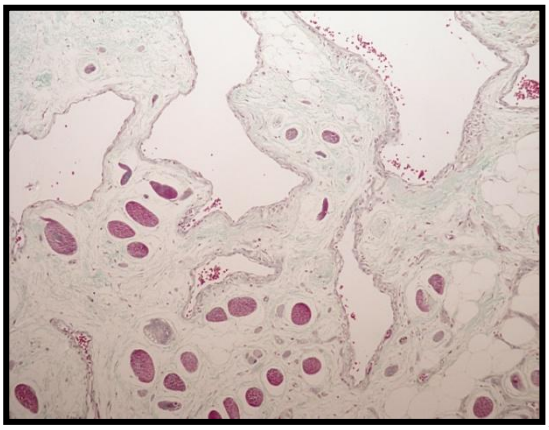
The Masson's Trichrome stained slides clearly identified the layers of the vessel wall, the presence of smooth muscle (purple) in the media, and the collagen (green) forming the adventitia and staining in variable proportion of inter-vascular space.



x40

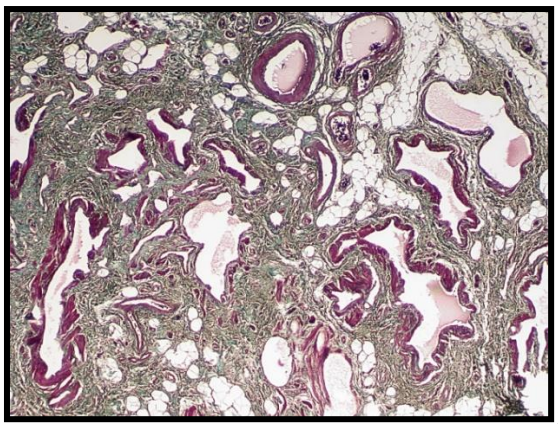
Figure 3. 9. Masson Trichrome stain of a section from skin control

The figure 3.9 shows circular SMC in tunica media and connective tissue, some in between muscle fibres but most of it forming the adventitia.



x40

Figure 3. 10. Masson Trichrome stain of aVM



x25

Figure 3. 11. Masson Trichrome stain of an AVM

Figures 3.10 and 3.11 demonstrate the smooth muscles and the proportion of connective tissue within the lesion, which was calculated using image analysis. This stain clearly showed the variable proportion of SMCs and connective tissue present in each specimen. As it can be seen in the VM specimen above, the SMCs are very scarce within the vessel wall, there were SMCs outside the vessel wall as this lesion was invading a muscle layer. The connective tissue predominates amongst the large, thin abnormal vessels of the CVM lesion. Sometimes vessels are so thin that it is difficult to clearly distinguish all three layers characteristics of a blood vessel. Within the next specimen (HF), the muscle layer is better represented, there are more thick vessels and they are appeared to be overall smaller in diameter comparing to the previous specimen. These represent simple observations but Masson's Trichrome stain allowed calculation of the collagen percentage present in CVM specimens in comparison to that found in controls.

3.4.1 Pilot study results

Demographic data of the population studied

The data consisted of 17 CVM patients and 10 control patients. The CVM population were represented by patients who underwent operations at Hammersmith Hospital between the years 1993-2003. The control patients underwent operations at Charing Cross Hospital between 2003 and 2007. The CVM group (n=17) consisted of both genders: 9 (52.9%) female and 8 (47.1%) male. The control group (n=10) were all female (100%). The mean age was 35.06 and 43.40, for the CVM and the control group respectively.

Tabel 3. 1. Descriptive statistics of age for CVM and control groups

Group	N	Median	Minimum	Maximum
CVM	17	33	3	74
Control	10	42	25	84

Mann-Whitney Test showed that there was no significant difference in age ($p = 0.20$) between the CVM and the control group. This may indicated that both groups are well matched. The CVM tissue analysed corresponded clinically and histologically to different CVM categories. This information was obtained from patient's medical records and histopathological reports. Twelve (70.6%) of the CVMs were LF malformations, 2(11.8%) were HF malformations, 2(11.8%) were lymphangiomas and one (5.8%) was glomangioma. The control tissue analysed was represented by normal skin vessels of which 4(40%) samples were obtained following breast reduction surgery, 3 following abdominoplasty (30%) and 3 following limb amputations (30%).

Table 3.2 showed there was significant difference in number of blood vessels ($p < 0.001$) between the CVM and the control groups.

Tabel 3. 2. Number of blood vessels in CVM and control groups

Variable	Group	N	Median	P-value
Number of blood vessels	CVM	17	7.60	< 0.0001**
	Control	10	4.50	

Mann-Whitney Test, ** Significant at 1% level

Table 3.3 showed that there was only significant difference ($p=0.05$) in diameter of blood vessels (field1) between the CVM group and the control group.

Tabel 3. 3. Diameters of blood vessels in CVM and control groups

Variables	Group	N	Median	P-value
Diameters of blood vessels (average of field 1)	CVM	17	65.85	0.05*
	Control	10	35.59	
Diameters of blood vessels (average of field 2)	CVM	17	72.77	0.45
	Control	10	37.86	
Diameters of blood vessels (average of field 3)	CVM	17	52.46	0.82
	Control	10	47.00	
Diameters of blood vessels (average of field 4)	CVM	17	51.22	0.86
	Control	10	57.27	
Diameters of blood vessels (average of field 5)	CVM	17	50.93	0.45
	Control	10	36.86	
Diameters of blood vessels (averages per specimen)	CVM	17	73.18	0.31
	Control	10	65.36	

Mann-Whitney Test, * Significant at 5% level

So far, the only significant results in the comparison between the CVMs and control vessels were concerning the number of blood vessels/field with magnification 40 and the diameter of blood vessels assessed at the same magnification. Spearman's rho correlation coefficient was used in the attempt to correlate these significant findings with the clinical categories of the CVM tissue analysed, as described earlier.

Tabel 3. 4. Vessel number and diameter and clinical types of CVMs - correlation

Variable	N	Correlation coefficient	P-value
Number of blood vessels	17	-1.00	0.71
Diameters of blood vessels (average of field 2)	17	0.41	0.12

Spearman's rho correlation coefficient

Spearman's rho correlation coefficient showed that there was no significant correlation between clinical categories and any of variables that showed significant difference between the CVM and the control groups. However, it is interesting to note that the highest correlation ($r = 0.41$) was between clinical categories and diameters of blood vessels (average of field 2). This finding was not statistically significant and it may be just by chance, due to the small numbers in this pilot study.

Tabel 3. 5. Collagen in CVM and control groups

Variable	Group	Median	P-value
Collagen (%)	CVM	19.82	0.47
	Control	16.07	
Collagen (Micro meters square)	CVM	850.55	0.13
	Control	408.51	

Collagen percentage showed no significant difference when CVM and control tissue were compared. This assessment considered stromal collagen found around the vessels within CVM and normal control tissue respectively.

Tabel 3. 6. S100 and EVG immune-staining score in CVM and control groups

Variable	Group	N	Median	P-value
	control	10	408.51	
S100	CVM	17	1.60	0.82
	control	10	1.70	
EVG score	CVM	17	1.00	0.09

Mann-Whitney Test, ** Significant at 1% level

Table 3.6 showed S100 and EVG score between the CVM and the control groups, demonstrating no significant difference between the two groups.

3.4.2 Results of morphological analysis of CVMs

Following the results of the pilot study, further numbers were added to increase the power of this morphological analysis. Therefore, an additional 16 prospective participants were added to the pilot study to make up a total of 33 CVM samples. As the results within the control group were uniform, the same number of 10 control samples was kept for comparison of the two groups.

Demography of the studied population is detailed in table below.

Within the CVM group we have looked at the significant findings in relation to the clinical confounding factors (i.e. type of flow – HF or LF, location of the lesion, previous interventional treatment of the lesion and presence of an ulcer in the vicinity of the lesion at the time of operation), in addition to the usual confounding factors such as age and sex. A more detailed analysis was performed on these data comparing to the basic analysis in the pilot study.

Tabel 3. 7. CVM morphological study - patient demographic and clinical data

Patients	Age/sex	Site	Clinical diagnosis	High flow/ low flow	Previous Endovascular therapy	Presence of ulcer at operation
1	51/F	torso	VM*	LF˜		
2	41/M	Lower limb	VM	LF		
3	28/F	Upper limb	VM	LF		
4	19/F	Upper limb	VM	LF		
5	49/F	Lower limb	VM	LF		
6	23/F	head	VM	LF		
7	3/M	Lower limb	LM**	LF		
8	25/M	Lower limb	LM	LF		
9	20/F	Lower limb	VM	LF		
10	31/M	Head	AVM°	HF^{+}		yes
11	43/M	Head	AVM	HF	yes	
12	40/F	Upper limb	AVM	HF	yes	
13	74/F	Head	VM	LF		yes
14	33/M	Head	VM	LF		
15	31/F	Upper limb	VM	LF		
16	36/M	Upper limb	VM	LF		
17	49/M	Lower limb	VM	LF		
18	20/M	Lower limb	VM	LF		
19	27/F	Upper limb	VM	LF		
20	26/F	Upper limb	VM	LF		
21	73/F	Upper limb	AVM	HF	yes	
22	64/M	Upper limb	AVM	HF		
23	24/F	Lower limb	AVM	HF	yes	yes
24	20/M	Lower limb	AVM	HF	yes	
25	28/F	Upper limb	AVM	HF	yes	yes
26	31/F	Lower limb	VM	LF		
27	18/M	Lower limb	VM	LF	yes	
28	32/M	head	VM	LF		
29	54/F	Lower limb	VM	LF		
30	19/F	Upper limb	VM	LF		

31	24/F	Upper limb	VM	LF	yes	
32	19/F	Lower limb	AVM	HF		yes
33	45/M	Lower limb	VM	LF		

* = venous malformation; ** = lymphatic malformation; ° = arteriovenous malformation; ˜ = LF; $^{+}$= HF

Vessel number (p=0.0004) and vessel diameters (p=0.0005) were significantly higher in the CVM group compared to controls. There was no correlation between the statistically significant morphological parameters resulted in this study, and any of the clinical parameters.

Comparison of histological findings in CVMs to control tissues

The control and test samples were matched in number although the control tissues have a higher female to male ratio; 3:1 than the CVM 3:2.

Tabel 3. 8. Demography and clinical data in CVM and control groups

Variable	Control group N=10	CVM group N=33	P-value from Mann-Whitney U test or Chi-squared test*
Age (Years)	42 [34-50]	31 [23-43]	0.064
Gender Number male (%)	0/10 (0%)	14/33 (42%)	0.012*
Number of vessels (mean of 5 fields x40/ mm^2)	3.3 [2.4-3.6]	4.8 [3.8-5.6]	0.0004
Vessel diameter (mean of 5 fields x40/ mm^2)	334 [258-419]	627 [508-734]	0.0005

Data quoted as median [interquartile range]

Tabel 3. 9. Vessel number and diameter, CVM demography and clinical data

Significant morphology parameters on comparing CVMs and controls*		Vessel Number/mm^2	Vessel Diameter µm
Demographic and clinical data CVMs			
Sex	Male female	5 4	594 643
Type of flow	High Low	6 5	628 625

Location	Lower limb Upper limb Head torso	5 4 4 3	591 657 613 475
Previous endovascular therapy	Previous therapy No previous therapy	5 5	661 624
Presence of ulcer at operation	Ulcer No ulcer	6 5	643 625

*Data quoted as median

3.5 DISCUSSION

This series of 33 'true' vascular malformations (meeting the diagnostic clinical, radiological and histological criteria), is one of the largest recorded in the world literature. Currently, there is no correlation between accepted clinical classifications and morphology of these lesions.

The aim of this part of the study was to identify morphological features characteristics to CVMs by comparing CVM tissue with similar normal control skin vessels.

Secondly, this study allowed morphological examination of a variety of CVM, which permitted more insight into the reasons as to why is still impossible to match these lesions to the recognisable clinical categories.

Since CVMs represent abnormal proliferation of blood vessels, we decided to assess the vessels present within CVMs specimens and objectively, the number of vessels present per field and the average vessel diameter, which were chosen as indicators of vessel proliferation. There are no similar studies in literature reporting figures for these chosen parameters, for morphological assessment of CVMs. The most important microscopic changes we demonstrated in this part of the study were related to the blood vessel as a whole since we have shown that CVMs contain considerably more blood vessel per field and they have a greater diameter in comparison with skin controls. It has been suggested that abnormal local concentrations of angiogenesis mediators are responsible for increased blood vessel diameter (Nakatsu, Sainson et al, 2003) but this possibility needs to be investigated in vascular malformations. Increased expression of angiogenesis markers such as VEGF, VEGFR-1, Tie-2, αVβ3 were found in this study (see results in chapter 5). The abnormal expression of these cytokines may be responsible for the increased blood vessel diameter within CVMs. Vessel size also changes following alterations in

smooth muscles proliferation – not demonstrated in CVMs - or matrix protein synthesis, which remains to be investigated.

CVMs vessels consist of three definable layers as seen in other vessels. Some of the morphological changes observed in a variety of CVMs specimens in this study appear to be located within the tunica media and adventitia. Although there were presumptions in the literature that CVMs have at the origin an abnormality of the ECs composing the abnormal vessels of CVMs, we could not demonstrate any microscopic abnormality or disruption of the endothelial lining, based on Factor VIII immunostaining. Certainly, the ECs in vessels comprising the vascular malformations in our study were sufficiently differentiated to express Factor VIII antigen and similar findings have been previously reported. These results however, do not exclude intrinsic abnormalities of the ECs composing CVM, a matter which does not represent the direct aim of this study.

Histological analysis of CVMs by comparison with controls demonstrated a deviation of the vessel morphology in CVM from what we considered normal vessel morphology in controls. Although both specimens had recognisable, demarcated vessel wall, CVMs varied considerably in respect to the presence of the circular layer of muscles in tunica media, the types of vessels (thick and thin, large and small), the amount of connective tissue forming the adventitia or interposing between the abnormal vessels. These features apart from confirming the pathological element of CVMs, also pointed out their wide variability, hence the difficulties in matching these variable but similar histological entities with a defined clinical lesion. Certainly, flow categories of CVMs cannot be distinguished by histological morphology so far. Many of the CVM specimens had the external elastic lamina disrupted, and the elastic fibres appeared to be multilayered and diffuse within the stromal tissue of the CVMs. Thrombus formation was noted within some CVM vessels. Leakage of capillaries or larger vessels was commonly observed.

Scanning all CVM specimens did not allow to confidently predict the site of the so called ‘nidus’, destruction of which would lead to a cure of CVMs. This ‘nidus’ is frequently reported by the interventional radiologists during embolization of AVMs and represents a cluster of prominent blood vessels. The ‘nidus’ is suspected to be amongst the most active parts of the specimens, featuring the common morphological elements described in relation to CVMs. However, morphology alone cannot establish exactly the location of the ‘nidus’ or whether this has been removed or not at the time of surgery.

The intralesional nerves noted in our study were also described in a study of 167 extracranial CVMs, of which 76 were vascular malformations. The nerve bundles were identified in 91% of the vascular malformations and it has been suggested as a diagnostic clue to differentiate vascular malformations from haemangiomas (Adegboyega and Qiu, 2005). The close relationship between the abnormal vascular channels and associated large nerve fibres may implicate the Ephrin family of molecules in the development of these lesions. Ephrins are known to have essential roles in both angiogenesis and neural crest cell and axonal growth guidance. Recent published work indicated expression of arterial markers ephrinB2 and EphB2 by the venous ECs of the malformed veins in a series of seven venous malformations. Whether this can be interpreted as a cause or consequence remains to be established.

CVMs are considered by some authors to represent focal persistence of primitive vascular elements but this statement remains unproven and this study found no results to support such a statement.

CVM vessels are embedded into a stromal tissue, to which they are closely related and with which they interact. This stromal tissue appears to vary from specimen to specimen in quantity, although by analysing the quantity of connective tissue present in CVMs in comparison with control tissue, there was no significant difference. Another important feature of the CVM stroma was the presence of a cell infiltrate amongst most of CVMs. The nature of this cell infiltrate is still to be determined and this represents the objective of the next chapter. Interestingly, previous studies reported that stroma of these lesions was unremarkable (Mulliken et al, 1982). This remark may be associated with the difficulties over the years in clearly defining CVMs and differentiating them from haemangiomas. The access to CVM tissue is still very limited and this explains the paucity of studies in the literature on CVM tissue. This emphasises the difficulty in interpreting the available studies in literature, since authors may not have a clear understanding of CVMs nomenclature, and therefore the lesions described may not be classified correctly.

To summarise, CVMs contained a higher number of blood vessels ($p=0.0004$) and these vessels had an average diameter higher that skin controls ($p=0.0005$). Alteration of vessel walls in CVMs was noted although, it is not possible to know whether these changes developed primarily within the vessels wall of CVMs or they are the result of the interaction between vessel wall and stromal constituents, which in our study points

out towards a stromal cell infiltrate which became the object of our next phase of the study.

This study demonstrates the necessity of: 1) finding a classification that is clinically reproducible and applicable and 2) establishing a common language for clinicians and histopathologists. It provides a basis for further work in understanding the aetio-pathology of vascular malformations. The morphological heterogeneity of the lesions studied here suggests that specific molecular patterns may exist within vascular malformations, which may result in further clinical sub-classification. Exploration of angiogenesis, hypoxia and related signalling pathways involved may further identify these patterns and may provide novel classifications, more therapeutically, and prognostically useful together with a common international terminology for all specialists dealing with these difficult lesions.

CHAPTER 4

4. HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL ANALYSIS OF INFLAMMATORY CELL INFILTRATION OF CVMs

4.1 INTRODUCTION

The morphological analysis described in Chapter 3 resulted in the observation of a stromal cell infiltrate within CVM specimens, which was clearly identified in most H&E sections of the CVMs.

Among the variety of inflammatory cells, which could represent the cell infiltrate identified, mast cells and macrophages have been the most commonly studied in literature and they are frequently present where cell infiltrates are identified. There is data in literature indicating that leukocytes can attach firmly to ECs and transmigrate into surrounding tissue (Mozzam, DeLano et al. 1997; Marschel and Schmid-Schonbein et al. 2002). It is also known that inflammatory cells can respond to flow changes. Altered blood flow triggers an inflammatory response that facilitates remodelling (Bakker et al, 2008) and vascular tone appeared crucial determinant of the direction of the remodelling response. Little is known about the blood flow within the abnormal vessels of vascular malformations.

CVMs are not considered inflammatory lesions but their natural course is complicated at times by elements involving inflammation such as ulcers and thrombosis. In addition, endovascular therapies, meaning introducing foreign bodies/substances within CVM vessels, could also introduce an element of local inflammation. In theory it is possible that these lesions may have an underlying chronic inflammatory process, which in certain conditions may become acute. However, in our series of 33 CVMs, only three CVMs had ulceration, six had endovalscular treatment and two had both ulcers and history of endovascular treatment. The abundant cell infiltrate was present in the majority of specimens and not to those described above.

Whilst there is sufficient data in literature regarding the presence of mast cells and macrophages in haemangiomas, as described below, there is only one study referring to their presence in vascular malformations (Mulliken et al, 1982), implying their absence in these lesions. This is why further clarification is required as to the nature of the cellular infiltrate we identified on analysing vascular malformations.

4.1.1 Mast cells in CVMs

Mast cells circulate as immature cells and mature once they settle in a tissue. They are considered resident cells of connective tissue and have multiple functions as described in Chapter 1.

The occurrence of mast cells in vascular tumours was observed by Dalion in 1965. Baroni in 1964 noted an increase of mast cells in haemangiomas, sclerosing haemangiomas

(dermatofibromas) and glomus tumours. Kim and Lee reported an increased number of mast cells within vascular tumours, particularly in capillary and mixed types of haemangiomas, whereas no significant increase was found in cavernous haemangiomas. Glowacki et al (1982) found abundant mast cells in the proliferating phase of haemangiomas, but very few in the involuting phase of haemangiomas as well as in vascular malformations and skin. They speculated that haemangiomas may arise or be maintained by very large concentrations of mast cells, but when stimuli from the mast cells disappear, the ECs and vascular channels may involute. The abundance of mast cells in the proliferating phase of haemangiomas suggests that they may play a role in the natural history of these lesions. Pasyk et al (1984) noticed the increase in the number of mast cells counts was associated with the formation of fibrous connective tissue, which appeared gradually within the stroma of these vascular tumours. The number of mast cells was proportional to the amount of fibrous tissue. In patients with intermediate or complete involution of strawberry haemangiomas, where haemangioma lesions in the majority of cases have been replaced by connective tissue, the number of mast cells was considerably decreased. The mast cell counts in lesions known to never spontaneously regress (port wine stains and AVMs) remained unchanged or were slightly increased in comparison with the number of mast cells in normal skin. Pasyk et al found a strikingly low number of mast cells in cellular haemangiomas that showed active growth as well as deficiency of the fibrous connective tissue inside the stroma of these vascular tumours. Therefore, they postulated that the concentration of mast cells in growing haemangiomas was connected with the gradual growth of fibrous connective tissue inside the tumour. Asboe-Hansen reported that mast cells occur during new connective tissue formation in pathological states. Since the lowest number of mast cells was found in cellular haemangiomas in which the ECs showed the largest proliferation, Pasyk (1983) suggested that mast cells are not responsible for the proliferation of the endothelium of blood vessels and that they are not the direct cause of the development of haemangiomas. This impression was further emphasized by the coincident appearance of increased mast cell counts and of fibrous connective tissue in growing stages of haemangiomas and the accumulation of mast cells in growing stages of haemangiomas prior to clinical regression.

Woodrow et al (1997) described the entity of ‘enlarging congenital haemangioma’ in an adult with histological evidence of numerous mast cells suggesting an aetiological role of these cells in pathogenesis of haemangiomas.

Enjorlas et al (2001) proposed the term ‘non-involuting congenital haemangiomas’, which may be a variant of common haemangioma of infancy or another hemangiomatous entity characterized by persistent fast-flow and associated with increased mast cells. These lesions

tested negative for GLUT-1, a marker for common haemangioma of infancy (North et al, 2000). Authors explained that this lesion does not carry a risk of excessive operative bleeding and recurrence and must be differentiated from vascular malformations because both exhibit fast-flow. However, to date there is no histological criteria towards this differentiation or sufficient data to prove the two lesions are different.

The roles of mast cells which are present in varying number in all stages of haemangiomas have not yet been defined but clearly they appear to be implicated in the patho-physiology of these lesions. Mast cells have been indicated as a source of various inflammatory cytokines, chemokines and growth factors. Certain effectors produced by mast cells may participate in the development of haemangioma. Mast cells may secrete both pro-angiogenic and anti-angiogenic mediators, some of them already quantified in haemangiomas and considered to be implicated in their development (see 'angiogenesis and CVMs'). The roles of mast cells in the life cycle of haemangioma are likely to be complex and may involve stimulators of angiogenesis in the proliferative phase and inhibitors in later phases (Tan et al, 2004). Histamine which is a mast cell product may stimulate angiogenesis through its direct effect on EC proliferation by acting on the histamine-1 receptors.

4.1.2 Macrophages in CVMs

Macrophages are monocytes differentiated on migration into tissue and they share a number of cell markers and features. Their life time varies between 6 and 16 days and they are two types: resident (normal) and inflammatory (activated).

Isik et al (1996) found macrophages to infiltrate haemangiomas during the proliferative phase but they were not found in vascular malformations. Macrophages have the potential to display, at different times, both inflammatory and anti-inflammatory activities. They have diverse functions, including phagocytosis, antigen presentation, antimicrobial cytotoxicity, and tissue remodelling as well as secretion of a wide range of growth factors, cytokines, complement components, prostaglandins and enzymes (Burke et al, 2002). They have the potential to initiate angiogenesis by release of proteases, growth factors (basic FGF, GM-CSF, TGF-alpha, IGF-I, PDGF, VEGF, TGF-beta), and other cytokines (IL-1, IL-6, IL-8, TNF-alpha, substance P, prostaglandins, interferons, thrombospodin 1). Activated macrophages have the capability to influence each phase of the angiogenic process, such as alteration of the local extra-cellular matrix, induction of ECs to migrate or proliferate, and inhibition of vascular growth with formation of differentiated capillaries (Boye et al, 2001). Monocytes recruitment to various tissues has been shown to be controlled by various chemokines including monocyte chemotactic protein (MCP-1) (Sunderkotter et al, 1994) which Isik et al (1996) found to be significantly raised during haemangima growth. However, no explanation as to the role of macrophages

infiltrates in proliferating haemangiomas has been given to date.

In summary, to date there is no knowledge about the composition or significance of the cell infiltrates in the stroma of CVM. This information could provide more insight into the pathophysiology of these lesions.

4.2 OBJECTIVES

Previous morphological analysis of CVMs demonstrated evidence of a cellular infiltrate within the CVM stromal tissue. This study aimed to identify the nature of infiltrate by investigating the constituent cells using markers for mast cells, macrophages, B lymphocytes, T lymphocytes, and a marker for cell proliferation. The cell infiltrate within CVMs was compared with that in control skin tissue.

4.3 MATERIALS AND METHODS

4.3.1 Selection of patients and tissue collection

CVM specimens were obtained retrospectively from archive and prospectively as participants were subjected to surgery for their CVMs. Control specimens were all obtained prospectively. Details of patient selection criteria, consent forms and tissue collection are given in Chapter 2.

All CVM lesions analysed in this section were negative for GLUT-1, therefore supporting the clinical observation that they were vascular malformations, not haemangiomas.

4.3.2 Assessment of cell infiltrate in CVMs

Mast cells were assessed histochemically using Toluidine blue dye as described in Chapter 2. Paraffin sections of CVMs and controls were stained with Haematoxylin and Eosin staining (H&E) and Toluidine blue. Toluidine blue identified mast cells by demonstrating the metachromatic substances found in their cytoplasm. CD68, CD3, CD20 and Ki67 were assessed in addition, using the ABC technique for immunostaining of tissue, described in detail in Chapter 2 and Appendix. Primary antibodies were applied to the sections for 1 hour at room temperature, at the following concentrations: CD68 (M0876-Dako) (1:200), CD3 (VP-C316-Vector Laboratories) (1:200), CD20 (M0755-Dako) (1:600), ki67 (VP-K542-Vector Laboratories) (1:200). For visualisation, the peroxidase reaction was developed using diaminobenzidine (Vector Laboratories).

4.3.3 Measurement of mast cell and macrophage count on a field (1mm^2) of CVM

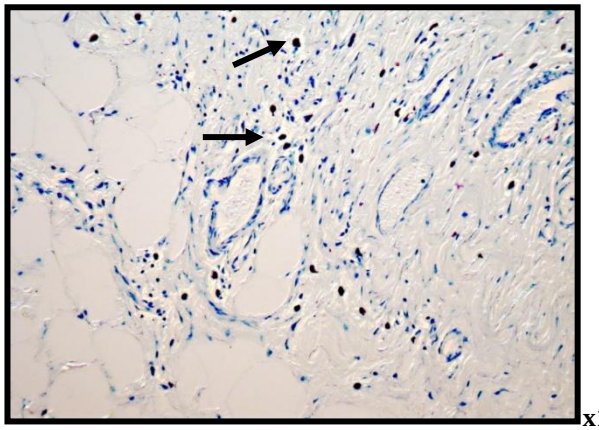
Once the tissue was stained for all markers, the numbers of cells was counted using AnalySIS software and expressed as cells per field, technique described in Chapter 2.

In addition to this technique, for the preliminary study detailed below, based on 17 CVM specimens obtained retrospectively, light microscopy method was used to count macrophages and mast cells by 2 observers. A 1mm^2 microscopic grid under light microscopy

was used to count the cells on high power, over 5 different fields as described in the image analysis technique. This second technique was employed to assess the quality of data collected by analysing the inter-observer agreement.

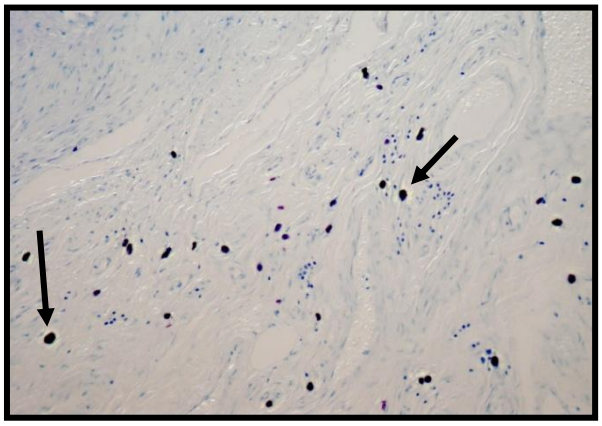
The selected variables (cell infiltrates), histological and immunohistochemical were analysed within the CVMs group and compared with their values in the control group. Within the CVM group, the significant findings in relation to the clinical confounding factors (i.e. type of flow, location of the lesion, previous interventional treatment of the lesion and presence of an ulcer in the vicinity of the lesion at the time of operation) were considered. Preliminary results were obtained as a result of analysing data obtained from the 17 CVM samples found in the archive, which were compared with the data obtained from control tissues. This study was performed concurrently using consecutive sections of tissue from blocks used for the pilot morphological analysis described in Chapter 3. As more prospective CVM samples became available, a database of 33 CVM specimens was consolidated. Below, the preliminary results are given in the first instance, followed by the results of the more comprehensive study based on the 33 CVMs.

4.4 RESULTS



x100

Figure 4. 1. Toluidine Blue stain showing mast cells in a HF AVM



x200

Figure 4. 2. Toluidine Blue stain showing mast cells in a LF AVM

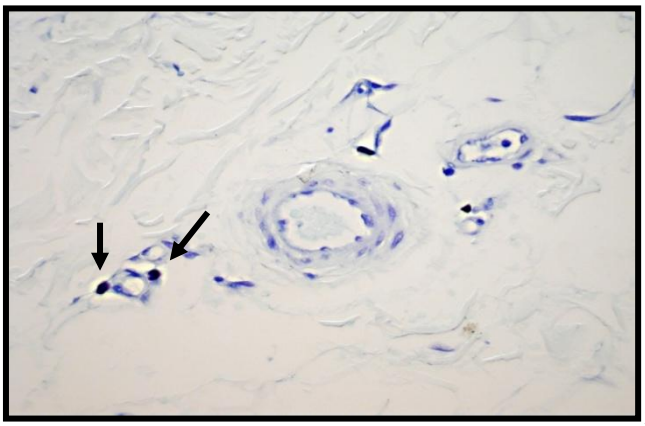


Figure 4. 3. Toluidine Blue stain showing mast cells in a skin control

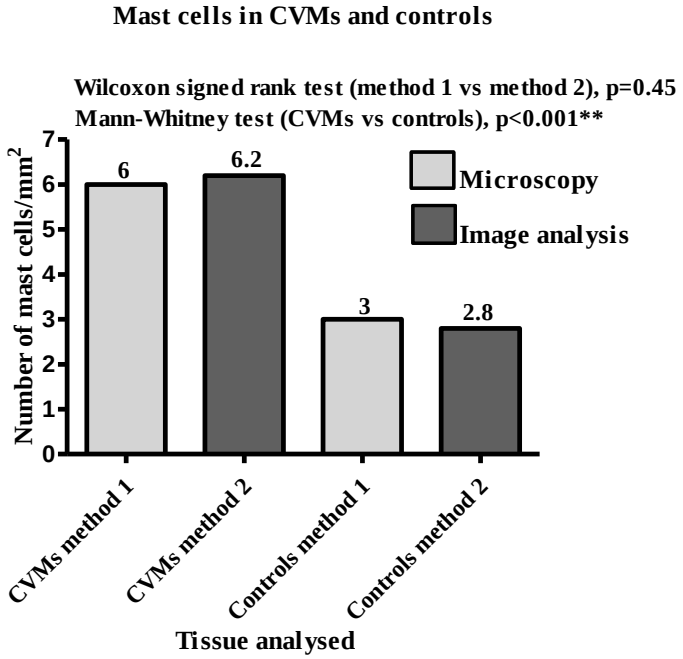
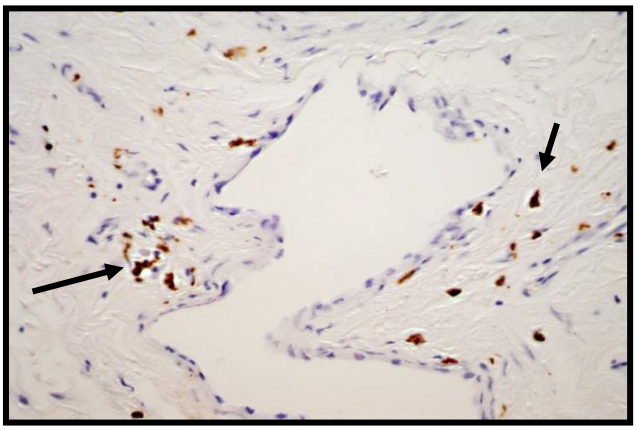


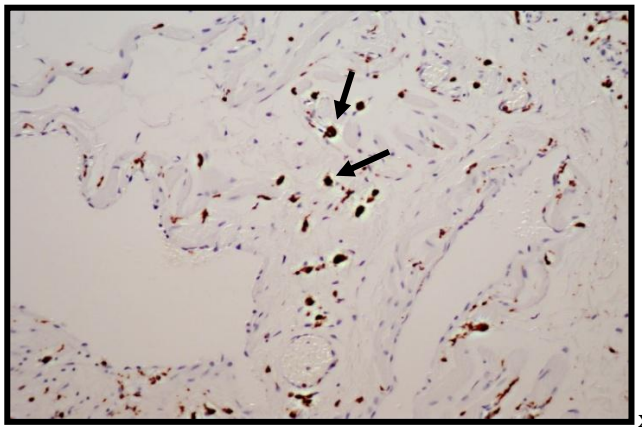
Figure 4. 4. Mast cells in CVMs and controls

The figure above shows there was a higher number of mast cells per microscopic field in CVMs comparing to skin controls, by both microscopy and image analysis as methods of quantification.



x200

Figure 4. 5. Immuno-staining expressed in stromal histiocytes (brown) in a HF AVM



x200

Figure 4. 6. Positive expression of CD68 in a LF AVM

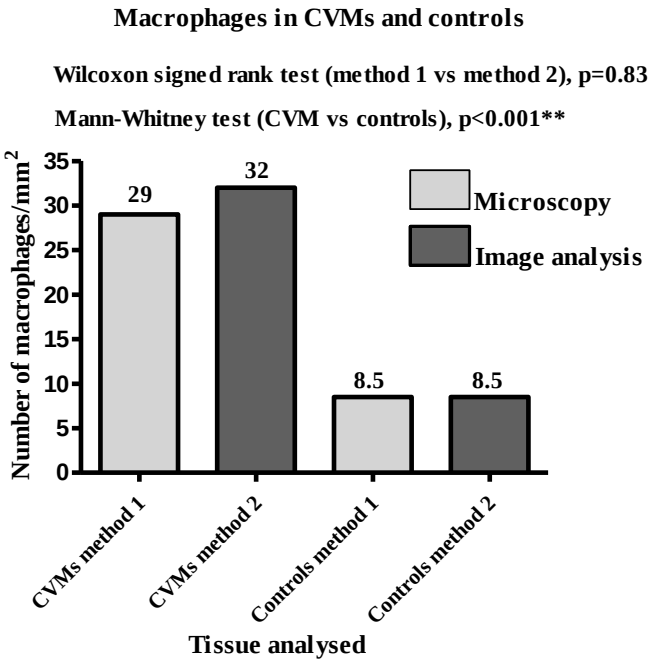


Figure 4. 7. Macrophages in CVMs and controls

The figure above shows there was a higher number of macrophages per microscopic field in CVMs comparing to skin controls, by both microscopy and image analysis as methods of quantification.

Tabel 4. 1. Macrophages and mast cells in CVM and control groups

Variables	Group	Count	Median	P-value
Macrophages CVM (microscopy)	CVM	17	29.2	< 0.001**
	Control	10	8.5	
Macrophages CVM (image analysis))	CVM	17	32.4	< 0.001**
	Control	10	8.5	
Mast cells (microscopy)	CVM	17	6	< 0.001**
	control	10	3	
Mast cells (image analysis)	CVM	17	6.2	< 0.001**
	Control	10	2.8	

Mann-Whitney Test, ** Significant at 1% level

The table 4.1 shows highly significant difference in macrophages ($p < .001$) and mast cells ($p < 0.001$) between the CVM and the control groups.

Tabel 4. 2. Macrophages and mast cells in CVMs by microscopy and IA

Variables	Median	P-value
Macrophages CVM (microscopy)	29.20	0.83
Macrophages CVM (image analysis))	32.40	
Mast cells (microscopy)	5.60	0.45
Mast cells (image analysis)	6.20	

Wilcoxon Signed Ranks Test

Wilcoxson test demonstrated no significant difference in CVM macrophages ($p=0.83 > 0.05$) between the microscopy and the image analysis method. No significant difference in mast cells ($p=0.45 > 0.05$) between the microscopy and the image analysis method was also proven.

Tabel 4. 3. CD3, CD20, ki67 in CVM and control groups

Variable	Group	Median	P-value
CD3	CVM	4.00	0.57
	Control	6.90	
CD20	CVM	5.40	< 0.0001**
	Control	.20	
KI67	CVM	.60	0.41
	Control	1.30	

Mann-Whitney Test, ** Significant at 1% level

Tabel 4. 4. Correlations between significant variables and HF-LF types of CVMs

Variable	Correlation coefficient	P-value
Macrophages (microscopy)	-0.14	0.58
Macrophages (image analysis)	0.04	0.88
Mast cells (microscopy)	-0.06	0.82
Mast cells (image analysis)	-0.06	0.81
CD20	0.05	0.86

Spearman's rho correlation coefficient

There was no significant correlation between clinical categories and any of variables that showed significant difference between the CVM group and the control group. Highest correlation (r = 0.41) was between clinical categories and diameters of blood vessels.

Tabel 4. 5. Results of comparison between CVM and control groups

Variable	Control group N=10	CVM group N=33	P-value from Mann-Whitney U test or Chi-squared test*
Age (Years)	42 [34-50]	31 [23-43]	0.064
Gender Number male (%)	0/10 (0%)	14/33 (42%)	0.012*
Macrophages (Number/mm^2)	8.5 [7.4-9.4]	26.2 [18.8-32.8]	<0.0001
Mast cells (Number/mm^2)	2.8 [2.0-3.4]	5.4 [4.4-7.0]	<0.0001
B-cells	0.2	2.2	0.003

(Number/mm^2)	[0-0.6]	[0.4-6.6]	
T-cells # (Number/mm^2)	6.9 [4.2-7.6]	4.0 [1.4-12.2]	0.547
Proliferating cells (Number/mm^2)	1.3 [1.0-1.6]	0.4 [0-1.4]	0.057

Data quoted as median [interquartile range]

T-cell calculation based upon N=17 in CVM group and N=10 in control group.

Tabel 4. 6. Macrophages, mast cells, demography and clinical data in CVMs

Significant parameters on CVMs and controls* comparison		Macrophages number/mm^2	Mast cells number/mm^2
CVM demographic and clinical variables			
Sex	Male	26	6
	female	26	5
Type of flow	High	27	27
	Low	25	5
Location	Lower limb	23	5
	Upper limb	24	5
	Head	34	8
	torso	23	4
Previous endovascular therapy	Previous therapy	20	5
	No previous therapy	26	5
Presence of ulcer at operation	Ulcer	27	5
	No ulcer	25	5

*Data quoted as median

4.5 DISCUSSION

CVMs are not considered to be inflammatory lesions and therefore, there is no research data available in this respect. Previous morphological study indicated the presence of an inflammatory infiltrate within the stroma of these lesions and this study aimed to to clarify the nature of the cellular infiltrate.

In this study the nature of the stromal cell infiltrate within CVMs was investigated using a panel of histochemical and immunohistochemical markers such as Toluidine blue for mast cells,

CD68 for macrophages, CD3 for T lymphocytes, CD20 for B lymphocytes, and Ki67 for proliferating cells. For the pilot study, two methods were used to count the mast cells and macrophages, light microscopy and image analysis, to determine the most precise and accurate method. There was no difference between the two methods as they rendered similar results (Wilcoxon Signed Ranks, $p = 0.45$ for mast cells respectively $p = 0.83$ for macrophages). Mann-Whitney test showed a significant increased infiltrate of mast cells and macrophages in CVM stroma when compared to control tissue with a $p<0.001$ for both mast cells and macrophages. There were no significant increase in numbers of T lymphocytes or proliferative cells in CVMs when compared to controls by Mann-Whitney test, $p = 0.57$, respectively 0.41). Although results indicated there was a significant infiltrate of B lymphocytes within CVM stroma, it appears that the data is skewed by a few CVM specimens involving lymph nodes, and therefore containing more B lymphocytes than the rest of the samples.

The majority of mast cells and macrophages were found to be clustered in close vicinity of the abnormal CVM channels or even within the vessel adventitia. This raises the possibility that these cells are not resident in the stromal tissue of the lesions and that they migrated from the vessel lumen into the stromal tissue. What attracted these cells to the stromal CVM tissue, what triggered and favoured their migration from the vessel vessel lumen through the endothelial lining and what actually is the role they fulfil within the CVM stroma, are all questions for which there are no answers available at present.

The finding of mast cell and macrophage infiltrates in CVMs has not been reported previously. On the contrary, there was a report suggesting that the number of these cells was not raised in vascular malformations and the stroma of these lesions was unremarkable (Mulliken et al. 1982).

Macrophages and mast cells have been shown to have important roles in angiogenesis and response to hypoxia. Their presence within CVMs is not accidental and suggests an aetiological or pathogenic role. This infiltrate, when active, could also act as a promoter for progression of the CVMs. Mast cell chemotaxis is stimulated by angiogenic factors like VEGF and bFGF (Gruber, Marchese et al. 1995). Given the accepted role of mast cells in angiogenesis, scientists looked at the parallel development of blood vessels and mast cells in the lateral geniculate nuclei and concluded that both exhibited similar developmental time course (Michaloudi, Grivas et al, 2003). Human mast cells are a recognised source of cytokines as detailed in Chapter 1. The importance and role of mast cell-derived cytokines in diseases is uncertain, but it is understandable that they might play an important role in both physiologic and pathologic conditions. The function of mast cells in tissues is still generally unknown, but most likely involves both homeostatic controls of nerves and blood vessels, as well as host immunity.

Mast cell mediators also are known to affect ECs by inducing vasodilatation and are capable of initiating the recruitment of inflammatory cells and possibly the recruitment of macrophages in this case.

The finding of a significant mast cells and macrophages infiltrate does not help with the classification of these lesions or help to improve communication between histopathologists and clinicians. There was no correlation between number of mast cells or macrophages found in CVMs with the clinical type of malformation, i.e. HF or LF, using Spearman's rho correlation coefficient.

In summary, there was a significant increase in mast cell and macrophages (CD68) cell infiltration in association with CVMs compared to control skin vessels. This finding could account for the morphological vascular changes observed in CVMs, described in previous chapter. On the other hand, the accepted role of these cells in angiogenesis raises the possibility that their presence may be the result of altered angiogenesis markers in CVMs or, the abnormal expression of such markers. As the following chapter describes, there appear to be increased expression of VEGF, VEGF-R1, Tie2 and αVβ3 within CVMs. Whether there is a direct link between the expression of these markers and the presence of an inflammatory infiltrate, it is not known and further research is required. However, we performed a correlation analysis between the cytokines found to be over-expressed in CVMs and mast cells and macrophages infiltration. The results of these analyses are detailed in Chapter 5.

To gain more insight into these mechanisms it is necessary to explore the complex signalling pathways associated with angiogenesis and hypoxia. This may identify patterns of disease and may provide novel useful classifications, which may act as therapeutic and prognostic indicators.

CHAPTER 5

5. ANGIOGENESIS MARKERS EXPRESSION IN VASCULAR MALFORMATIONS

5.1 INTRODUCTION

CVMs are composed of abnormal vessels and are associated with an inflammatory cell infiltrate represented by mast cells and macrophages within the stroma, cells which have a recognised role in angiogenesis. In addition, the morphological findings in CVMs such as increased number of vessels per field, increased vessel diameter, along with changes in vessel wall, may all be related to abnormal angiogenesis which may be implicated in the aetio-pathogenesis of these lesions.

5.1.1 VEGF in CVMs

Very little is known about factors which regulate VEGFR expression in vascular ECs. High levels of VEGF have been associated with several disorders, including CVMs. Elevated levels of VEGF have been found in the proliferative phase of haemangiomas (Mulliken et al, 2000). Takahashi et al (1994) stated that vascular malformations do not express VEGF. There is no research data quoting VEGF levels in peripheral vascular malformations but there is some data in relation to cerebral AVMs. The recurrent childhood cerebral AVMs have increased EC expression of VEGF, whilst non-recurring lesions have a low expression of VEGF (Sonstein et al, 1996). There is increased expression of VEGF mRNA in the parenchyma adjacent to brain AVMs with increased levels of VEGF protein in the AVM endothelium, compared to those in normal brain tissue and vessels (Hatva et al, 1996). Cutaneous and leptomeningeal vascular malformations were also reported to have increased expression of VEGF A, VEGFR-1 and VEGFR-2 (Comati et al, 2007). These data may not be applicable to peripheral CVMs for which no similar data exist. The expression of angiogenic cytokines such as VEGF in the vicinity of CVM could explain their tendency to recur when abnormal vessels are left behind after embolisation or debulking of the lesion.

5.1.2 Tie-2/angiopoietins in CVMs

Vikkula et al, 1996 reported venous malformations occurring in two families where a mis-sense mutation in the Tie-2 receptor resulted in an arginine to tryptophan substitution. They indicated that patients carrying this mutation develop vein-like structures that are deficient in non-ECs, mainly SMCs. Therefore, these malformations are comprised of vein-like lumina lined by a monolayer of ECs, with reduction in the smooth muscle layers, compared to normal vessels with similar sized lumina. There is a proportional increase in the number of smooth muscle cell layers as the diameter of the normal vessels increases but, venous malformations exhibit wide variation in luminal size without a complementary increase in smooth muscle layers with increasing vessel diameter. The abnormally large lumina in venous malformations

suggest that there must have been EC proliferation. Vikkula et al, 1998 stated that it would be interesting to determine whether sporadic venous malformations are caused by the same Tie-2 mutation, as in the familial cases or whether other Tie-2 mutations can have the same functional effects. Folkman et al, 1996 suspected that the increased EC proliferation in the venous malformation is secondary to absence of SMCs. Vikkula speculated that the Tie-2 receptor-ligand system is coupled with chemotaxis and proliferation of mesenchymal cells and with their differentiation into smooth muscle cells. Mutations result in 'uncoupling between proliferation and differentiation of ECs and SMCs' and to a 'disproportionate number of ECs and SMCs in VMs'. Tie-2 was reported to have an increased immunohistochemical expression in the blood vessels of cerebro-vascular malformations (Comati et al, 2007).

5.1.3 Integrins αVβ3 and αVβ5 in CVMs

αVβ3 is reported to be expressed on blood vessels in human wound granulation tissue but not in normal skin (Brooks, Clark et al, 1994). αVβ3 is prominently expressed on the surface of ECs and platelets (Shattil, 1995). Expression of αVβ3 is known to be up-regulated on ECs involved in the new blood vessel formation such as quiescent ECs lining blood vessels express minimal amount of αVβ3, whilst angiogenic vessels show increased expression. Complex formation of integrins and MMPs is crucial for wound healing and angiogenesis. In ECs αVβ3 induces the production of MMP-2 and subsequently interacts with the synthesized MMP-2 to stimulate vascular invasion (Brakebusch, Bouvard et al, 2002).

EC interactions with the surrounding ECM are mediated by the integrin family of adhesion receptors as detailed in the Introduction chapter. ECs have been shown to express a variety of integrins, including αVβ3 and αVβ5.

Whilst αVβ3 is frequently reported to be critically important in angiogenesis (Nisato, Tille et al, 2003), there is surprising data in the literature reporting normal vascular development in absence of all αV integrins (Bader, Rayburn et al, 1998). This raises the question whether different angiogenic processes have differential dependency on integrins. Tumour angiogenesis could be different from developmental angiogenesis and CVM angiogenesis. In conditions where angiogenesis process is dependent on integrins, these mediators may be a useful therapeutic target for those conditions. αVβ3 antagonists downregulate αVβ3 expression and inhibit ECs and vascular proliferation (Singh, Fu et al, 2000). A lot of integrin data in literature is derived from studies on tumour angiogenesis. Studies on rat lung implicated the αVβ3 integrin in the inflammatory responses by increasing the capillary permeability (Singh, Fu et al, 2000).

There are no studies in the literature assessing any of the integrins in CVMs. However, there is data reporting αVβ3 expression (Lim, Guccione et al, 2005) or both αVβ3 and αVβ5

expression (Askn, Ozlem et al, 2006) in cerebral vascular malformations, suggesting these molecules contribution to cerebral AVM pathology and indicating αVβ3 as a potential target for treating CVMs.

Thus αVβ3 together with αVβ5 appear to be important mediators to be investigated in CVMs, given the abnormal angiogenesis theories surrounding CVMs pathogenesis. In addition an understanding of integrin activation mechanisms is crucial to the understanding regulation of cell adhesion in CVMs.

5.1.4 Endothelins in CVMs

ET-1 is one of the most potent vasoconstrictor peptides, produced and released by ECs. It has many roles in the homeostatic mechanisms in different organs as modulator of vascular tone, tissue differentiation and development, cell proliferation and hormone production (Bagnato et al, 2004).

The role of ET-1 in pathological states is unclear. Experiments have shown animals with ET-1 mutations develop cardiovascular mutations (Kurihara et al, 1995). Multiple sources data suggests that ET plays a crucial role in cardiovascular diseases such as chronic heart failure, ischaemic heart disease, hypertension, atherosclerosis, pulmonary hypertension among others. In these diseases the circulating level of ET-1 is usually high. Data also suggests that ET-1 participates in the growth and progression of a variety of tumours. Chronic hypoxia has been shown to be an important stimulus for ET-1 release.

5.2 OBJECTIVES

As discussed, extensive data in the literature has suggested that CVM may be the result of disregulated angiogenesis. In order to support this statement it is necessary to demonstrate what chemical mediators are participating in CVM angiogenesis. A whole array of mediators plays a crucial role in the process of angiogenesis. It was impossible to analyse all of them, within the scope of this project, so a few markers for angiogenesis mediators were selected to be assessed in this work. This selection was based on an extensive literature review with respect to vasculogenesis, angiogenesis and the known chemical mediators involved in these processes. In addition, the morphological analysis detailed in chapter 3 demonstrated a potential role of an abnormal stromal cellular infiltrate found in CVMs. That may alter the expression of various chemical mediators with angiogenic roles. VEGF A, VEGFR-1, Tie-2, αVβ3, αVβ5, and ET-1 were chosen to be analysed in this project as each of them in part were considered to play key roles in the blood vessel development and, represented different pathways. The purpose of this component of my research work was to determine if the presence of abnormal stromal cellular constituents altered immunohistochemical expression of the following pro-angiogenic markers in CVMs: VEGF A, VEGFR-1, Tie-2, αVβ3, αVβ5, and ET-1.

5.3 MATERIAL AND METHODS

5.3.1 Selection of patients and tissue collection

CVM specimens were obtained retrospectively from archive and prospectively as participants were subjected to surgery for their CVMs. Control specimens were all obtained prospectively. This tissue was selected based on the same criteria as discussed in Chaper 2 and originated in the same blocks of tissue as those used for the studies detailed in Chapters 3 and 4. All CVM lesions analysed in this section were negative for GLUT-1, which supported the diagnosis of vascular malformations, rather than haemangiomas.

The following table gives the number of CVM and control specimens used in this component of research for each proposed immunostain.

Tabel 5. 1. Immunostaining for angiogenesis markers and number of specimens analysed

Antibody / Tissue	VEGF A	VEGFR1	Tie2	αVβ3	αVβ5	ET-1
CVM	29	11	11	11	11	10
Control	10	7	7	7	7	x

ET-1 immunohistological stains were also performed on additional 3 haemangioma specimens selected randomly from the Tissue Bank. Two of these were capillary haemangiomas and one was cavernous haemangioma. There were no additional demographic or clinical data known for these patients.

5.3.2 Immunohistochemical analysis

The immunostaining with VEGFR-1, Tie-2, αVβ3, αVβ5 antibodies required frozen tissue technique and this explains why these stains were fewer in comparison to VEGF A immunostain, which could be performed on paraffin embedded tissue. Frozen tissue was available only those prospective cases where sufficient tissue was available. The immunostaining for VEGF A and ET-1 were performed on fixed paraffin embedded tissue.

The ABC peroxidase technique and immunostaining protocol used here has been described in Chapter 2.

CVM tissue immunostained for VEGF A, VEGFR-1, Tie-2, αVβ3, αVβ5, and ET-1 was analysed by 2 observers for the distribution of expression of all markers expression using image analysis and light microscopy. The immunopositive slides were quantified using a simple scoring system from 0 to 3 as described in Chapter 2. On interpreting and reporting the data, the mean score of the two observers was considered. The staining was assessed as to whether this

was diffuse or focal, confined to ECs or within the vessel wall. The data obtained was interpreted for agreement using the kappa coefficient analysis.

The results of the analysis of immunostaining of CVM tissue for each antibody considered was compared to the results from control tissue using the Mann-Whitney test for un-paired non-parametrical data as this could not be assumed to have a Gaussian distribution. Correlations were made between the expression of each mediator in CVMs and the significant stromal constituents represented by macrophages and mast cells using Spearman's coefficient for non-parametrically distributed data.

5.4 RESULTS

Tabel 5. 2. Demographic data for angiogenesis markers in CVMs study

Antibody	Control	CVM	Control	CVM
	Age (Years) Mean±SD		Gender Number male (%)	
VEGF A	43±17	30±17	0(0%)	13(45%)
VEGFR-1	42±20	39±19	0(0%)	3(27%)
Tie-2	42±20	39±19	0(0%)	3(27%)
αVβ3	42±20	39±19	0(0%)	3(27%)
αVβ5	42±20	39±19	0(0%)	3(27%)
ET-1	x	32±17	x	4(40%)

Tabel 5. 3. Angiogenesis markers in CVMs and relationship to clinical features

Antibody	Flow type LF:HF	CVM location	Number (%) Embolised CVMs
VEGF A	3.8:1	Upper limb 11 (38%) Lower limb 11 (38%) Abdomen 1(3%) Head and neck 6 (21%)	4 (14%)
VEGFR-1 Tie-2 αVβ3 αVβ5	1.2:1 1.2:1 1.2:1 1.2:1	Upper limb 4 (36%) Lower limb 6 (55%) Abdomen 1 (9%)	4 (36%)

ET-1	9:1	Upper limb 4 (40%) Lower limb 3(30%) Head and neck 3(30%)	3 (30%)

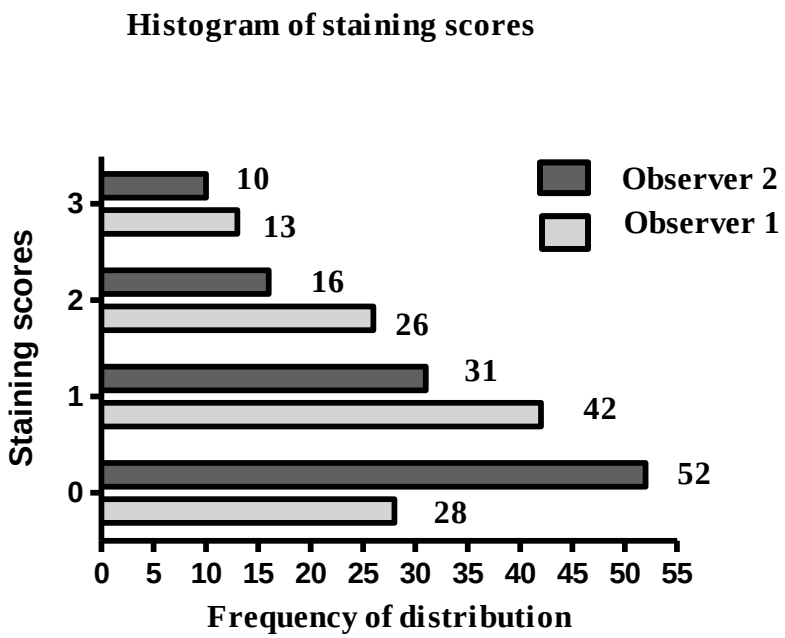


Figure 5. 1. Staining score for angiogenesis markers in CVMs

The figure above shows the scoring distribution (from 0 to 3) for all angiogenesis markers analysed by the two independent observers.

Inter-observer agreement was acceptable (kappa=0.33). For perfect agreement kappa=1 and for no agreement better than chance, kappa=0. The p-value of <0.0001 indicates that this agreement is not occurring by chance.

Tabel 5. 4. Kappa coefficient data analysis

		Observer 2				Total
		0	1	2	3	
Observer 1	0	24	4	0	0	28
	1	24	15	2	1	42
	2	4	8	11	3	26
	3	0	4	3	6	13
Total		52	31	16	10	109

Tabel 5. 5. Results of kappa coefficient analysis

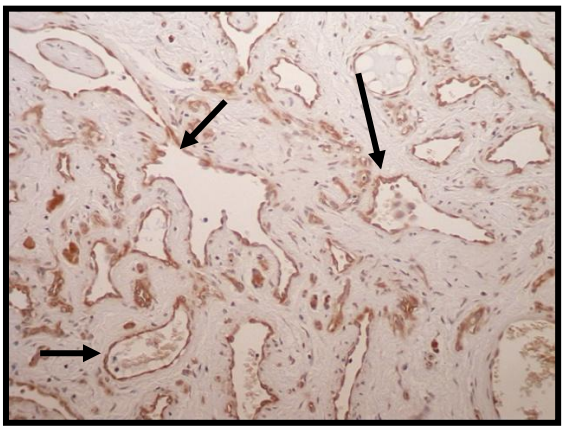
Agreement	Expected Agreement	Kappa	Std. Err.	Z	Prob>Z
51.38%	27.81%	0.3265	0.0557	5.86	0.0000

5.4.1 VEGF A/VEGFR-1

5.4.1.1 Immunohistochemical localisation of VEGF A/VEGFR-1

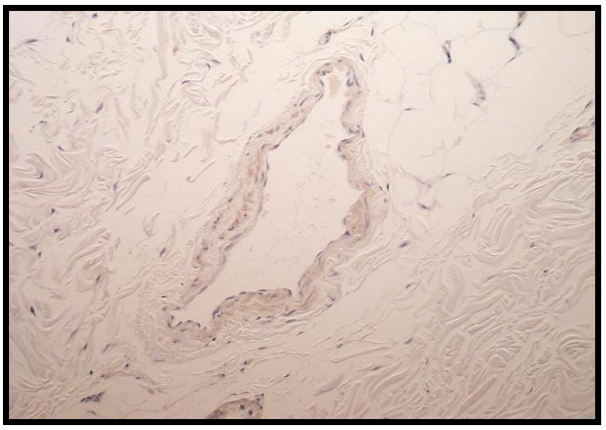
CVM specimens and control tissue were immunostained for VEGF A and VEGFR-1 using the ABC technique described previously. The stained slides were analysed using light microscopy and image analysis software. Both VEGF A and VEGFR-1 were localised to EC of abnormal blood vessels comprising the CVM. VEGF A was expressed in all categories of CVMs. VEGFR-1 was present in most CVMs and was highly expressed by the ECs of the clinical category of CVMs known as HF, which are a more aggressive entity.

5.4.1.2 Image analysis of of VEGF A/VGFR-1 immunostained sections of CVMs



x100

Figure 5. 2. Positive VEGF A Immuno-staining in a CVM



x40

Figure 5. 3. Negative VEGF A Immuno-staining in a skin control

Fourteen % of cases showed moderate expression of VEGF-A (score 2). Twenty one % of CVMs showed focal expression of VEGF-A (scored 1) and 66% were negative for VEGF-A. VEGF-R1 was highly expressed by the ECs in some CVMs (18%). Eighteen % of lesions scored 2, 45% scored 1 and 18% were negative. Tie-2, which controls vascular remodelling, was moderately expressed by the ECs in all CVMs. All lesions expressed Tie-2. Eighteen % of lesions scored 3, 64% scored 2 and 18% scored 1. From the adhesion molecules assessed, αVβ3 was highly expressed by ECs in CVMs. Eighteen % of lesions scored 3, 18% scored 2, 45% scored 1 and 18% were negative for αVβ3. αVβ5 which mediates angiogenesis induced by VEGF A was also expressed. Eighteen % of cases scored 3, 27% scored 2, 36% scored 1 and 18% were negative for αVβ5.

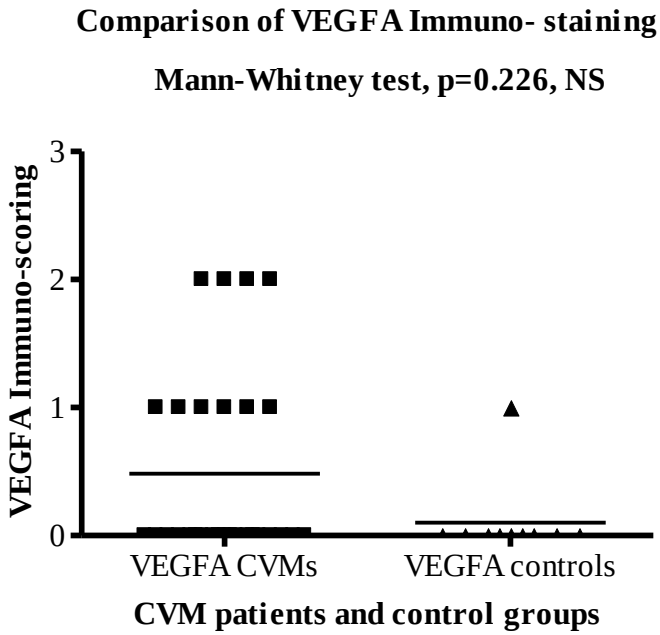


Figure 5. 4. Comparison of VEGF A immuno-staining in CVM and control groups

The figure 5.4 shows that there was no statistically significant difference between the immunohistochemical expression of VEGF A in CVMs and controls (p=0.226).

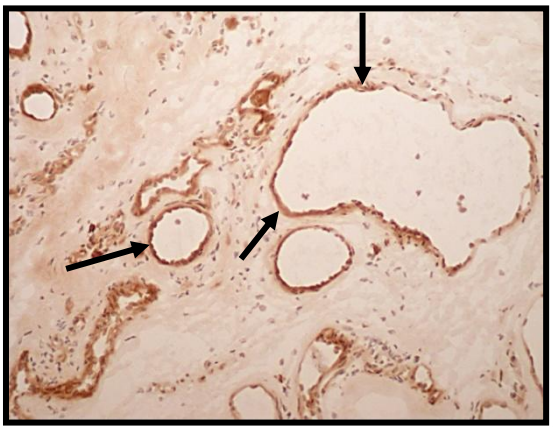


Figure 5. 5. Positive VEGFR-1 Immuno-staining in a CVM

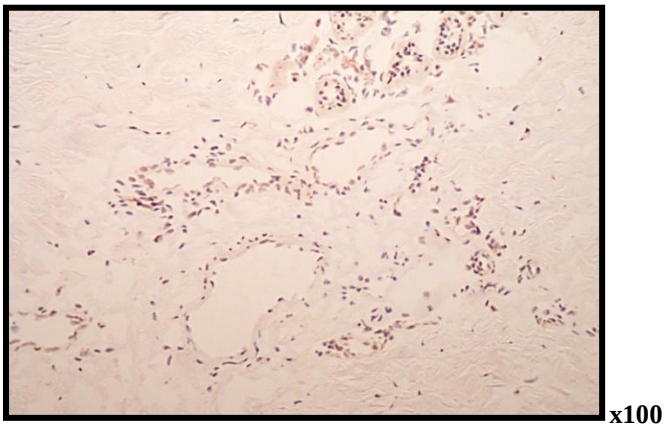


Figure 5. 6. Negative VEGFR-1 immuno-staining in a skin control

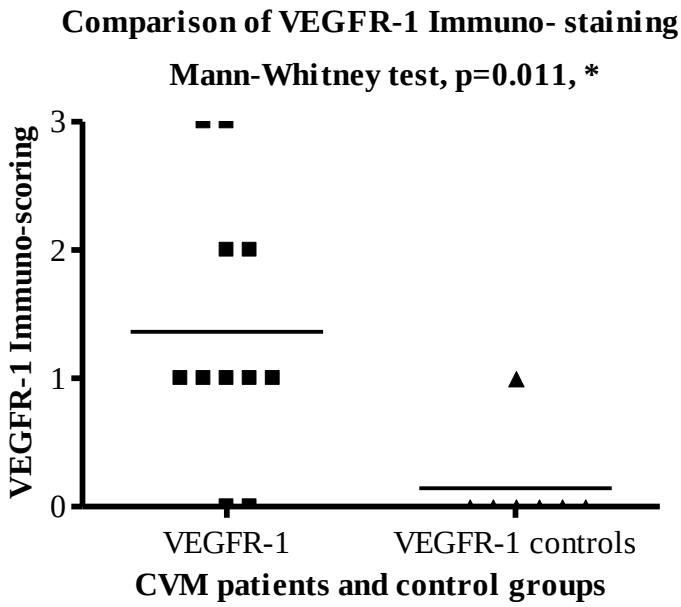


Figure 5. 7. Comparison of VEGFR-1 immuno-staining in CVM and control groups

The figure 5.7 shows that there was statistically significant difference between the immunohistochemical expression of VEGFR-1 in CVMs and controls (p=0.011).

5.4.1.3 Correlation of VEGF A/VGFR-1 expression with the significant morphological stromal changes in CVMs

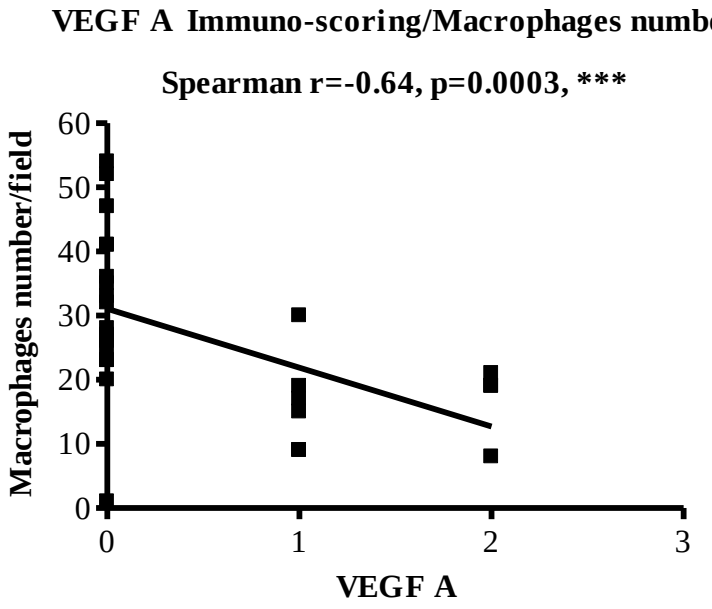


Figure 5. 8. VEGF A immuno-scoring and macrophages number in CVMs

The figure 5.8 shows that there was statistically significant correlation between the immunohistochemical expression of VEGF A and macrophages number in CVMs (p=0.0003).

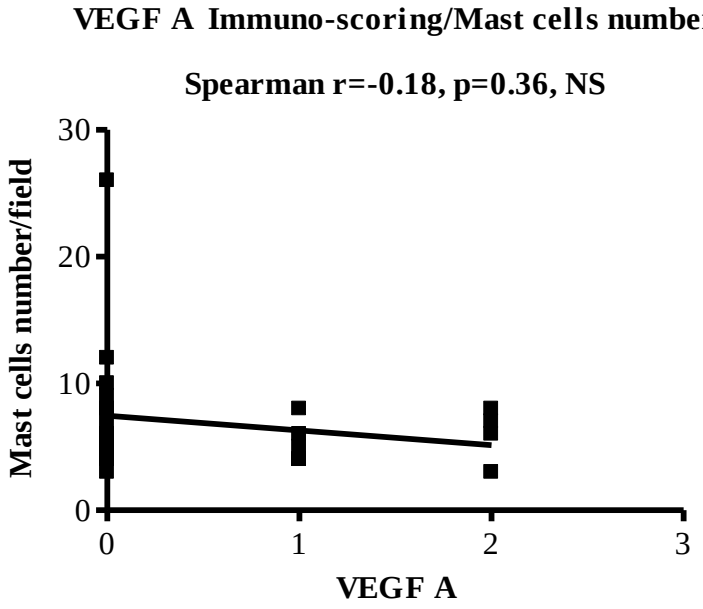


Figure 5. 9. VEGF A immuno-scoring and mast cells number in CVMs

The figure 5.9 shows that there was no statistically significant correlation between the immunohistochemical expression of VEGF A and mast cell number in CVMs (p=0.36).

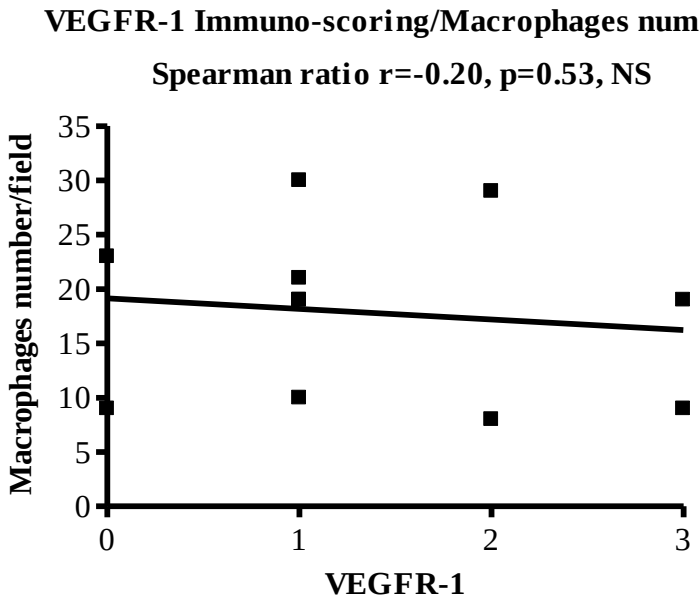


Figure 5. 10. VEGFR1 immuno-scoring and macrophages number in CVMs

The figure 5.10 shows that there was no statistically significant correlation between the immunohistochemical expression of VEGFR-1 and macrophages number in CVMs (p=0.53).

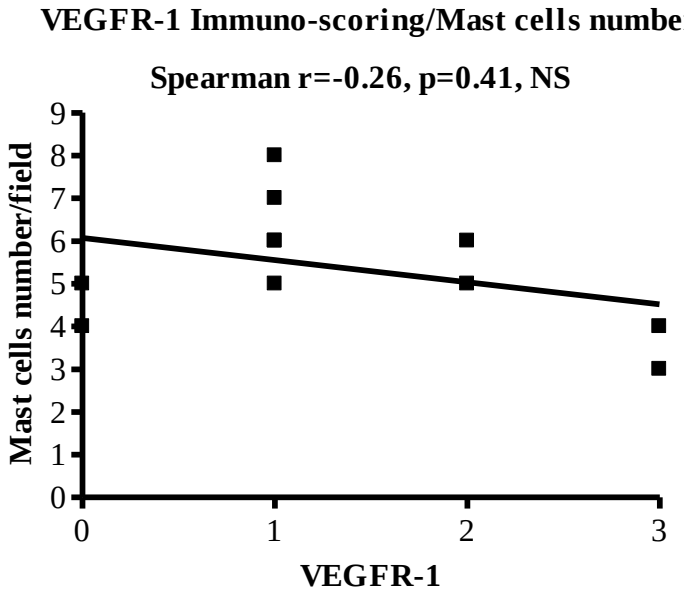


Figure 5. 11. VEGFR-1 immuno-scoring and mast cells number in CVMs

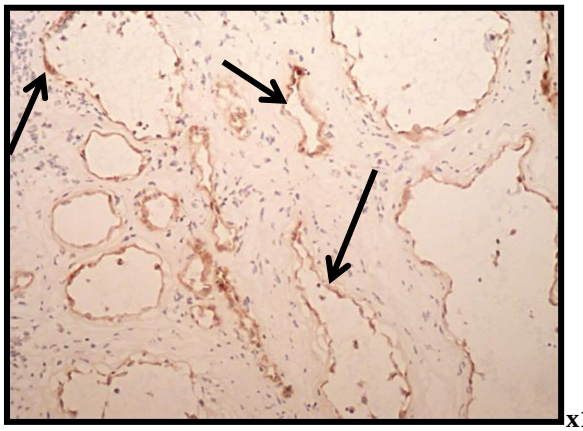
The figure 5.11 shows that there was no statistically significant correlation between the immunohistochemical expression of VEGFR-1 and mast cells number in CVMs (p=0.41).

5.4.2 TIE-2

5.4.2.1 Immunohistochemical localisation of Tie-2

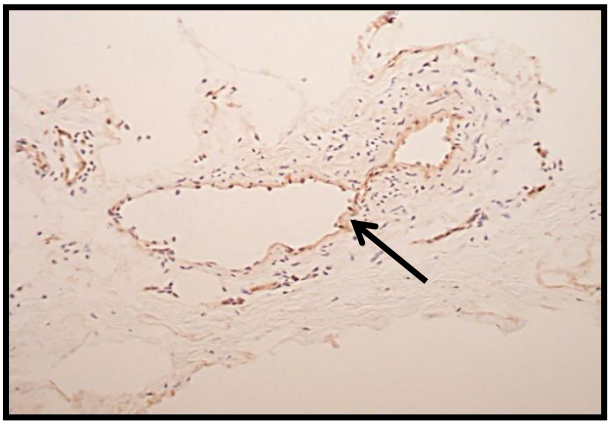
Tie-2 which controls vascular remodelling was moderately expressed in all CVMs and it was localised to EC.

5.4.2.2 Image analysis of Tie-2 sections of CVMs



x100

Figure 5. 12. Positive expression of Tie-2 Immuno-staining in a CVM expressed in ECs



x40

Figure 5. 13. Weak expression of Tie-2 on Immuno-staining in a skin control

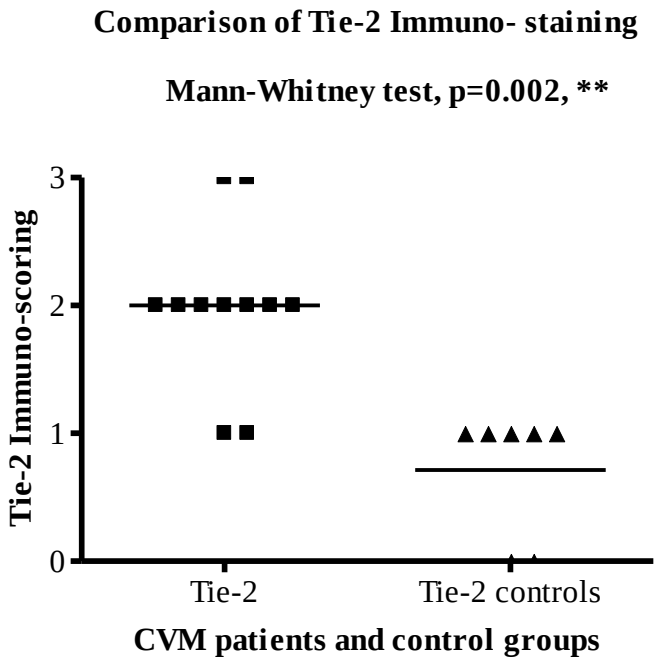


Figure 5. 14. Comparison of Tie-2 immuno-staining in CVM and control groups

The figure 5.14 shows that there was statistically significant difference between the immunohistochemical expression of Tie-2 in CVMs and controls (p=0.002).

5.4.2.3 Correlation of Tie-2 expression with the significant morphological changes in CVMs (number of macrophages and mast cells per field).

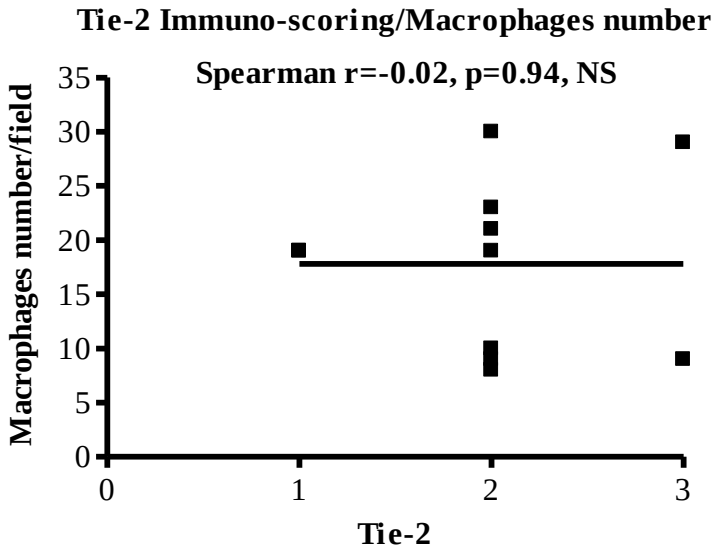


Figure 5. 15. Tie-2 immuno-scoring with macrophages number in CVMs

The figure 5.15 shows that there was no statistically significant correlation between the immunohistochemical expression of Tie-2 and macrophages number in CVMs (p=0.94).

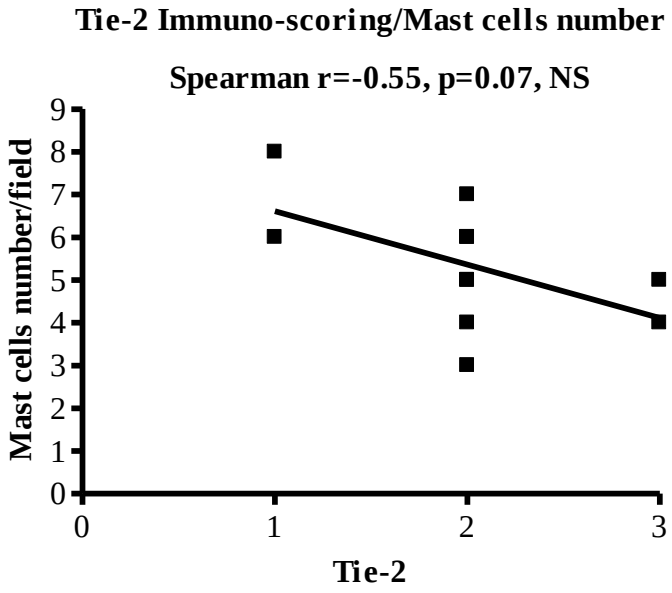


Figure 5. 16. Tie-2 immuno-scoring with mast cells number in CVMs

The figure 5.15 shows that there was no statistically significant correlation between the immunohistochemical expression of Tie-2 and mast cells number in CVMs (p=0.07).

5.4.3 Integrins αVβ3 and αVβ5

5.4.3.1 Immunohistochemical localisation of αVβ3 and αVβ5

αVβ3 is known to be exclusively expressed on activated ECs. αVβ5 is also an endothelial integrin which promoted VEGF (but not FGF2) induced angiogenesis. αVβ3 was highly expressed in CVMs but αVβ5 was absent.

5.4.3.2 Image analysis of αVβ3 and αVβ5 sections of CVMs

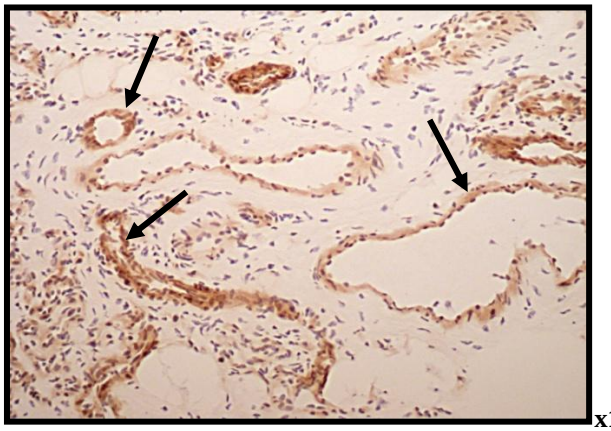
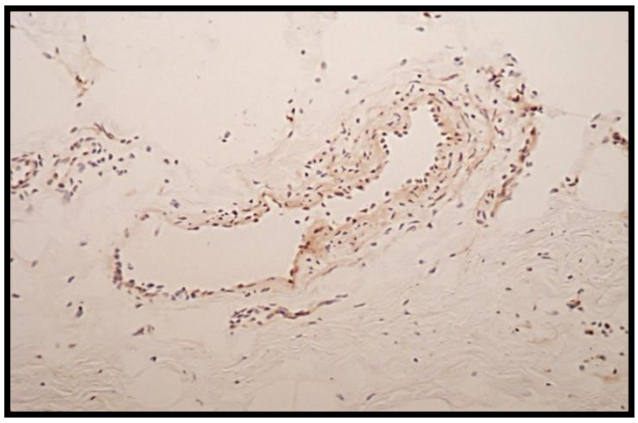
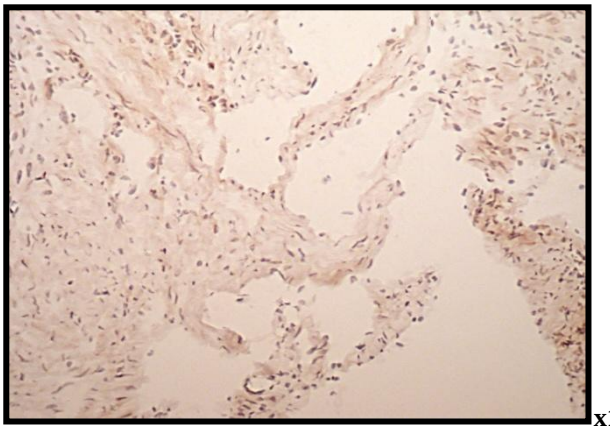


Figure 5. 17. Positive αVβ3 immuno-staining in a CVM expressed in ECs



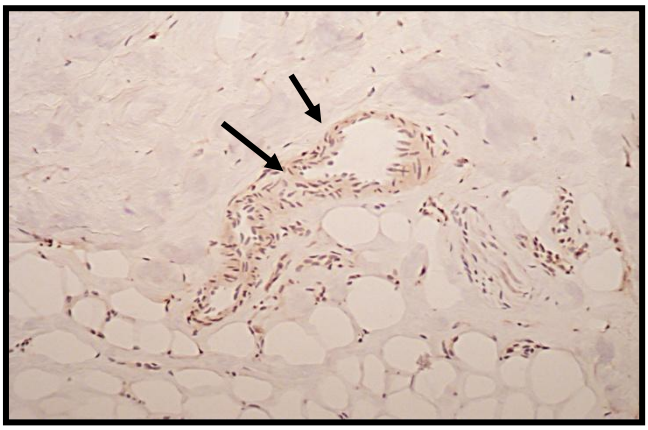
x100

Figure 5. 18. Negative αVβ3 Immuno-staining in a skin control



x100

Figure 5. 19. Negative αVβ5 Immuno-staining in a CVM



x40

Figure 5. 20. Weak expression of αVβ5 in a skin control

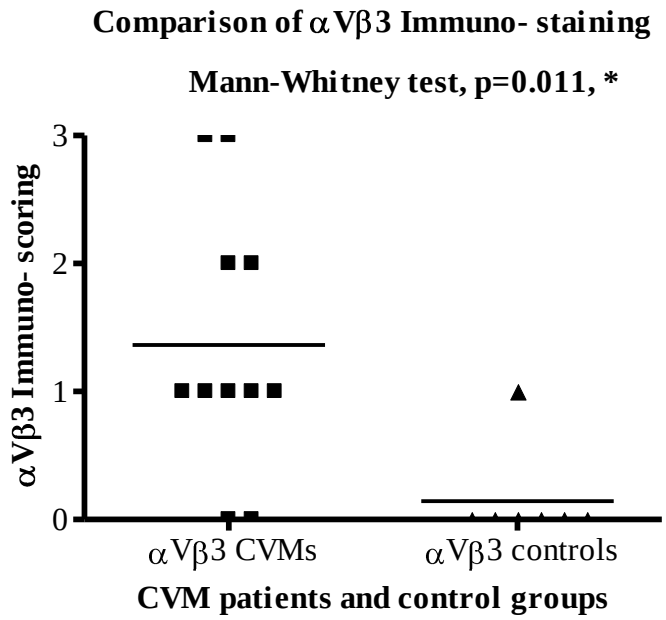


Figure 5. 21. Comparison of αVβ3 immuno-staining in CVM and control groups

The figure 5.21 shows that there was statistically significant difference between the immunohistochemical expression of αVβ3 in CVMs and controls (p=0.011).

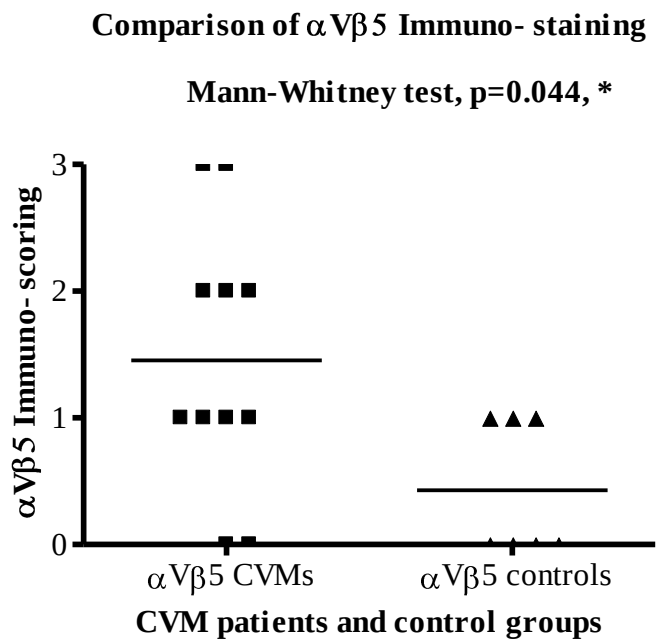


Figure 5. 22. Comparison of αVβ5 immuno-staining in CVM and control groups

The figure 5.22 shows that there was statistically significant difference between the immunohistochemical expression of αVβ5 in CVMs and controls (p=0.044).

5.4.3.3 Correlation of αVβ3 and αVβ5 expression with the significant morphological changes in CVMs (number of macrophages and mast cells per field).

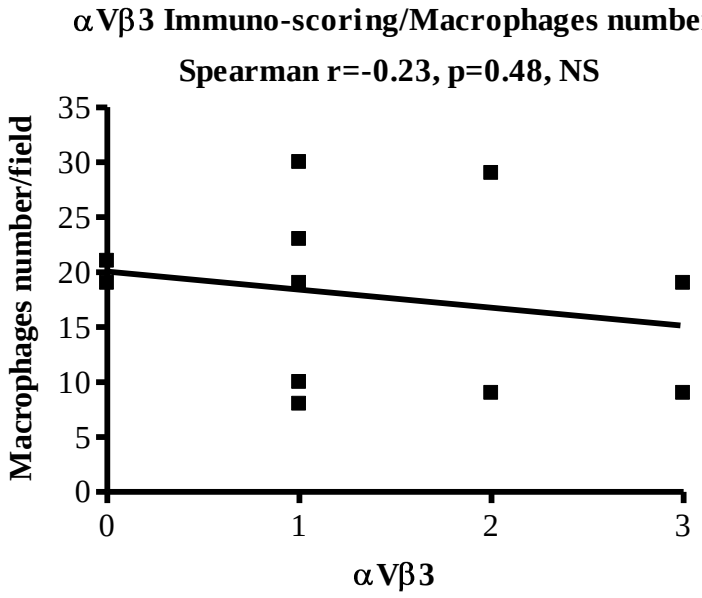


Figure 5. 23. αVβ3 immuno-staining and macrophages number in CVM

The figure 5.23 shows that there was no statistically significant correlation between the immunohistochemical expression of αVβ3 and macrophages number in CVMs (p=0.048).

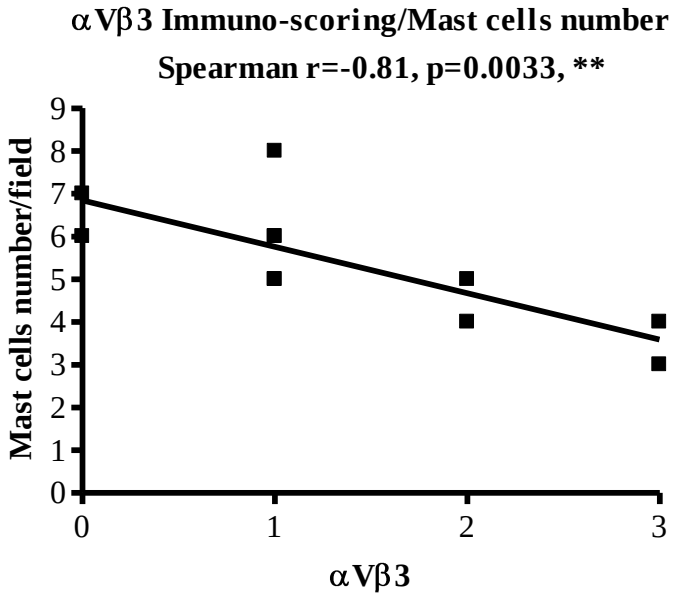


Figure 5. 24. αVβ3 immuno-staining and mast cells number in CVM

The figure 5.24 shows that there was statistically significant correlation between the immunohistochemical expression of αVβ3 and mast cells number in CVMs (p=0.0033).

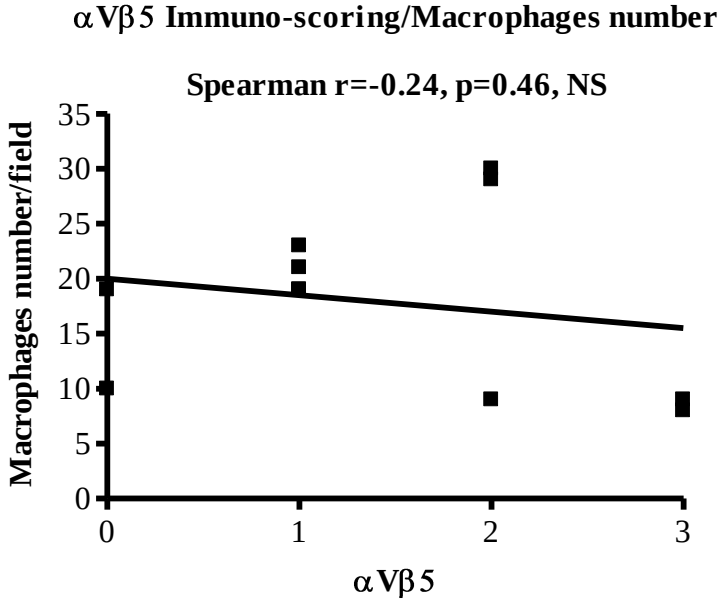


Figure 5. 25. αVβ5 immuno-staining and macrophages number in CVM

The figure 5.25 shows that there was no statistically significant correlation between the immunohistochemical expression of αVβ5 and macrophages number in CVMs (p=0.46).

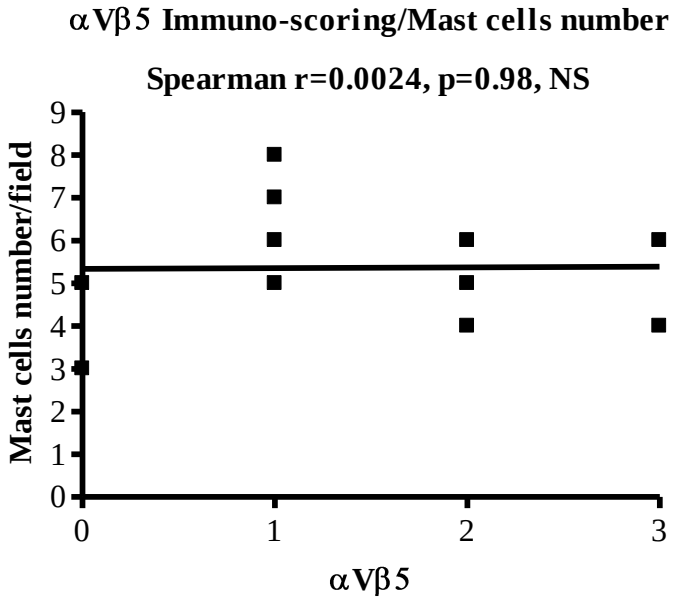


Figure 5. 26. αVβ5 immuno-staining and mast cells number in CVM

The figure 5.26 shows that there was no statistically significant correlation between the immunohistochemical expression of αVβ5 and mast cells number in CVMs (p=0.98).

5.4.4 ET-1/ET_A/ET_B

Endothelin-1 and its receptors ETA and ETB have recognised roles in direct and indirect modulation of angiogenesis. As detailed in Chapter 1, ET-1 promotes EC proliferation, migration, protease formation, tube formation and invasion. Previous studies implied ET-1 association with microvessel density and VEGF expression. Data in literature reports a pathogenic role for ET-1 in disease states. Interestingly, cerebral AVM vessels lacked ET-1 although ET-1 expression was prominent in vessels distant from these lesions (Rhoten, Comair et al, 1997). This molecular phenotype was thought to be responsible for the lack of haemodynamic auto-regulation present in cerebral AVMs. The pattern of expression of ET-1 has not been previously reported to be assessed in any of the CVMs. ET-1 presence or absence in CVMs would be informative in the context of the other angiogenic markers investigated in this chapter, as well as hypoxia markers and NEP investigated in Chapter 6. NEP is a metallopeptidase known to modulate bioavailability of peptide mediators and growth factors. NEP degrades ET-1 and controls inflammatory responses.

5.4.4.1 ET-1 immunohistochemical expression in CVMs

The immunohistochemical staining of CVM tissue was technically sub-optimal but the majority of vascular malformations were negative for ET-1. Ten vascular malformations were analysed immunohistochemically for ET-1 and 8 of them had negative expression of ET-1. One lesion which was ET-1 immuno-positive was embolised pre-operatively, had an intra-muscular component and it was located on the forearm. Another vascular malformation had equivocal expression of ET-1, but the corresponding slide had areas of technical errors (tissue has lifted) and it was difficult to interpret results accurately. It was interesting to note that from the 3 haemangiomas (two capillary haemangiomas and one cavernous haemangioma) immunostained for ET-1, all of them showed positive expression of ET-1. ET-1 was localised in the ECs and inflammatory cells in haemangiomas. Small bowel was used as a positive control for ET-1 immunostaining and the figure 5.27 shows ET-1 expression in the ECs and inflammatory cells in a section of small bowel.

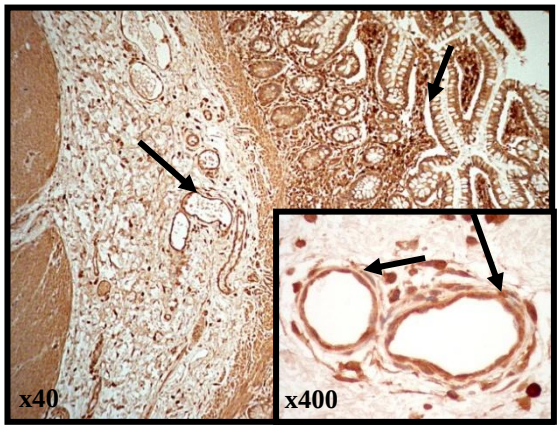


Figure 5. 27. Positive expression of ET-1 in small bowel

5.5 DISCUSSION

The first aim of this part of study was to determine the expression of angiogenic mediators in CVMs. Secondly the aim was to explore the relationship between the identified angiogenic markers and the CVM stromal inflammatory cell infiltrate represented by macrophages and mast cells. The immunohistochemical assessment of the chosen angiogenic mediators – VEGF A (p=0.22), VEGFR-1(p=0.01), Tie-2 (p=0.002), αVβ3 (p=0.01) and αVβ5 (0.04) – indicated that there was increased expression of all these mediators in CVM tissue in comparison to normal control tissue. The exception was VEGF A which, although present in CVMs, showed no statistically significant difference in its expression in CVMs in comparison to control tissue, in the cases analysed. Also, ET-1expression in vascular malformations appears to be absent, although there is positive expression of ET-1 in haemangiomas. The statistical analysis was performed using the Mann-Whitney U test. The findings suggest that all the angiogenic mediators found to be up-regulated in CVM tissue could be implicated in the pathogenesis of these lesions. Clinical correlation showed the highest immuno -score was noted in lower limb CVMs for VEGFR-1, Tie-2 and αVβ3. The score for αVβ5 was similar in lower limb and upper limb lesions. There was no significant difference in the immuno-score for HF or LF lesions but the majority of embolised HF lesions were positive for all investigated markers, except in two instances: one where VEGFR-1 was negative in an embolised upper limb HF lesion and, one where αVβ5 was negative in a lower limb HF lesion with a history of multiple embolisations. All these clinical parameters (location of lesion, type of flow and history of embolisation) could influence the expression of the angiogenic mediators in CVMs. The embolic material introduced at the time of embolisation theoretically could lead to tissue hypoxia, which in turn could act as a stimulus for the angiogenic mediators. The shear stress caused by the pattern of flow or even location of the CVM could potentiate any hypoxic effects.

There was a statistically significant negative correlation between the immunohistochemical expression of αVβ3 and the number of mast cells per microscopic field with a Spearman correlation ratio r= – 0.81 and p=0.0033. The highest number of mast cells per microscopic field was found in CVMs where there was no expression of αVβ3. Similarly, there was a statistically significant negative correlation between the immunohistochemical expression of VEGF A and the number of macrophages per field, with a Spearman correlation ratio r= -0.64 and p=0.0003. Again, the highest number of macrophages per field was found in specimens of CVMs where there was no immunohistochemical expression of VEGF A. No other correlation was identified between any of the up-regulated angiogenic markers in CVMs and stromal macrophages or mast cells, although the numbers compared were small.

HIF-1α activity is known to result in the transcriptional up-regulation of many angiogenic factors including VEGF A and VEGFR-1. VEGF induction may be secondary to activation of HIF-1α, either by inhibiting its degradation, or by increasing its rate of synthesis. HIF-1α and especially HIF-2α (see Chapter 6), were present in CVMs, although little is known about the HIF-2α signalling pathways in comparison to HIF-1α signalling pathways. Recently it has been shown that macrophage production of VEGFR-1 in response to hypoxia was dependent on HIF-2α, whilst HIF-1α specifically regulated VEGF production (Sumner et al, 2011), by neutralising VEGF and inhibiting its biological activity.

Macrophages are known to be capable of producing and expressing VEGF A along with other angiogenic factors as discussed previously. They are also reported to become angiogenic in conditions of hypoxia and respond to hypoxic conditions by up-regulating HIFs expression, with subsequent increase in VEGF A expression. In this study VEGF expression appears to be the result of hypoxia/HIFs up-regulation, rather than mediated by macrophages. The immunohistochemical localisation of the VEGF A was on the ECs of the CVM vessels and not on macrophages, although macrophages in many instances were intimately related to the abnormal vessels of CVMs. These results suggest at least two different categories of CVMs: those with higher number of macrophages and no expression of VEGF A and those with no macrophages or few numbers and increased expression of VEGF A. VEGF expression was not detected in macrophages themselves despite the accepted ability of macrophages to produce and express VEGF. This finding could represent a snap picture of the CVM disease status, meaning some lesions may be more active than others or simply, CVMs have different molecular mechanisms involved in their patho-physiology. All CVMs specimens were obtained at the time of surgery and as previously indicated, it was difficult to assess disease activity clinically, except in very few cases. Macrophages also have the ability to modulate angiogenesis by releasing factors that inhibit migration and mitoses of ECs and can down-regulate the formation

of new capillary blood vessels. CVMs with no expression of VEGF and increased number of macrophages may well represent a remission disease status or a pre-activation status or simply a quiescent status. Usually in tumours the increased number of macrophages is associated with on-going growth, neo-vascularisation and poor prognosis (see Introduction). These findings suggest that angiogenic pathways in CVMs may be different from those in tumours.

A high number of mast cells in CVMs correlated with no staining for αVβ3 and absent or low number of mast cells in CVMs correlated with over-expression of αVβ3.

Integrins mediate adhesion between cells and their surrounding matrices and transmit signals regulating cell survival, differentiation and migration. There have been studies reporting integrins to be mediators of mast cell inflammatory responses although, these reports were related to different integrins (α2β1) from those analysed in this study. It was postulated that mast cells express integrin and their co-stimulatory function may contribute to mast cell activation and cytokine production (Edelson et al, 2003). There are other studies confirming that mast cells express fibronectin-receptor integrins, which are involved in cell activation (Ra, Yasuda, et al, 1994) and other studies report that αVβ3 is expressed by most mast cells (Lorentz et al, 2002).

αVβ3 expression in this study was localised to the CVM ECs and did not appear to be expressed by mast cells, although mast cells were very closely related to the CVM blood channels. Some categories of CVMs may have a 'quiescent' (asymptomatic) status and contain a significantly higher number of macrophages and mast cells. Eady et al, 1997 demonstrated a significant correlation between the number of mast cells and number of blood vessels in the dermis. CVMs are characterised by a significantly higher number of blood vessels/mm^2 and this may also signify a higher number of macrophages and mast cells/ mm^2 in quiescent states.

To conclude, CVMs are not classified as inflammatory lesions but this study demonstrated increased stromal macrophages and mast cells (see Chapter 4). Although it is not possible to prove the role of this stromal infiltrate in the phase of active angiogenic process in CVMs it is plausible that the infiltrate must play some role in the pathogenesis of CVMs. There is heterogeneity in expression and up-regulation of the selected angiogenesis markers in CVMs and, this suggest an angiogenic pathway characteristic to CVMs. Hypoxia or other mechanisms may be more important than the inflammatory cells in the up-regulation of angiogenic markers. However, this data supports the general statement that aberrant angiogenesis in CVMs appears to be the result of complex interactions between vessel EC and surrounding stroma. Different clinical categories of CVM appear to exhibit different angiogenic markers, which imply a different pathogenic mechanism and therefore a means for more accurate assessment and treatment.

CHAPTER 6

6. ANALYSIS OF HYPOXIA IN CVMs

6.1. HYPOXIA INDUCIBLE FACTORS EXPRESSION IN CVMs

6.1.1 INTRODUCTION

6.1.1.1 Hipoxia inducible factors and CVMs

A variety of pathological conditions exists where the affected tissues are hypoxic or exhibit a low oxygen tension and CVMs may be amongst them, since these lesions appear to exhibit disregulated angiogenesis which is enhanced by hypoxia. Oxygen starved cells can recruit new blood vessels to their vicinity by the production of vascular endothelial growth factor (VEGF). Angiogenesis does not occur in normal adult tissue under normoxic conditions.
Data in literature indicates that hypoxia-inducible factor 1 (HIF-1α) is a critical mediator of physiological responses to acute and chronic hypoxia. HIF-1α is required for the development of the systems that mediate these responses including the heart, blood and blood vessels.

Studies have shown that hypoxia is marked during tumour development (Carreau et al, 2011). Hypoxia develops in tumors because of the vascularisation with less ordered, chaotic and leaky vascular beds compared with that in normal tissues. In laboratory models, hypoxia has been shown to be associated with treatment resistance and increased malignant potential. Clinical studies have shown the presence and extent of tumor hypoxia as a negative prognostic indicator. Tumour-associated angiogenesis is partly regulated by the HIF pathway.

CVMs are not vascular neoplasms and the significance of HIF expression in CVMs is still to be demonstrated.

The association between hypoxia and inflammation is well established, and experimental models of ischaemia and reperfusion injury have proved the expression of certain inflammatory mediators is regulated by hypoxia.

Recent experiments have shown that HIF-1α alters the expression of many of the key angiogenic growth factors that are produced by hypoxic cells in tissues including VEGF (Kelly et al 2003), and these bind to receptors on ECs and SMCs. In addition, HIF-1α also controls cell-autonomous responses to hypoxia in ECs by regulating the expression of hundreds of genes (Manalo et al 2005). Loss-of-function and gain-of-function studies indicate that HIF-1α plays a critical role in vascularisation both during development and in postnatal life (Carmeliet et al 1998, Iyer et al 1998, Kelly et al 2003, Ryan et al 1998).
There are some data to suggest that the prolyl hydroxylases (PHDs) increase during hypoxia. This would suggest that chronic hypoxia may cause reduced expression of HIF-1α.

HIF-1α is universally expressed in all cell types, and coordinates responses to hypoxia in all tissues in which it is expressed. Wang and Semenza (1993) suggested that HIF-1α played

a more general role in oxygen homeostasis. HIF-1α also controls remodelling of pre-existing vessels in response to hypoxia. HIF-1α is required for embryonic survival (Carmeliet et al 1998, Iyer et al 1998, Maltepe et al 1997, Ryan et al 1998). The absence of HIF-1α activity results in lethality at mid-gestation with defective development of the heart, blood and vessels. HIF-1α protein levels and transcriptional activity were found to respond dramatically to cellular O_2 concentration (Jiang et al, 1997). O_2-dependent hydroxylation of proline and asparagine residues in HIF-1α represent the mechanism for transducing changes in cellular oxygenation into changes in HIF-1α activity (Epstein et al 2001, Ivan et al 2001, Lando et al 2002, Yu et al 2001).

The HIF-2α subunit shows properties similar to HIF-1α, but is mainly expressed in stromal macrophages, and may mediate a different response to hypoxia. HIF-2α has a more specialized role, and appears to be much more cell-type specific (Talks et al, 2000).

Arterial thrombosis is clinically linked to low or absent oxygen content within the thrombus and therefore, it could be argued that HIFs should be expressed. The process of arterial thrombosis may be acute or chronic but there are no reports in the literature regarding HIFs expression in thrombi in either situation. Many CVMs are characterised by thrombus formation.

Animal experiments have shown that chronic hypoxia results in increased total vessel length, volume, endothelial surface area and number of endothelial cells in vivo, demonstrating a process of hypoxia-induced angiogenesis (Howell et al, 2003).

Comati et al, 2007 reported markedly elevated nuclear immunohistochemical expression of HIF-1α and HIF-2α in cerebro-vascular malformations. It was emphasised that EC-specific HIFs activation provides a setting which supports and sustains angiogenesis and they could be used for developing therapeutic strategies aiming to treat incurable lesions.

6.1.2. OBJECTIVES

Whether or not hypoxia represents a permanent or intermittent feature of CVMs, is unknown. Since it appears CVMs are lesions characterised by disregulated/active angiogenesis (see Chapter 5), it is logical to expect CVMs to express HIFs as hypoxia induces angiogenesis. Therefore, the possibility of hypoxia-mediated responses relevant to CVMs was explored assessing expression of HIFs in CVMs and correlating results with the expression of the selected pro-angiogenic markers in CVMs.

6.1.3. MATERIAL AND METHODS

6.1.3.1 Selection of patients and tissue collection

To determine hypoxia-mediated responses in CVMs two techniques were used on CVM tissue: immunohistochemistry and Western blot analysis. In the immunohistochemistry part of

this study 12 and 20 CVM specimens for HIF-1α and HIF-2α respectively, were analysed, using tissue excised over a period of 3 years, as well as 10 control specimens. HIF-1α and HIF-2α expression were also assessed on 12 specimens of arterial thrombi (received from 8 patients). In addition, 22 CVM specimens (from prospective, newly acquired CVM patients) and 10 controls were immunostained for CAIX. Preliminary immunohistochemical analyses showed the selected HIFs antibodies did not work on paraffin sections from processed CVM tissue. They did work on thrombi. The immuno- technique to assess HIFs did work on sections of frozen tissue. Therefore, all CVM cases in the HIF immunohistochemistry part of this study were prospective and staining was performed on frozen tissue.

For the Western blot analysis samples from 7 patients were suitable for HIF-1α analysis and 8 patients for HIF-2α analysis. The Western blot analysis was also performed on frozen tissue only.

All tissue specimens were selected, collected and stored as described in Chapter 2.

6.1.3.2 Immunohistochemical analysis

Sections of CVM and control non-diseased tissue were stained with H&E, and immunohistochemical stains for HIF-1α and HIF-2α were used to determine whether HIF regulated hypoxia is a feature of CVMs. Additional stains were done for CAIX. A routine ABC peroxidase protocol used for surgical pathology specimens at Charing Cross Hospital was followed (described in Chapter 2 and Appendix). The immunostained slides were analysed for the distribution and expression of HIF-1α and HIF-2α and the immunopositivity was quantified using both image analysis and light microscopy.

6.1.3.3 Immunohistochemical localisation of HIF-1α and HIF-2α

HIF stained slides were analysed for localisation of staining by two independent assessors and a simple scoring system was used to interpret data. Slides were scored for the intensity and distribution of the staining using the traditional scoring system from 0 to 3 (as described in Chapter 2). On interpreting and reporting the data, the mean score of the two observers was considered. The staining was assessed as to whether this was diffuse or focal, localised to endothelial, within the vessel wall, stromal or within the macrophages.

6.1.3.4 Western Blotting

This study was to determine the presence or absence of downstream proteins in the HIF pathway in CVMs using Western Blot analysis. The protocol is described in detail in Chapter 2 and it was optimised in the Renal Research Laboratories at Hammersmith Hospital, London. HIF immunostaining has been very difficult technically but with optimal tissue fixation the protocol has proven reliable and reproducible for the renal research team at Hammersmith Hospital. The Western blot analysis of CVM samples was done in collaboration with the team at

Hammersmith Hospital. The 'in house' HIF-2α monoclonal antibody was also used in this work, as described in Chapter 2.

The protein levels of HIF-1α and HIF-2α are determined mainly by their rate of proteasomal degradation. Although other mechanisms, including positive and negative regulation of co-activator recruitment, phosphorylation, cellular redox state, and intracellular compartmentalization may also have a role in determining HIF activity, the dominant mode of regulation is HIF-1α stabilisation.

6.1.4 RESULTS

The following table presents the number of CVM and control specimens, analysed immunohistochemically categorised clinically according to site, whether there was pre-operative embolisation and the type of flow within CVM vessels, i.e. HF or LF.

Tabel 6. 1. HIFs staining of CVMs and clinical data

Immunostaining	Number specimens	Type of CVM flow	Location CVM	Location control
HIF-1α	12 CVMs 10 Controls	High flow 2 Low flow 10	Lower limb 6 (2 embolised) Upper limb 4 (1 embolised) Head and neck 2	Breast 4 Amputation 3 Abdomen 3
HIF-2α	20 CVMs 10 Controls	High flow 5 Low flow 15	Lower limb 8 (3 embolised) Upper limb 8 (3 embolised) Head and neck 3 Abdomen 1	Breast 4 Amputation 3 Abdomen 3

Western blot analyses for both HIF-1α and HIF-2α were performed on two HF lesions and five LF lesions. Westen blot for HIF-2α was also performed on a skin control specimen. The HF lesions and two of the LF lesions had been previously embolised or underwent previous sclerotherapy. Four specimens analysed originated in lower limbs, one in upper limb, one was abdominal and one was located in soft tissue of the head.

The next table gives a summary of the localisation of HIF-1α and HIF-2α staining for the samples analysed immunohistochemically.

Tabel 6. 2. HIFs staining of CVM specimens and localisation of staining

<table>
<tr><th>Immunostaining</th><th>Number specimens</th><th colspan="2">Predominant staining area CVMs</th><th>Predominant staining area controls</th></tr>
<tr><td rowspan="6">HIF-1α</td><td rowspan="3">12 CVMs</td><td rowspan="3">EC</td><td>Focal 1 (8%)</td><td rowspan="3">EC – Focal 0 (0%)
– Diffuse 4 (40%)

Macrophages – Focal 0 (0%)
– Diffuse 4 (40%)

No staining – 6 (60%)</td></tr>
<tr><td>Diffuse 7 (58%)</td></tr>
<tr><td rowspan="3">8 Controls</td><td rowspan="2">Macrophages</td><td>Focal 1 (8%)</td></tr>
<tr><td>Diffuse 6 (50%)</td></tr>
<tr><td colspan="2">No staining – 3 (25%)</td></tr>
<tr><td rowspan="6">HIF-2α</td><td rowspan="3">20 CVMs</td><td rowspan="3">EC</td><td>Focal 1 (5%)</td><td rowspan="3">EC – Focal 4 (50%)
– Diffuse 2 (25%)
Macrophages – Focal 0 (0%)
– Diffuse 3 (37%)
Vessel wall – Focal 1 (13%)

No staining – 2 (25%)</td></tr>
<tr><td>Diffuse 17 (85%)</td></tr>
<tr><td rowspan="3">8 Controls</td><td rowspan="2">Macrophages</td><td>Focal 1 (5%)</td></tr>
<tr><td>Diffuse 15 (75%)</td></tr>
<tr><td colspan="2">No staining – 2 (10%)</td></tr>
</table>

The figures above do not add up because in most specimens HIF was expressed by both EC and macrophages. The most common overlapping picture of HIF expression seen in CVMs or controls was either focal or diffuse HIF expression by EC and diffuse expression by macrophages.

The mean age ± SD of the patients recruited for immunohistochemical analysis was 27.42 ± 11.80 for HIF-1α and 32.65 ± 11.80 for HIF-2α. The median age for HIF-1α group was

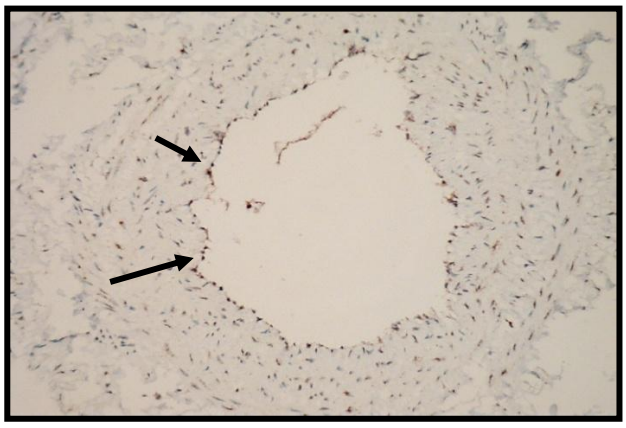
24 with a minimum of 13 and maximum of 54 and the median age for HIF-2α group was 26.5 with a minimum of 13 and maximum of 73. The sex ratio was 3:1/female: male in the HIF-1α group and 2:1/female: male in the HIF-2α group.

The mean age ± SD of the patients recruited for Western blot analysis was 28.14 ± 13.83 for HIF-1α and 27.88 ± 12.82 for HIF-2α. The median age for both groups was 24 with a minimum of 18 and maximum of 58. The sex ratio was 6:1/female: male in the HIF-1α group and 7:1/female: male in the HIF-2α group.

Within the HIF-1α group on the scoring scale from 0 to 3 (as described in the Methods sub-chapter), three lesions scored 0, seven lesion scored 1, one lesion scored 2 and one scored 3. Within the HIF-2α group, one lesion scored 0, three lesions scored 1, ten lesions scored 2 and six lesions scored 3. These represented the mean score assessed by two independent observers. The kappa coefficient analysis of the data showed that the inter-observer agreement was acceptable (kappa 0.33) and the p value of <0.0001 indicated that this agreement was not occurring by chance.

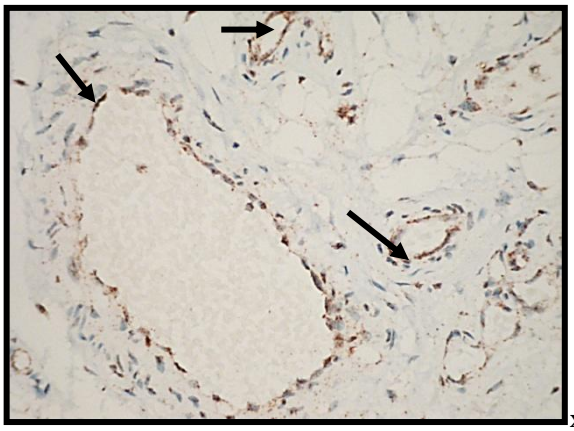
6.1.4.1 Image analysis of HIF-1α in CVMs

The following slides demonstrate the immunostaining results for HIF-1α of various types of CVMs. HIF-1α was present in the ECs of abnormal channels of CVMs. HIF-1α was also expressed to a less extent in some of the control tissue samples.



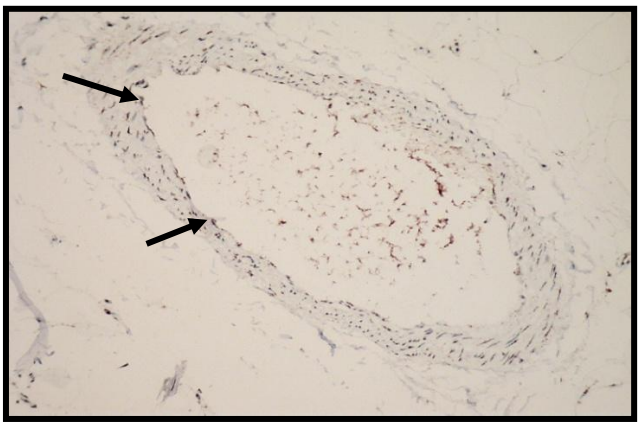
x100

Figure 6. 1. Weak expression of HIF-1α immuno-staining in a skin control



x200

Figure 6. 2. HIF-1α immuno-staining in a CVM



x200

Figure 6. 3. Weak expression of HIF-1α immuno-staining in a skin control

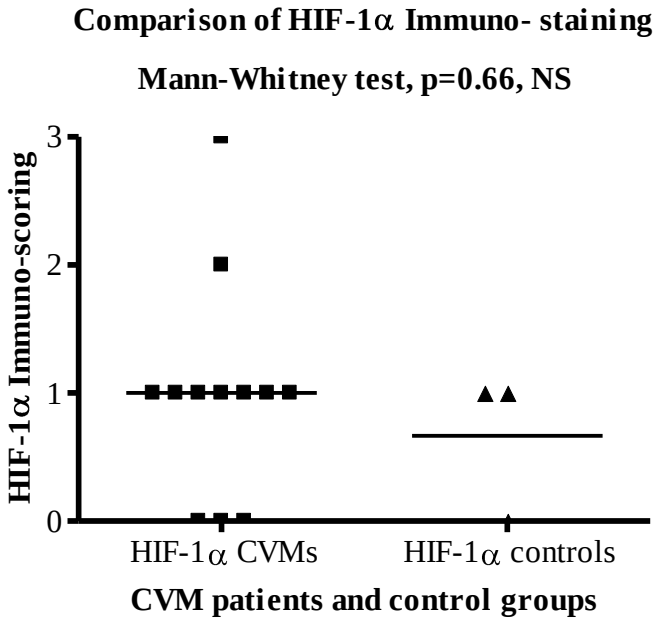


Figure 6. 4. HIF-1α immuno-staining in CVM and control groups

The figure 6.4 shows that there was no statistically significant difference between the immunohistochemical expression of HIF-1α in CVMs and controls (p=0.66).

6.1.4.2 Absence of immunohistochemical staining for carbonic anhydrase IX

CA IX has been shown to be induced by hypoxia. HIF-1α has a binding site on CA IX. Previous reports indicated HIF-1α to be essential for CAIX transcription under hypoxia. CX IX was negative in all 22 CVM specimens analysed.

6.1.4.3 Correlation of HIF-1α and pro-angiogenic markers expression in CVMs

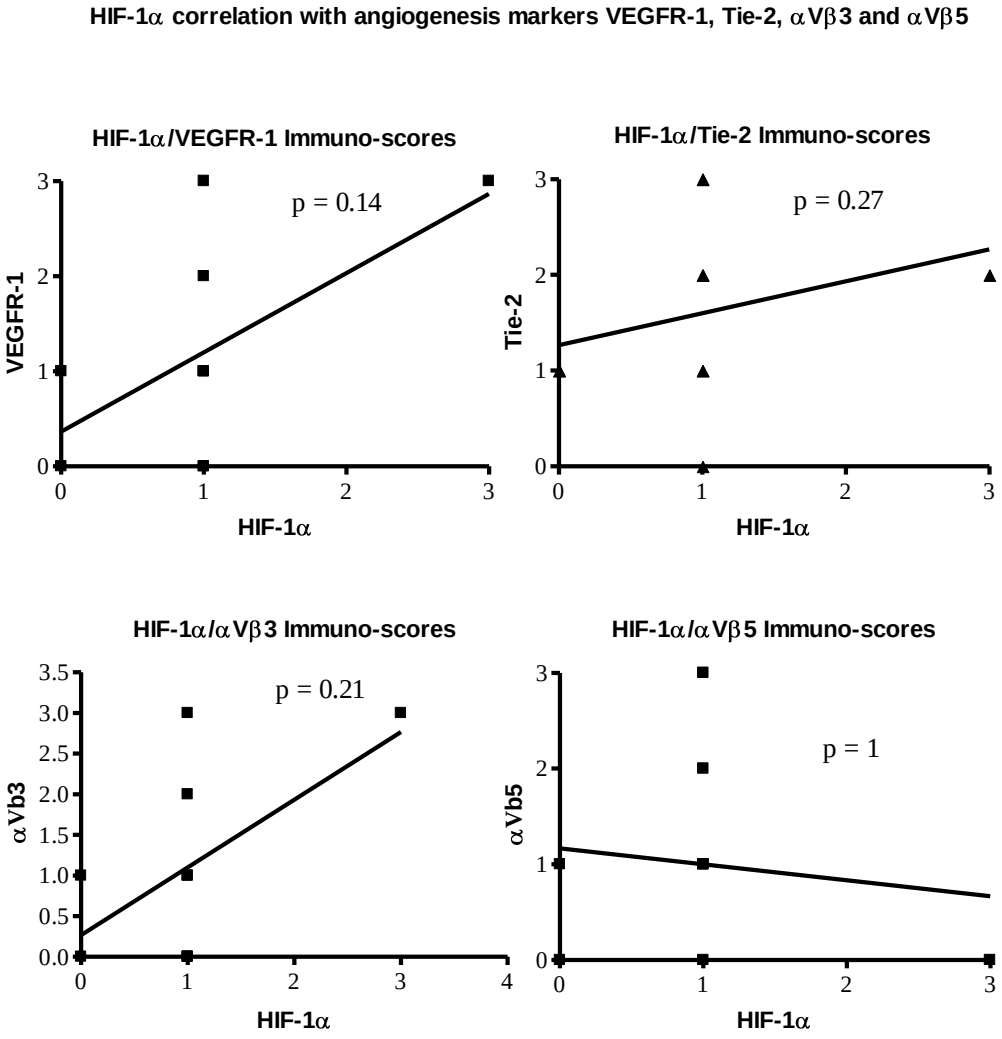


Figure 6. 5. HIF-1α and angiogenesis markers immuno-staining in CVMs

The figure 6.5 shows that there was no statistically significant correlation between the immunohistochemical expression of HIF-1α and VEGFR-1, Tie-2, αVβ3 and αVβ5 in CVMs (p=0.14; p=0.27; p=0.21; p=1).

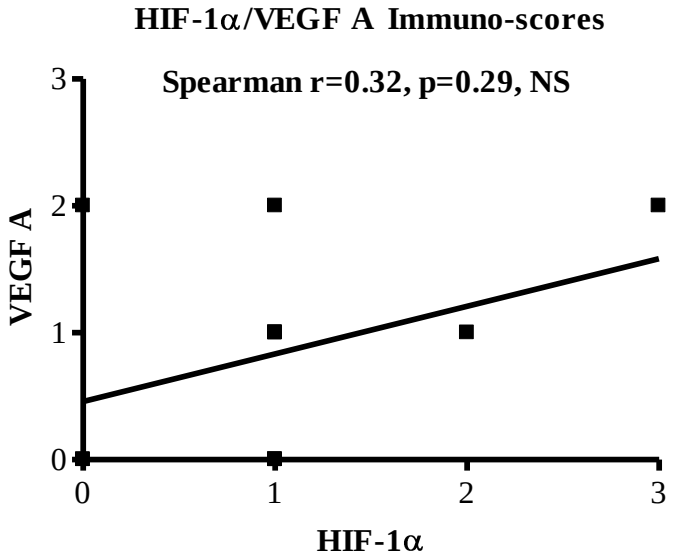


Figure 6. 6. Correlation of HIF-1α and VEGF A immuno-staining in CVMs

The figure 6.6 shows that there was no statistically significant correlation between the immunohistochemical expression of HIF-1α and VEGF A in CVMs (p=0.29).

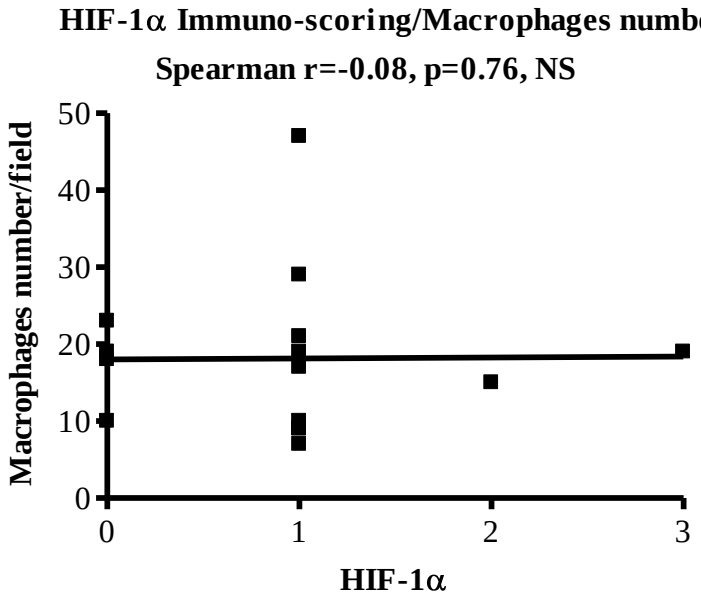


Figure 6. 7. Correlation of HIF-1α and macrophages number/field in CVMs

The figure 6.7 shows that there was no statistically significant correlation between the immunohistochemical expression of HIF-1α and macrophages number in CVMs (p=0.76).

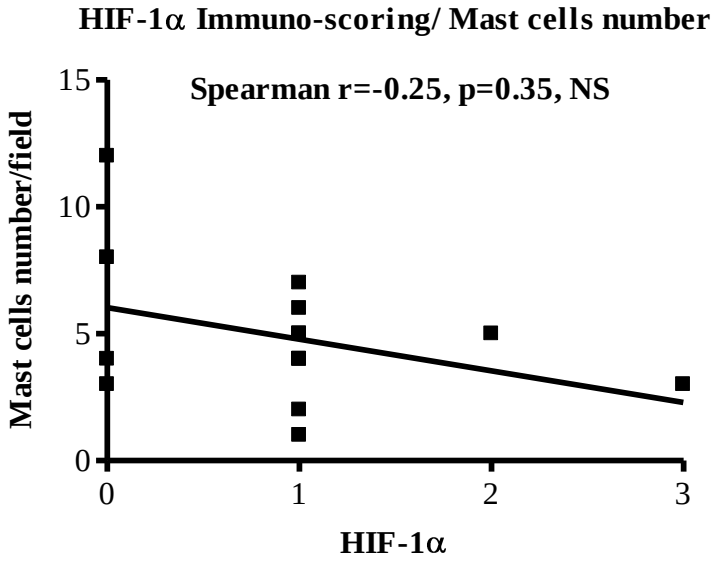
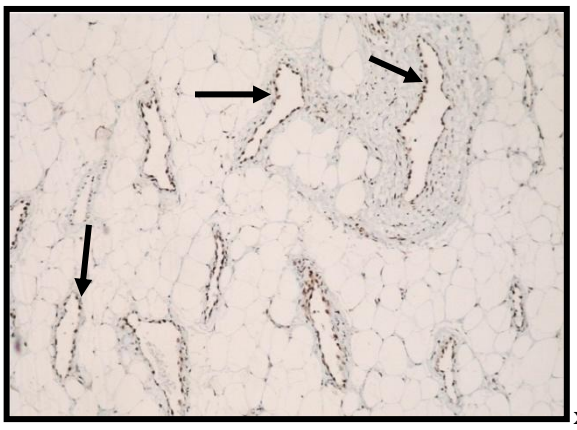


Figure 6. 8. Correlation of HIF-1α and mast cells number/field in CVMs

The figure 6.8 shows that there was no statistically significant correlation between the immunohistochemical expression of HIF-1α and mast cells number in CVMs (p=0.35).

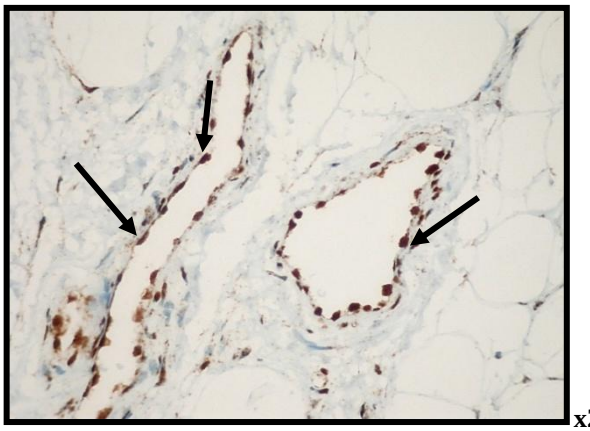
6.1.4.4 Image analysis of HIF-2α

HIF-2α was even more prominent in CVMs and mostly absent in controls. HIF-2α was demonstrated in macrophages and co-localisation of CD68 and HIF-2α positive cells in serial sections was confirmed. HIF-2α stained EC nuclei.



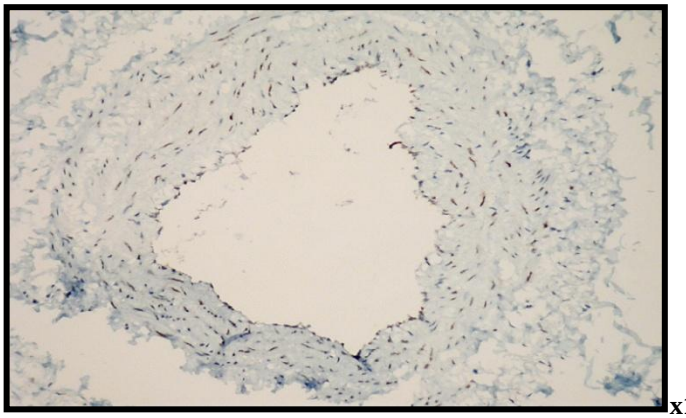
x40

Figure 6. 9. Positive expression of HIF-2α immuno-staining in a CVM



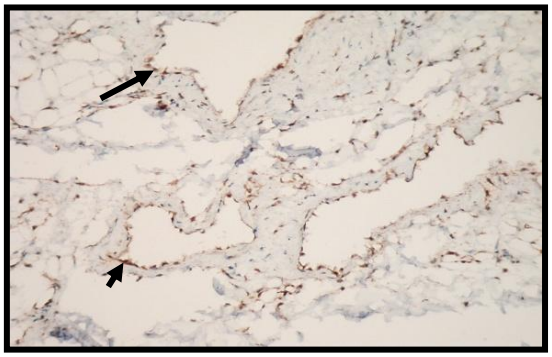
x200

Figure 6. 10. Positive expression of HIF-2α immuno-staining in a CVM

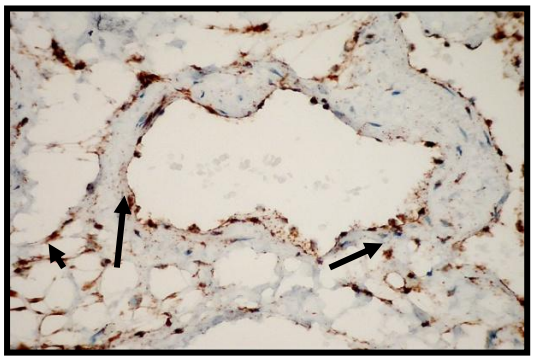


x100

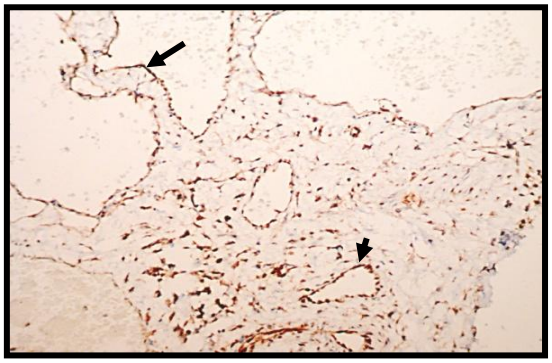
Figure 6. 11. Negative expression of HIF-2α immuno-staining in a skin control



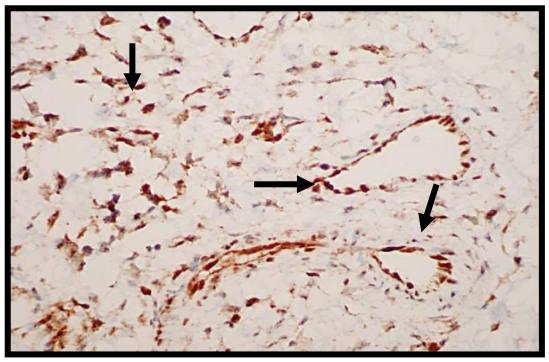
HIF-1α x100



HIF-1α X200



HIF-2α x100



HIF-2α X200

Figure 6. 12. Positive HIF expression in CVMs – macrophages and EC nuclear staining

The picture above brings together HIF-1α and HIF-2α staining and suggests that although both are expressed in CVMs, HIF-2α is more extensively expressed than HIF-1α.

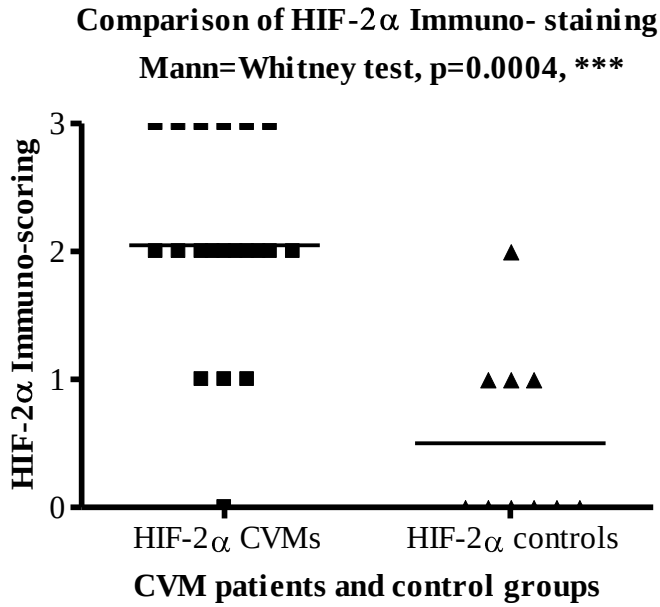


Figure 6. 13. Comparison of HIF-2α immuno-staining in CVM and control groups

The figure 6.13 shows that there was statistically significant difference between the immunohistochemical expression of HIF-2α and in CVMs and controls (p=0.0004).

6.1.4.5 Correlation of HIF-2α and pro-angiogenic markers expressions in CVMs

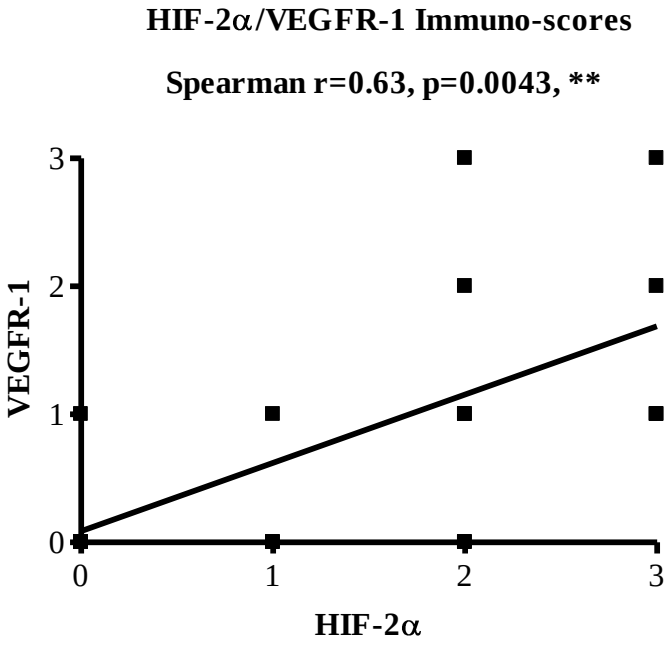


Figure 6. 14. Correlation of HIF-2α and VEGFR-1 immuno-scores in CVMs

The figure 6.14 shows that there was statistically significant correlation between the immunohistochemical expression of HIF-2α and VEGFR-1 in CVMs (p=0.0043).

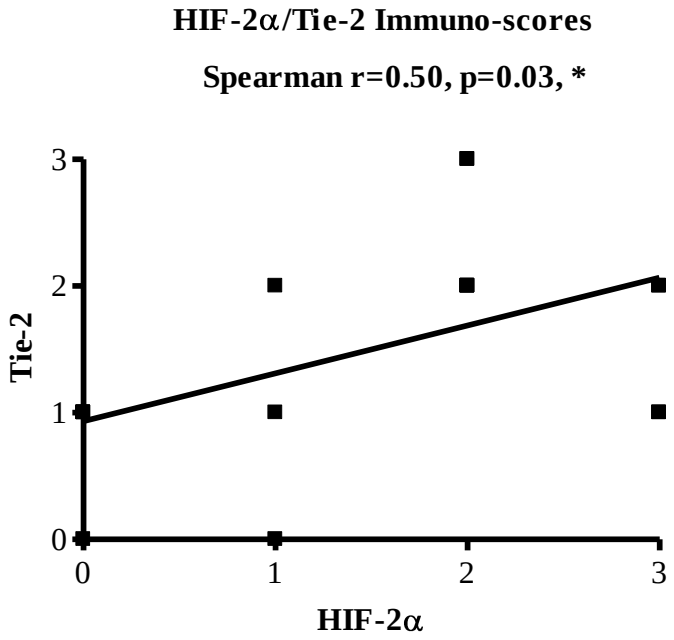


Figure 6. 15. Correlation of HIF-2α and Tie-2 immuno-scores in CVMs

The figure 6.15 shows that there was statistically significant correlation between the immunohistochemical expression of HIF-2α and Tie-2 in CVMs (p=0.03).

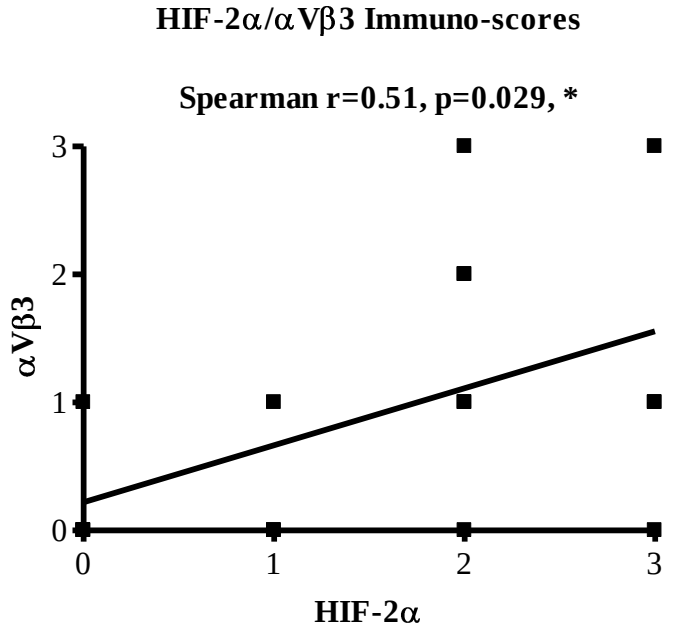


Figure 6. 16. Correlation of HIF-2α and αVβ3 immuno-scores in CVMs

The figure 6.16 shows that there was statistically significant correlation between the immunohistochemical expression of HIF-2α and αVβ3 in CVMs (p=0.029).

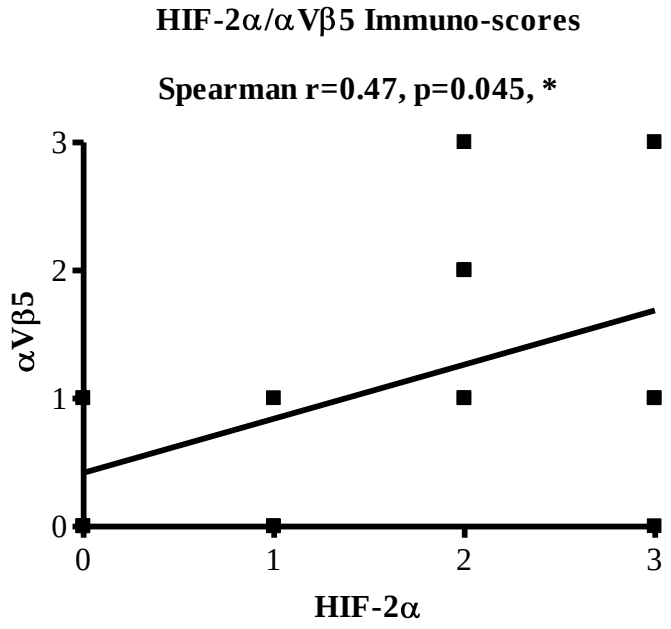


Figure 6. 17. Correlation of HIF-2α and αVβ5 immuno-scores in CVMs

The figure 6.17 shows that there was statistically significant correlation between the immunohistochemical expression of HIF-2α and αVβ5 in CVMs (p=0.045).

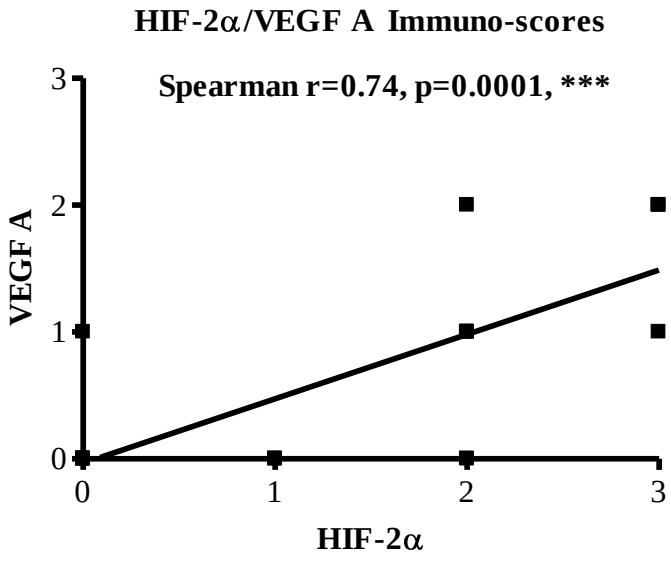


Figure 6. 18. Correlation of HIF-2α and VEGF A immuno-scores in CVMs

The figure 6.18 shows that there was statistically significant correlation between the immunohistochemical expression of HIF-2α and VEGF A in CVMs (p=0.0001).

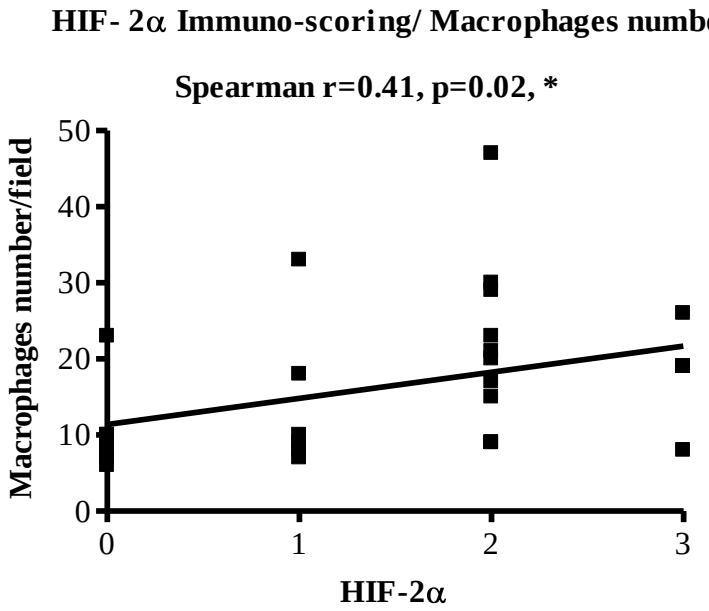


Figure 6. 19. HIF-2α immuno-scoring and macrophages number in CVMs

The figure 6.19 shows that there was statistically significant correlation between the immunohistochemical expression of HIF-2α and macrophages number in CVMs (p=0.02).

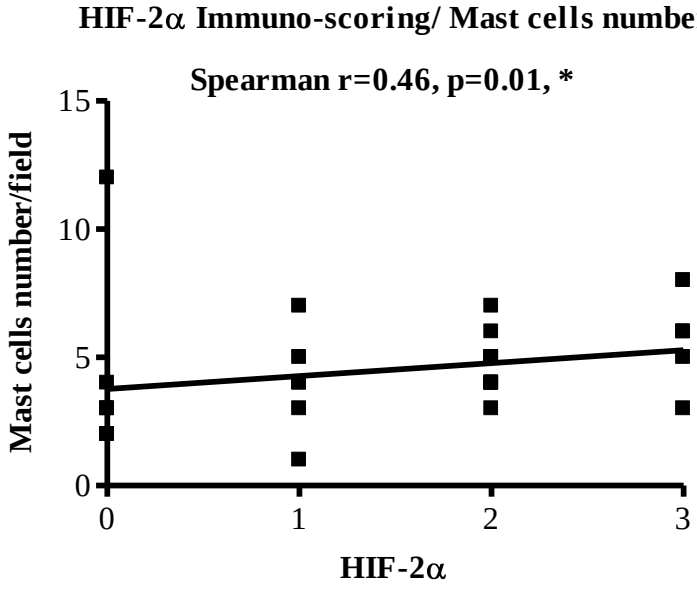


Figure 6. 20. HIF-2α immune-scoring and mast cells number in CVMs

The figure 6.20 shows that there was statistically significant correlation between the immunohistochemical expression of HIF-2α and mast cells number in CVMs (p=0.01).

6.1.4.6 HIFs expression in thrombi

HIF-2α co-localised with CD68 staining for macrophages in CVMs.

HIF-2α expression was negative in 5 out of 12 specimens and the rest of 7 specimens had little and few areas of staining.

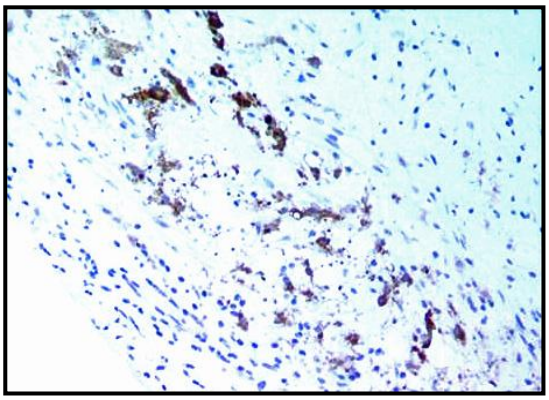


Figure 6. 21. Positive expression of HIF-2α immuno-staining in a RCC specimen

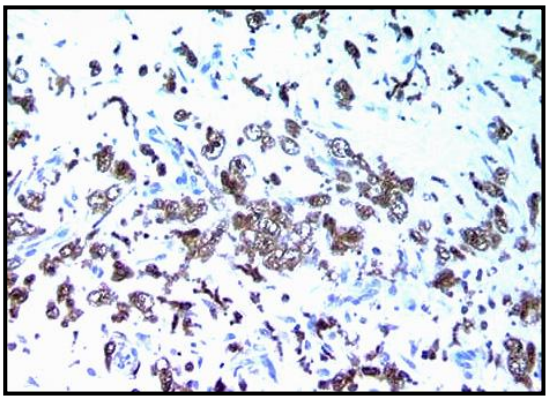


Figure 6. 22. Positive expression of HIF-2α immuno- staining in thrombi

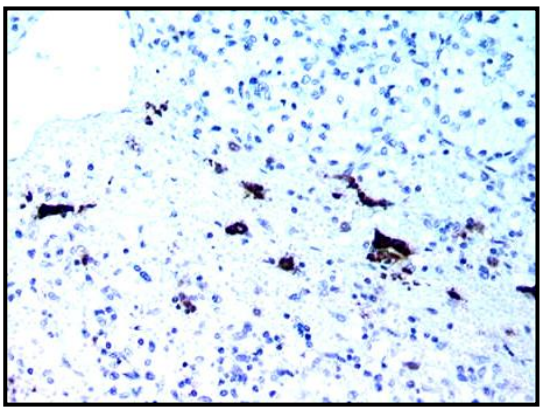


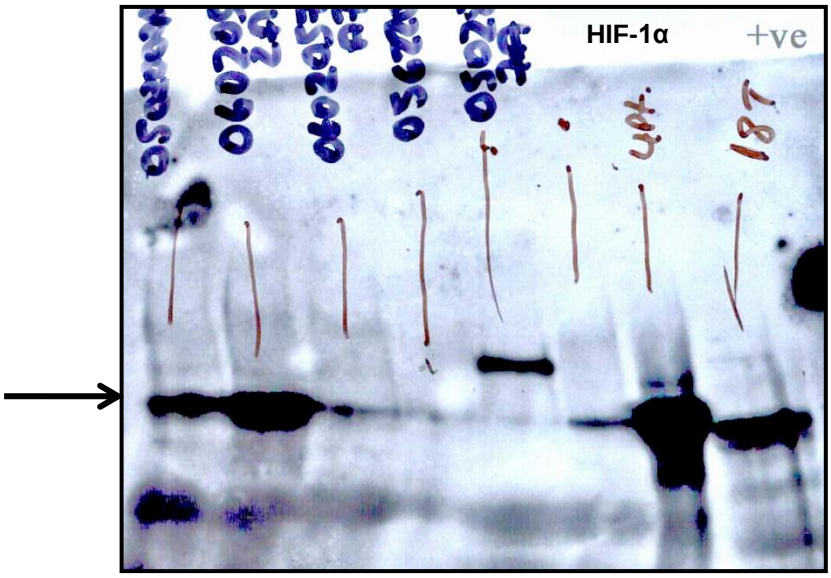
Figure 6. 23. Positive expression of CD68 showing macrophages in the same thrombi specimen

Western Blot analysis results in CVMs

The analysis was technically difficult because of the tissue composition of CVMs. The two HF specimens analysed (originating from lower limbs) were strongly positive for both HIF-1α and HIF-2α. In addition, one LF specimen (originating from upper limb) was strongly positive for both HIF-1α and HIF-2α. All these specimens have in common the fact that they have been embolised pre-operatively. One LF specimen (originating in the lower limb) was negative for HIF-1α and strongly positive for HIF-2α. One LF lesion (abdominal) was negative for HIF-1α and weakly positive for HIF-2α. Another LF lesion (head and neck) was weakly positive for HIF-1α and strongly positive for HIF-2α. The seventh lesion was weakly positive for both antibodies and this lesion in particular underwent pre-operative sclerotherapy. The HIF-2α was negative in the skin control. The positive control was represented by RCC, known to be positive for HIFs. These results indicate variability in HIF-1α and HIF-2α presence in CVMs specimens, with a clear tendency for the specimens to show a stronger positivity for HIF-2α rather than HIF-1α, results similar to those obtained by immunohistochemistry. There was correspondence between HIFs expression on immunohistochemistry and HIFs positivity on Western blot as showed in the following table. These results have not been analysed statistically given the small numbers.

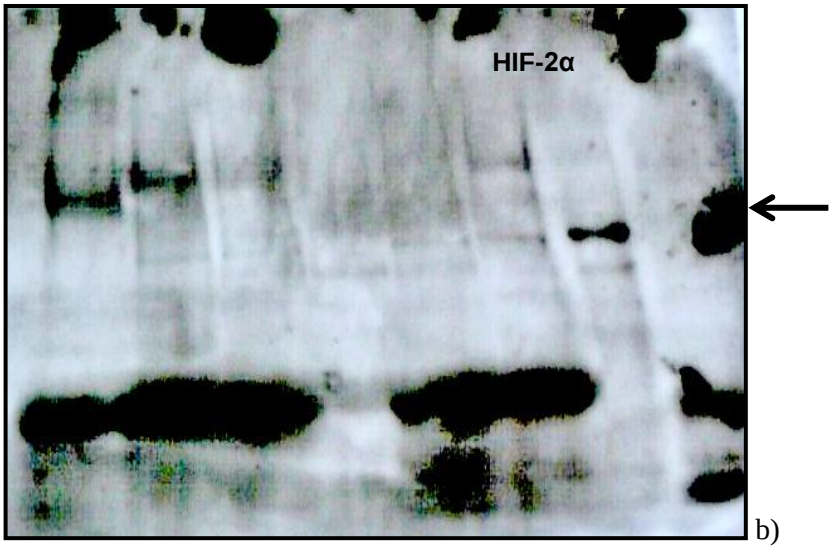
Tabel 6. 3. HIFs immune-scoring and Western blot analysis results in CVMs

Immuno-scoring HIF-1α	Western blot HIF-1α	Immuno-scoring HIF-2α	Western blot HIF-2α
1	Strong	2	Strong
x	0	2	Weak
0	0	3	Strong
1	Weak	2	Weak
1	Strong	3	Strong
3	Strong	3	Strong
1	Weak	2	Strong
		0	0



a)

Figure 6. 24. Western blot analysis of CVMs showing results of HIF-1α in CVMs



b)

Figure 6. 25. a) and b)-Western blot analysis of CVMs showing HIF-2α results in CVMs

6.1.5 DISCUSSION

Both HIF-1α and HIF-2α were found to be present in CVM tissue analysed both immunohistochemically and using the Western blot. The expression of HIF-2α was significantly higher in CVM tissue than in control tissue (p=0.0004) but, there was no significant statistical difference between the immunohistochemical expression of HIF-1α in CVMs and control tissue. In theory these results are expected in CVMs, which are considered 'highly angiogenic' lesions. CVMs in which angiogenesis is taking place are said to be 'active'. This phase is variable between lesions. Areas of hypoxia are common in places of active angiogenesis. HIF-1α component has a short half-life, but it is stabilised by hypoxia. HIF-1α has a low expression in

normal tissues. HIF-1α also directly potentiates migration and tube formation in ECs with potency similar to FGF2 (Eccles, 2004). Less is known about the contribution HIF-2α makes to the angiogenesis process. The contribution of HIF-1α versus HIF-2α to VEGF expression and other HIF regulated target genes under different conditions is also unclear. HIF-1α is known to regulate the transcriptional activation of VEGF in response to hypoxia. HIF-2α appears to enhance HIF-1α mediated VEGF expression in response to hypoxia (Carroll et al, 2006). The later authors also reported that in RCCs that constitutively express HIF-1α and HIF-2α due to loss of VHL function, high basal VEGF expression was predominantly dependent on HIF-2α. Other studies have shown that HIF-2α regulates a variety of hypoxia-inducible genes suggesting that its function is not restricted to the EC as it was initially thought (Hu, Wang et al, 2003).

Another recent study showed opposing roles for HIFs in regulation of angiogenesis by tumour associated macrophages, suggesting that administration of GM-CSF induced VEGFR-1 and inhibited tumour growth and angiogenesis in patients with melanoma (Sumner et al, 2011).
I have shown a statistically significant correlation between the immunohistochemical expression of HIF-2α and VEGF A (p=0.0001), VEGFR-1 (p=0.0043), Tie-2 (p=0.03), αVβ3 (p=0.029) and αVβ5 (0.045) suggesting a direct or indirect connection between HIF-2α expression and the up-regulated angiogenesis markers. HIF-2α appears to be up-regulated in CVMs when the angiogenesis process is 'active' and it is possible that HIF-2α could up-regulate the expression of VEGF A, VEGFR-1, Tie-2, αVβ3 and αVβ5 in CVMs. HIF-1α was diffusely localised on ECs in 58% of cases and diffusely localised on macrophages in 50% of CVMs analysed here. HIF-2α was diffusely localised on ECs in 85% of case and diffusely localised on macrophages in 75% of cases.
There was a positive correlation between HIF-2α and the number of stromal macrophages and mast cells in CVMs. Recently, the connection between hypoxia and inflammation has been demonstrated to be mediated by HIFs (Imtiyaz et al, 2011). In macrophages, HIFs regulate glycolytic energy generation, optimize the innate immunity, control pro-inflammatory gene expression, mediate bacterial killing and influence cell migration. In mast cells HIFs contribute to inflammatory functions (Imtiyaz et al, 2011). Macrophages themselves are known to be responsive to hypoxia leading to the up-regulation of HIFs.

The angiogenic markers in our study (Chapter 4) did not correlate with the stromal inflammatory infiltrate in CVMs, although they did correlate with HIF-2α expression which in turn correlates with the stromal inflammatory infiltrate. Other studies (Sluimer et al, 2008) demonstrated that hypoxia correlated with the presence of macrophages, angiogenesis, thrombus formation, and the expression of HIF and VEGF in human atherosclerotic lesions. It was speculated that hypoxic macrophages stimulate HIF and angiogenesis as well as the increase in

cytokine production. In other studies (Chen, et al, 2005) no correlation was found between HIF and angiogenesis in coronary intraplaque, and there was an inverse correlation with carotid and femoral intraplaque angiogenesis (Vink et al, 2007). The results from these studies contradict the well known positive correlation between HIF and angiogenesis in tumours (Semenza, 2003). Furthermore, in this study we also tested 12 thrombi for HIFs expression and 58% of specimens were positive with HIF staining co-localised with CD68. All studies so far indicate that the HIF signalling pathway and its involvement in angiogenesis and inflammatory processes may be different in different tissues/organs.

In this study hypoxia related markers were expressed in macrophages but not all macrophages were hypoxic. Hypoxia may develop from an increased oxygen demand, resulting from the high oxygen demand of metabolically active inflammatory cells (Murdoch et al, 2005). On the other hand, hypoxia causes macrophages to accumulate in affected areas. It is not clear what the chronology of events in CVMs is but the likely possibility is that macrophages are attracted to CVM stroma, the lesion becomes active. Two facts point towards this possibility: 1) macrophages and mast cells are present in the stroma of CVMs (Glowacki, 1982); 2) inflammatory cells were close to the abnormal vessels in CVMs, suggesting they have migrated there from the blood vessel lumen. There are some clinical features of CVMs (as described in the introductory chapter) which may indicate an active CVM but they are not always accurate.

In this study, there was variability in the HIFs expression in different clinical types of CVMs (HF or LF), with different locations. There was up-regulation of both HIF-1α and HIF-2α in all HF lesions analysed by both, immunohistochemistry and Western blot. HF lesions are the most challenging clinically and notorious for exacerbation after any trauma including surgical intervention. HIF-1α and HIF-2α were also strongly expressed in a LF arm lesion which was previously embolised. Weak expression of both antibodies was noted in a LF lesion subjected to sclerotherapy pre-operatively. Another LF VM was negative for HIF-1α and strongly positive for HIF-2α. This lesion had no history of embolisation or sclerotherapy. Taking into account these pre-operative interventional techniques is important as the embolic material or the sclerosant would locate into the CVM blood vessel, occluding the vessel and subsequently depriving the CVM tissue from O_2. Therefore, it is expected that lesions with previous radiological intervention would be hypoxic and express HIFs. Overall, all HF lesions analysed in this work and all CVMs with pre-operative radiological intervention over-expressed HIFs. In this case, HIFs and especially HIF-2α, which was more widely expressed could represent an index of activity of the CVM lesion and could possibly aid in predicting operative outcomes or simply to aid in the selection of which lesions would be most suitable for operative treatment. All patients from whom the CVM tissue in this study was derived had surgical

management and they had an excellent outcome from their operative management. However, the two lesions that were negative for HIF-1α and positive for HIF-2α required repetitive interventions and suffered exacerbations of symptoms between operations. How best to interpret these findings is difficult but it could be argued that more chronically hypoxic lesions tolerate better external intervention, even if these lesions are HF. The lesions that express less HIFs or no HIF-1α may be more vulnerable to external intervention. Looking at our clinical results, it is possible that even lesions less tolerant to external intervention may become more tolerant in time with successful repetitive interventions. This hypothesis cannot be proven as no tissue is available from lesions assessed to be high risk for surgery or inoperable and their immuno-profile cannot be ascertained. There may be other stimuli influencing HIFs expression, particularly HIF-2α expression in CVMs, which were not addressed in this study.

The results suggest that HIF-2α expression may play an important role in CVM related angiogenesis and that the combined expression of HIF-1α and HIF-2α may be significant in CVM progression and prognosis. There was no expression of CAIX, although CAIX is also a marker of hypoxia. However, absence of CAIX may be significant. HIF-1α is part of the signalling pathway for CAIX, and the absence of CAIX may indicate a less important role for HIF-1α in CVM pathogenesis in comparison to HIF-2α.

The results suggest HIF expression could be a useful target for therapeutic intervention and thus provide a route to cure these lesions and to avoid what may be life-threatening surgery.

6.2 NEP/CD10 EXPRESSION IN CVMs

6.2.1 INTRODUCTION

Neutral endopeptidase (NEP, neprilysin), also known as common acute lymphoblastic leukemia antigen, represents a cell surface zinc metallopeptidase involved in the extracellular metabolism responsible for the regulation of a variety of biologically active vasoactive peptides. Therefore, the presence of NEP may contribute to the regulation of vascular tone and local inflammatory responses in the vascular endothelium and surrounding stroma. Recent studies suggest that NEP may possess anti-angiogenic activity (Sumitomo et al, 2004).

NEP is a membrane protein consisting of three domains: an N-terminal short cytoplasmic domain, a hydrophobic transmembrane domain and a large extracellular domain, which is responsible for the catalytic activity (Sumitomo et al, 2004). NEP has various functions depending on cell type or tissue origin. NEP is implicated in the regulation of the cardiovascular system and is protective in a number of cancers. NEP can influence signal transduction pathways that regulate cell growth (Ganju et al, 1996, Angelisova et al, 1999) and apoptosis (Cutrona et al, 1999). The regulation of NEP expression is not well characterised at the cellular

level but studies suggest a cell type-specific event (Graf et al, 1995). These studies report that NEP is inducible by thrombin via activation of the PKC pathway. NEP is constitutively expressed in a variety of human EC types (Graf et al, 1995) suggesting a systemic as well as a local regulatory role.

In the vascular system, ET-1 is amongst the well known and best researched bioactive peptides reported to be degraded by NEP. ET-1 has an important role in vascular diseases as well as angiogenesis (see sub-chapter 5.4.4) but ET-1 expression or role in CVMs is unknown, as is the expression or role of NEP in CVMs. The cellular concentration of NEP is critical to tissue homeostasis (Turner, 2003). NEP up or downregulation can favour a range of pathological conditions such as cardiovascular, neurodegenerative or malignant, depending on the availability of the peptides acting as NEP substrate.

Another reason to examine NEP expression in CVMs is the growing evidence that up-regulation of NEP expression may relate to tumour progression. Loss or decrease in NEP expression has been reported in a variety of malignancies. Reduced NEP may promote peptide-mediated vascular proliferation by allowing accumulation of higher peptide concentrations, with angiogenic roles (Sumitomo et al, 2004). Tumour progression is based on active angiogenesis, and disregulated angiogenesis appears to be the underlying cause of CVMs. ET-1 absent expression in CVMs was demonstrated in this study (chapter 5) and this could represent a substrate for NEP. NEP is a membrane bound enzyme and can be detected with CD10 antibodies.

6.2.2 OBJECTIVES

The expression of NEP in CVM specimens was investigated using immunostaining techniques with CD10 antibodies. The aim was to elucidate the role of NEP in CVM pathogenesis.

6.2.3 MATERIALS AND METHODS

6.2.3.1. Selection of patients and tissue collection

CVM specimens were obtained retrospectively from archive and prospectively as participants were subjected to surgery for their CVMs. Control specimens were all obtained prospectively. Details of patient selection criteria, consent forms and tissue collection are given in Chapter 2.

In this study, 38 CVM specimens and 16 control specimens excised over a period of 15 years were immunostained for NEP with CD10 antibodies.

Twelve CVM specimens were also stained for HIF-1α and 20 specimens for HIF-2α as described previously and the specimens used to assess HIFs expression were prospectively collected. The majority of these specimens showed expression of HIF-1α and HIF-2α in ECs and stromal cells.

6.2.3.2. Immunostaining for NEP with CD10 antibodies

All specimens were analysed immunohistochemically using standard methods as described in Chapter 2 with the difference that all immunohistochemistry for CD10 was performed using an automated immunostaining machine (see Methods, Chapter 2), which helped to standardise the results. CD10 immunostaining was performed on formalin fixed and paraffin embedded tissue samples. Glomus tumor was used as negative control for immunostaining. For HIFs, the staining technique was developed for frozen sections as it did not work on paraffin embedded tissue.

CD10 stained slides were analysed for localisation of staining and were scored using the traditional scoring system from 0 to 3 as described in Chapter 2. Scoring was based on the proportion of cells with positive surface membrane and cytoplasmic staining. The staining was assessed as to whether was diffuse or localised to endothelial cells, within the vessel wall, stroma or adventitia. Descriptive statistics were calculated for baseline demographic and clinical characteristics of CVMs.

6.2.4 RESULTS

6.2.4.1 NEP/CD10 in CVMs

All CVM specimens analysed in this section were vascular malformations as they did not express GLUT-1 on immunostaining.

Tabel 6. 4. Demographic data of participants in NEP/CD10 analysis

Variable	Control group N=16	CVM group N=38
Age (mean±SD) (Years)	42±18	31±17
Gender Number male (%)	5/16 (31%)	18/38 (47%)

Endothelial cells lining the abnormal vessels of CVMs consistently expressed CD10. This marker was mostly absent or weakly expressed focally by the EC or vessel wall in our control tissues. CD10 was expressed in different types of CVMs regardless of their location (see

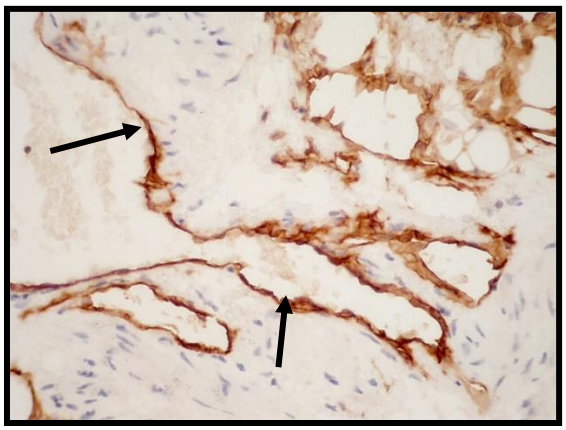
table 6.5). Sixteen % of cases scored 3, 16% scored 2, 63% scored 1 and 5% of cases were negative for CD10.

Tabel 6. 5. NEP/CD10 number and location of specimens analysed

Immunostaining	Number specimens	Location CVMs and numbers	Location controls and numbers
CD 10	38 CVMs	Lower limb 16 Upper limb 14 Head and neck 7 Abdomen 1	Breast 4 Amputation 4 Abdomen 3 AVM 3 Flap 2 Varicose veins 1
	17 control specimens		

In three cases the CD10 staining appeared different from the majority of CVMs, where the CD10 was located at the EC, either diffuse or focal. In these aberrant CVMs the CD10 was located at the vessels adventitia and the vessel itself had no staining.

The expression of CD10 in CVMs was correlated with the number of stromal macrophages (expressing CD68 immunostain) and mast cells (demonstrated with Toluidine Blue stain) but the results were not statistically significant.



x200

Figure 6. 26. Positive expression of NEP/CD10 Immuno-staining in a CVM

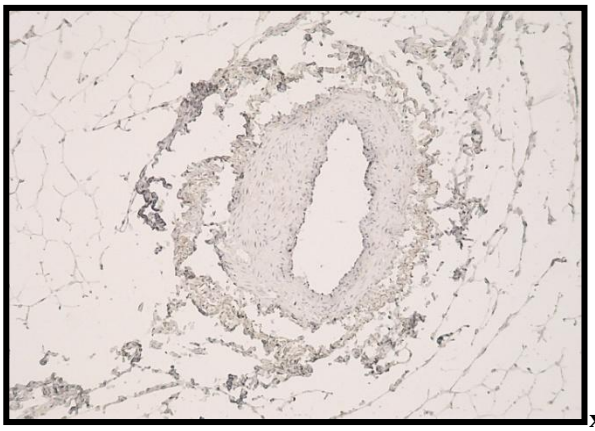


Figure 6. 27. Negative expression of NEP/CD10 Immuno-staining in a skin control

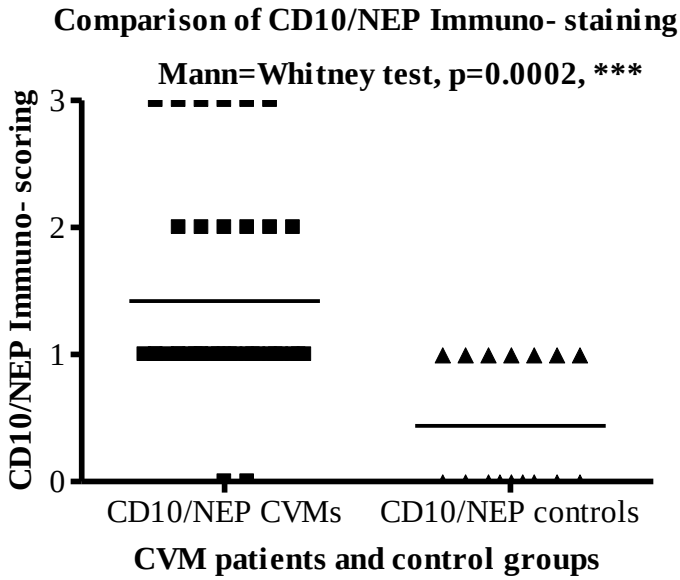


Figure 6. 28. CD10/NEP immuno-staining in CVM and control groups

The figure 6.28 shows that there was statistically significant difference between the immunohistochemical expression of CD10 in CVMs and controls (p=0.0002).

6.2.4.2 Correlation of HIFs with CD10/NEP in CVMs

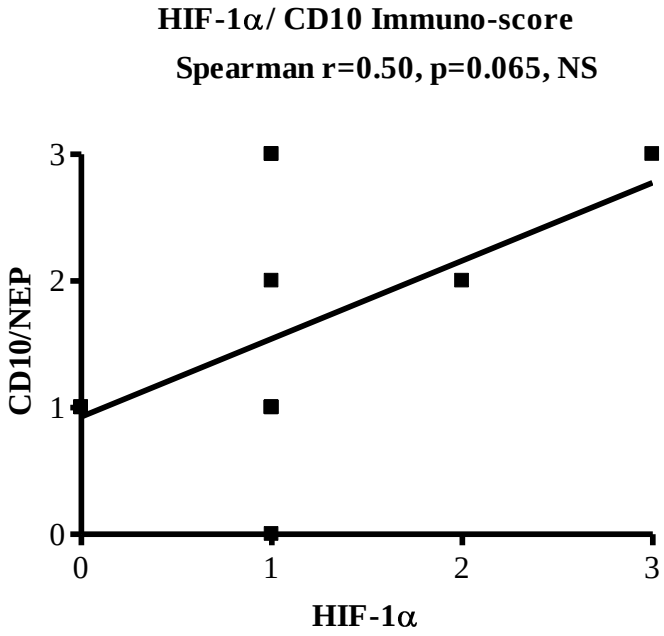


Figure 6. 29. Correlation of HIF-1α with CD10/NEP in CVMs

The figure 6.29 shows that there was no statistically significant correlation between the immunohistochemical expression of HIF-1α and CD10/NEP in CVMs (p=0.065).

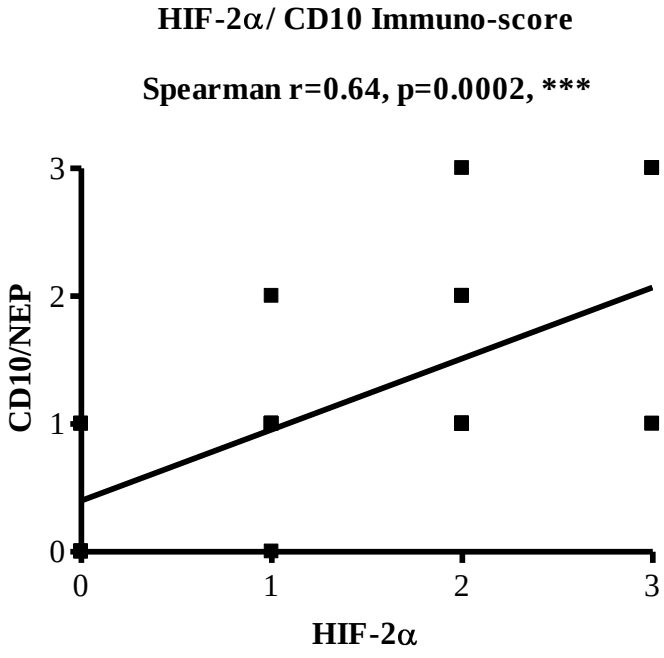


Figure 6. 30. Correlation of HIF-2α with CD10/NEP in CVMs

The figure 6.30 shows that there was statistically significant correlation between the immunohistochemical expression of HIF-2α and CD10/NEP in CVMs (p=0.0002).

6.2.4.3 Correlation of CD10/NEP with the significant stromal inflammatory cells in CVMs

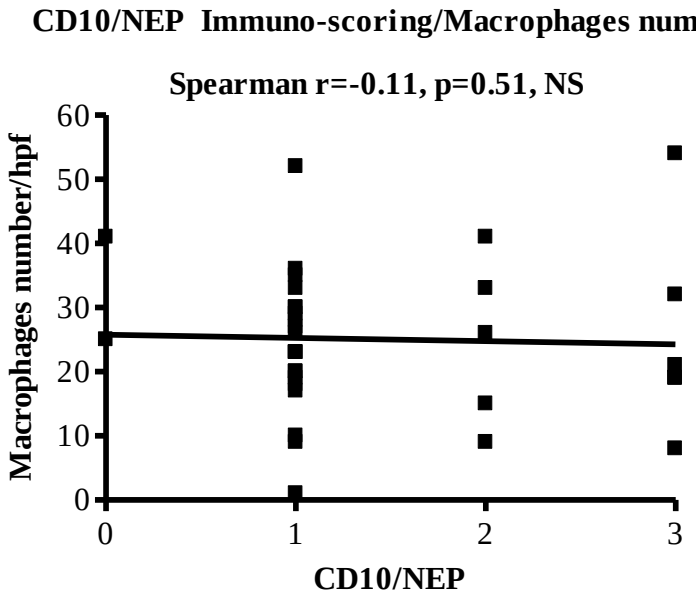


Figure 6. 31. CD10/NEP immuno-scoring and macrophages number in CVMs

The figure 6.31 shows that there was no statistically significant correlation between the immunohistochemical expression of CD10/NEP and macrophages number in CVMs (p=0.51).

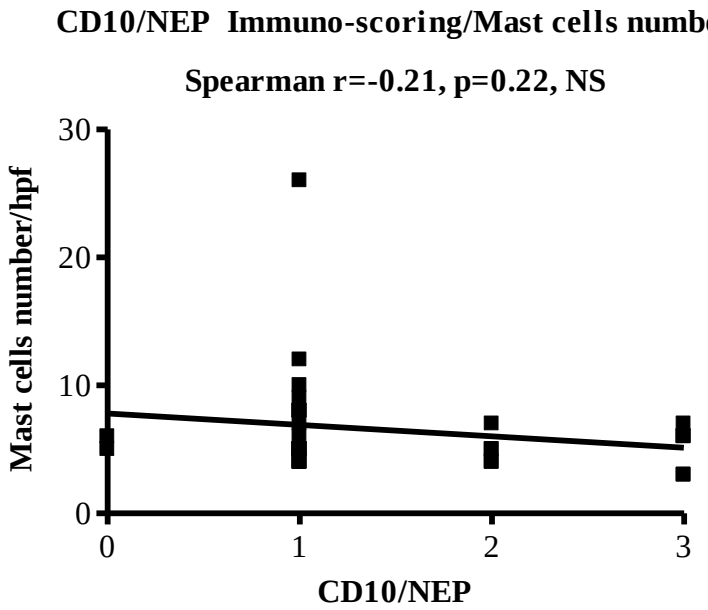


Figure 6. 32. CD10/NEP immuno-scoring and mast cells number in CVMs

The figure 6.32 shows that there was no statistically significant correlation between the immunohistochemical expression of CD10/NEP and mast cells number in CVMs (p=0.22).

6.2.5 DISCUSSION

The results from this study indicated NEP over-expression in CVMs, implicating NEP in the pathophysiology of CVMs and possibly suggesting a role for NEP inhibitors in CVM treatment. Recently, there has been a considerable interest in NEP inhibitors due to physiological importance of NEP in the modulation of nociceptive and vasoconstrictor responses.

The over-expression of CD10 coexisted with a prominent stromal inflammatory infiltrate and expression of HIFs, especially HIF-2α in CVMs.

It is not known if hypoxia is the cause of HIF expression in CVMs. HIFs could also be expressed as a result of tumor suppressor gene inactivation or other conditions inducing expression (Talks et al, 2000). The results of the analysis of blood gases in CVMs are given in the sub-chapter 6.3. If hypoxia is a feature of CVMs, this is more likely to be chronic or at most, acute on chronic, given the chronicity of these lesions. Recent studies have shown the effect of chronic hypoxia on the expression of HIF-1α and HIF-1α target genes in various tissues (Tipoe et al, 2006). HIF-1α regulates the gene expression of several vasoactive substances, including ET-1. Since the transcriptional regulation of several genes involved in angiogenesis induced by hypoxia is mediated by HIF-1α, it is conceivable that ET-1 expression might be regulated by the same oxygen-dependent mechanism. Chronic hypoxia may be an important stimulus for ET-1 release (Ferri et al, 1995) and this study has demonstrated ET-1 negative expression by CVMs (see Chapter 5). This raises the possibility that NEP may have a role in ET-1 degradation in CVMs but this is unknown and NEP may have more than one function in these lesions.

NEP/CD10 expression has been shown to be absent in some poorly differentiated tumours, allowing mitogenic peptides to drive cell division. In absence of NEP in malignant tumors is linked to poor outcomes. This would suggest that angiogenesis in CVMs is controlled by specific signalling pathways, which are different from those governing angiogenesis in tumours.

NEP up-regulation in CVMs in the presence of a pro-inflammatory infiltrate, expression of HIFs and absent expression of ET-1 in CVMs implies that the hypoxia/NEP pathway may play a part in the physio-pathology of CVMs. Stimulation of NEP expression by thrombin, which is produced during acute vascular events, may limit the local effects of pro-inflammatory or other vasoactive peptides, within CVMs.

It is clear that further work is necessary to establish clearer pathways. Animal studies in the literature have shown that hypoxia may down-regulate NEP (Wang et al, 2010) but clearly, this is not the case in CVMs.

At present it is not possible to judge whether NEP has a positive or negative effect or not. NEP expression may be in response to the need for ET-1 degradation, so that one of the angiogenesis signalling pathways is blocked and thus the CVM is maintained in a quiescent or less active status. If this is the case NEP has a positive role in CVM pathophysiology and its inhibitors will have no place in the management of these lesions. The importance of ET-1 in CVM angiogenesis in comparison to other pro-angiogenic markers is not known. The outcome of prevention of ET-1 degradation by NEP in CVMs is also unknown. NEP expression may be part of a different signalling pathway in CVMs and its effect whether advantageous to benefit the patient or not is unknown. The physiological relevance of NEP in CVMs is speculative.

6.3 BLOOD GASES ANALYSIS IN CVMs

6.3.1. INTRODUCTION

Oxygen is necessary for cells/tissue to survive. It is transported by arterial blood to the peripheral tissue after gas exchange at the pulmonary level following ventilation and diffusion of the oxygen from the atmosphere into the arterial blood. Oxygen is delivered to the tissue (by a process dependent on many local factors) usually following a gradient at the capillary level. Therefore, venous blood transported from periphery to heart and lungs will contain less oxygen. The exchange will take place again at the lungs level and the process is continuously repeated.

In AVMs there is shunting of the capillaries. From the arterioles, blood is drained directly through the abnormal communications to the venules. Therefore, it is possible that oxygen extraction by the peripheral tissues does not take place, nor the usual gradient. Random sampling over the years of the CVM blood during their operative treatment and as part of their management, by the surgeon supervising this study (NJS), indicated that the blood within the vessels of a CVM has an arterial pattern. This observation was valid even in VMs where there is no apparent blood shunting from the arterial to the venous system to explain such findings. Blood from the abnormal vessels of CVMs, showed results within the normal range for arterial oxygen pressure (10.7-14kPa). This occurred even when blood was obtained from predominantly venous lesions, where oxygen pressure will be expected to be reduced at 5.3-6.7 kPa. Furthermore, the blood from CVMs had the macroscopic features of bright red colour associated with arterial blood.

This finding may be explained by blood shunting capillaries where gas exchange normally takes place or oxygen delivery is hampered in abnormal blood vessels of CVMs (perhaps CVMs are well oxygenated lesions and there is no oxygen gradient to facilitate diffusion to the tissues, etc)

There are no studies in the literature assessing how well oxygenated is the blood within the CVM vessels or to assess the degree of oxygenation of CVM tissue. Presuming that CVMs have an underlying mechanism of disregulated or 'increased' angiogenesis, one would expect CVMs to have hypoxic features since angiogenesis and hypoxia are directly linked.

It is not known if hypoxia is a feature of CVMs, if blood within CVM vessels is oxygenated or whether hypoxia-regulated molecules such as HIFs are expressed by all CVMs. This work aimed to establish if hypoxia is an element of CVMs by directly measuring the oxygen pressure within abnormal blood vessels of CVMs during their operative management. This information was correlated with the expression of hypoxia-regulated transcription factors (HIF-1α and HIF-2α) and with the expression of the selected angiogenesis markers (VEGF A /VEGFR-1, Tie-2, ET-1, and the integrins αVβ3 and αVβ5), detected immunohistochemically.

6.3.2 OBJECTIVES

1) To identify hypoxia-mediated responses relevant to CVMs by establishing whether hypoxia is a feature of CVMs by measuring oxygen pressures within abnormal blood vessels of these lesions and as well as arterial and venous systemic blood.

2) To correlate oxygen pressures within CVMs with the expression of HIFs by CVM tissue.

Three comparisons were made in patients with CVMs:

a) Paired comparison of arterial oxygen pressure in the radial artery and the CVM blood vessels in patients undergoing surgical correction of their CVM.

a) Paired comparison of venous oxygen pressure in the venous systemic blood (ipsilateral to the CVM as well as from the contra lateral side to the CVM) and the CVM blood vessel in patients scheduled for surgical correction of their CVM.

6.3.3 MATERIALS AND METHODS

6.3.3.1. Selection of patients and blood sample collection

This was a prospective study involving blood samples collected from subjects affected by CVMs between 2006 until 2009. A total number of 13 participants were recruited for this study. All details regarding selection of patients, consent forms and blood samples collection are given in Chapter 2.

6.3.3.2. Partial oxygen pressure (pO_2) comparisons

Comparisons were made between the partial oxygen pressures within CVM blood and controls represented by systemic blood from the same patient from an artery and two veins with different locations – one on the same side as the CVM and one on the opposite side. The pO_2 does not reveal how much O_2 blood contains but only the pressure exerted by dissolved O_2 molecules against the measuring electrode. The pO_2 compared were considered in mmHg and therefore the results obtained in kPa were converted in mmHg (760mmHg = 101.325kPa). The pO_2 were measured for each patient at different fractions of inspired oxygen (FiO_2), due to anaesthetics protocols. Therefore, the pO_2 were adjusted for a fraction of inspired oxygen of 100% knowing that each 10% increase in the fraction of inspired oxygen wil increase the partial oxygen pressure with 50-60mmHg. At any altitude, the FiO_2 is 0.21.

6.3.4 RESULTS

The demographic data of the patients recruited in this study is given in the following table.

Tabel 6. 6. Demographic data of patients recruited in blood gas analysis study

Variable	CVM group N=13
Age (mean±SD) (Years)	41±23
Gender Number male (%)	9/13 (69%)

Tabel 6. 7. pO_2* within CVM and systemic blood and HIFs staining

Patient	CVM type	pO_2 CVM	pO_2 arterial blood	pO_2 venous blood same side with CVM	pO_2 venous blood opposite side with CVM	HIF-2α	HIF-1α	CAIX
1	LF	74	207	170	85	+		0
2	HF	506	577	371	509			0
3	HF	200	344	369	206	+++		0
4	LF	462	402	316	418	+++	0	0
5	LF	410	339	186	292			0
6	HF	412	374	311	330			0
7	HF	485	568	374	363			0
8	HF	451	479	434	413			0

9	LF	449	324	375	321			0
10	LF	419	487	451	388			0
11	LF	453	369	246	241	+	+	0
12	LF	425	277	288	260			0
13	HF	385	290	214	196			0

* Normal pO_2 at FiO_2 100% is 500-600mmHg; * pO_2 are calculated / adjusted at FiO_2 of 100%.

The comparisons between the adjusted pO_2 within CVM blood vessels and arterial blood as well as venous blood on the same side as CVM and venous blood on the opposite side to CVM are given in the following three graphs.

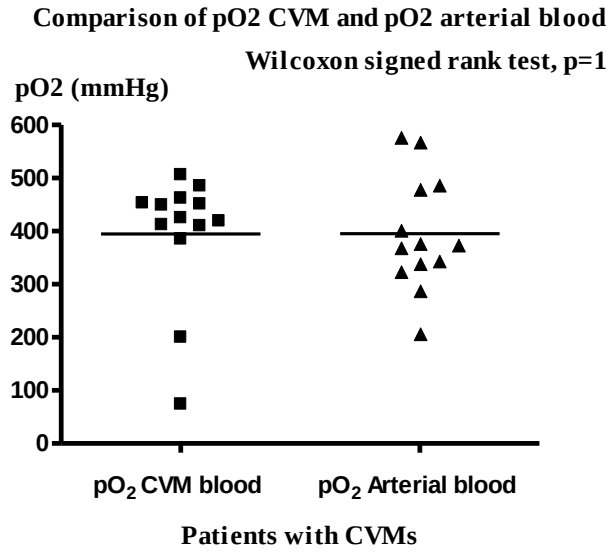


Figure 6. 33. Comparison of CVM pO_2 and arterial blood pO_2 data

The figure 6.33 shows there was no statistically significant difference between pO_2 in CVMs and arterial blood (p=1)

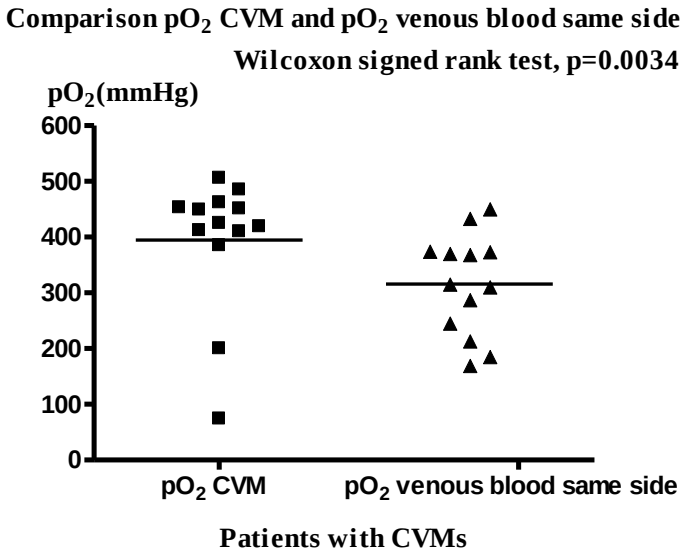


Figure 6. 34. pO_2 CVM and venous blood on the same side with CVM

The figure 6.34 shows there was statistically significant difference between pO_2 in CVMs and venous blood ipsilateral to CVMs (p=0.0034).

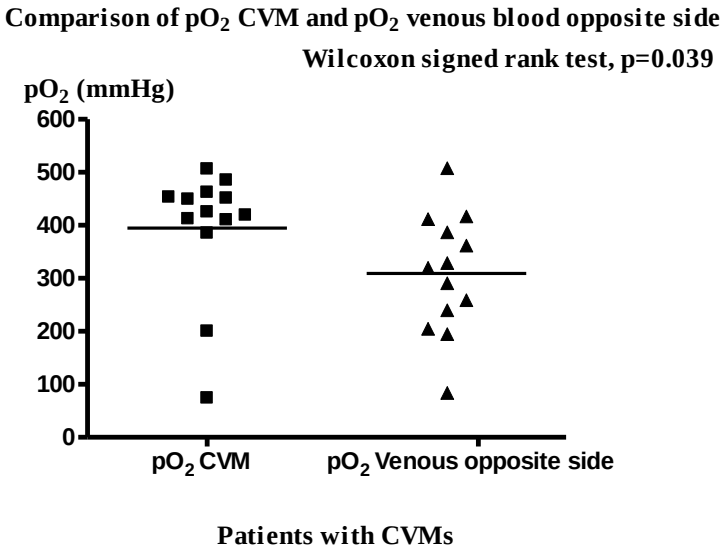


Figure 6. 35. pO_2 CVM and venous blood opposite side to CVM

The figure 6.35 shows there was statistically significant difference between pO_2 in CVMs and venous blood contra lateral to CVMs (p=0.039).

6.3.4.1 Correlation of pO_2 within CVM vessels with HIF-1α and HIF-2α immunostaining of the corresponding tissue.

Since the numbers are small it was not possible to perform a statistical analysis of the correlation between pO_2 within CVM vessels and HIF-1α and HIF-2α immunostaining. The two

studies were performed at different times and this allowed only 2 and 4 paticipants respectively, to have had HIF-1α and HIF-2α immunostaining as well as pO_2 measured. However, the results are presented here. HIF-1α and HIF-2α immunostaining were interpreted using a scoring system from 0 to 3 as described earlier. HIF-1α was expressed in one of the two cases tested for both HIF and pO_2 and scored 1. Both specimens tested were clinically LF vascular malformations. Overall HIF-1α was expressed by CVM tissue, although most specimens scored one. The corresponding pO_2 within CVM vessels, for the two common specimens were similar (462 and 453mmHg). Four cases tested in the oxygen study had HIF-2α immunostaining and the scores were 1, 3, 3 and 1. One specimen was clinically a HF CVM and the rest were LF vascular malformations. The corresponding pO_2 for the four cases stained for HIF-2α varied between 74 and 462 mmHg. There appeared to be no correlation between the intensity of the HIF-2α staining and pO_2 within the CVM vessels. HIF-2α appeared to be over-expressed by CVMs, in HIF study, despite there being a pO2 range in these lesions comparable with arterial systemic pO_2. These data indicate that there is no correlation between pO_2 in CVM vessels and HIF-1α or HIF-2α expression on immunostaining or the clinical type of CVM.

6.3.5 DISCUSSION

There was no statistical difference between the pO_2 within CVM vessels and arterial vessels ($p = 1$). The CVM blood appears to be oxygenated even in LF vascular malformation where there should be no vascular shunting. The ratio LF: HF vascular malformations was 7:6, and the values of pO2 within CVM vessels were even. This supports the clinical suspicion that CVM blood resembles arterial blood. There appears to be no relationship between the pO_2 within CVM vessels and the clinical type of CVM, i.e. HF or LF due to similar values of the pO_2 within CVMs.

Comparison of pO_2 within CVM vessels with that in venous blood ipsilateral and distal to the lesion, showed there was a statistically significant difference with $p = 0.039$. This difference was even higher when the comparison was made with the pO_2 within the venous blood on the contra lateral side ($p = 0.01$). The further away the vein, from which the blood sample was taken the higher the difference between the pO_2 within the CVM and the venous blood. This suggests hyper oxygenation of blood within the CVM as well as surrounding vessels. However, expression of HIF-2α in CVM tissue obtained from patients in whom the pO_2 was measured, suggested the tissue was hypoxic. The HIF-1α was weak or absent. HIF-2α was tested in our study in CVM lesions from a number of sites and regardless of the location, patient's age or sex, it was over-expressed in the majority of lesions. HIF-1α was also tested but this was generally weaker than HIF-2α or even absent. Since CVMs represent a network of abnormal blood

vessels, which are arguably the product of abnormal angiogenesis, it is theoretically possible that CVMs are hypoxic when active, given the close relationship between hypoxia and angiogenesis. All CVM lesions included here were clinically considered to be in their quiescent phase as surgery to active lesions could exacerbate the morbidity, especially in AVMs. The assessment of cell proliferation using ki67 immunostaining showed no significant expression in CVM, when compared to controls which supported the clinical assessment of the quiescent phase. The question arises whether the observed HIF expression in CVMs reflects the in vivo situation or not and whether CVMs are hypoxic lesions or not. HIF expression in CVM tissue could take place in absence of hypoxia if VHL gene expression is disregulated. VHL gene expression in CVMs is unknown. Blood shunting capillaries where the gas exchange normally takes place at tissue level could explain the discrepancy between high pO_2 in CVM vessels and HIF expression in some CVMs where there is shunting, but not in all lesions. As CVMs are composed of abnormal blood vessels, the O_2 delivery to the tissue could be deficient if CVMs are well oxygenated lesions, there may be no oxygen gradient to facilitate O_2 diffusion to the tissues, which is the main mechanism of O_2 delivery. It is not possible from these data to determine which mechanism if any applies to CVMs or whether multiple mechanisms are responsible. Nevertheless, HIF-2α overexpression in CVMs could be investigated further and possibly exploited as target therapy for CVMs.

CHAPTER 7

7. FINAL DISCUSSION

CVMs are rare lesions but they pose serious challenges in their clinical management and therefore, these patients are usually seen in specialist centres by consultants. The decision whether or not to treat CVMs and especially AVMs is crucial because if the lesion is inappropriately subjected to any treatment – radiological or surgical- symptoms may be exacerbated, leading to complications, increased morbidity and even mortality. This course of events occurs due to lack of experience with these lesions. Medical personnel specialised in the management of CVMs have learnt by experience which CVMs are 'not to be touched', although not with 100% accuracy. Better understanding of the biology of CVMs is required to achieve better therapeutic results and to find alternative management possibilities.

The exact cause of CVMs is not clear but it is likely to be a multifactorial process. What signalling pathways are altered in the process of angiogenesis leading to CVMs or what is the primary causation it is not yet known. Most theories regarding the aetio-pathogenesis of CVMs (Chaper 1.8) are addressing haemangiomas and not the AVMs, which represent the real problem. It has been suggested the original defect involves the intrinsic properties of EC but no research has proven this theory. From our research data it resulted that the stromal infiltrate was associated with the imbalance of the angiogenic and hypoxia mediators, implicated in the development of vascular malformations. The paucity of AVM data in the literature is partly caused by the lack of AVM tissue available for research all over the world. This is due to the fact that true AVMs often rapidly progress when operated by un-skilled hands and there is a general acceptance, although not universal, amongst specialists that AVMs should be managed non-surgically. To avoid exacerbation or activation of AVM, the whole lesion has to be removed at one operation, or debulking in a few serial surgical procedures performed until the whole lesion is removed. This is often quite difficult as the lesion tipically invades important or vital structures. Torrential bleeding is one of the most fearful encounters for any surgeon when embarking on such surgery. Fig 7.1 illustrates the extent of haemorrhage surgeons encounter when operating CVMs, and represents 'a sample' of the clinical experience of one senior surgeon and expert in the field (NJS). In this series of 73 operated CVMs, 16 were complicated by major haemorrhage (Maftei et al, 2009).

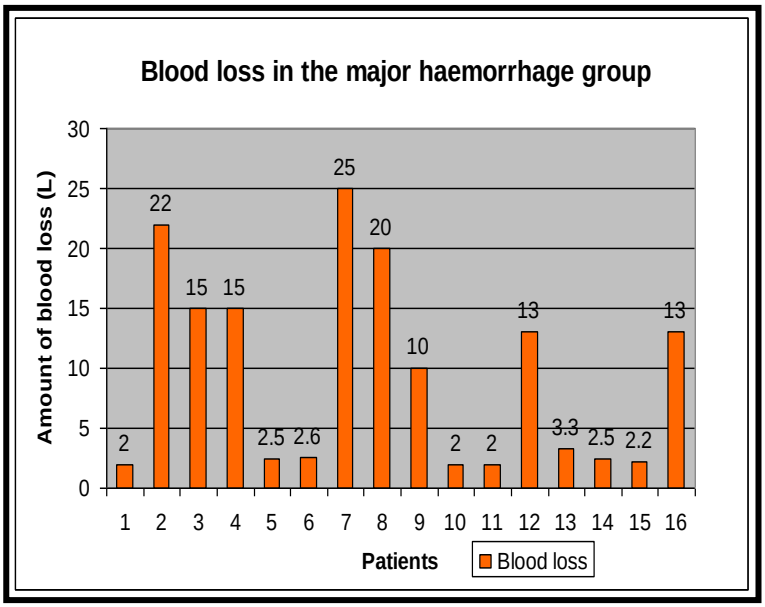


Figure 7. 1. Report of a major haemorrhage group at surgery

The use of cell-saver (equipment allowing patient own blood to be transfused back to the patient after a process of filtration) is common when operating on these lesions, as well additional transfusion of blood and blood products and the requirement of a very experienced anaesthetist with CVMs.

Figure 7. 2. Cell-saver used during a CVM operation

There was and still is confusion about the classifications of CVMs and what some authors describe in their papers as AVMs are in fact haemangiomas, which are usually self-limiting and do not pose as much clinical difficulties as AVMs. Previous research work has addressed mostly haemangiomas. The classification of CVMs has changed extensively over the years (see

Chapter 1.2) and although the Hamburg classification is now generally accepted and also the current ISSVA is increasingly accepted, many authors do not adhere to either of these classifications. Therefore, it is difficult to know what category of lesion they are referring to and often the terminology used for the lesions described is erroneous or mis-leading, leading to mis-interpretation of research work.

The samples analysed for this study came from CVM tissue carefully collected through the Tissue Bank at Hammersmith Hospitals NHS Trust over many years. The results sustain the hypothesis that CVMs are most likely the result of aberrant vessel angiogenesis. The embryonic vascular system develops during the 3rd week of foetal life from mesodermal cells and represents the field of complex interactions between pro- and anti-angiogenic factors, which are numerous. To date, there is no reported study investigating pathways of abnormal angiogenesis in CVMs. This work does not scientifically overlap with any work reported in the literature.

The detailed histological analysis of CVM morphology clearly demonstrated that the number of blood vessels per mm^2 and the vessel diameter (μm) in CVMs were significantly higher than in control tissue with $p=0.0004$ and $p=0.0005$ respectively. These findings represent the effect of the prominent inflammatory infiltrate in the CVM stroma (Chapter 4) or may be due to the increased expression of various categories of pro-angiogenic mediators (Chapter 5). There were no morphological features to distinguish different clinical categories of CVMs but the morphological criteria continue to remain the basis for the confirmation of the clinical diagnosis of CVM. This study demonstrates the necessity of a novel classification of CVMs that ideally will close the perceived gap between the histopathologist and clinician. This classification is likely to be a molecular one and it should also have prognostic value.

Abundant macrophages and mast cells were found in the stromal tissues of CVMs, and both cells types have recognised roles in angiogenesis. Macrophages have an important role in sustaining angiogenesis partly by producing VEGF. They are likely to act as promoters of angiogenesis when exposed to a hypoxic environment, by interacting directly with HIF or with other angiogenic pathways such as the VEGF pathways. Previous studies have shown that mast cells accumulate at sites of angiogenesis and this study showed increased number of mast cells in close vicinity to abnormal blood vessels constituting the CVM. Changes in the mast cells density at the time of capillary proliferation indicate a special role for these cells in the formation of new vessels. Angiogenic mediators secreted by mast cells such as VEGF have been implicated as mast cells chemotactic factors.

CVMs are represented by dilated abnormal blood vessels and they may be more permeable vessels, allowing cells and chemical mediators to escape from the blood stream. The leaky vessels may also be the reason for the increased oxygen pressure (demonstrated in Chapter 6)

within the CVM vessels. The presence of the stromal inflammatory infiltrate may be reactive in response to hypoxia or tissue expression of HIFs. It is also possible that areas of hypoxia in CVM stroma develop as a result of increased metabolism associated with the inflammatory cells. The vessels of the CVM and the infiltrate of mast cells and macrophages in the stroma must be related. The following figure suggests a possible relationship between the inflammatory infiltrate, tissue hypoxia and blood vessel morphology.

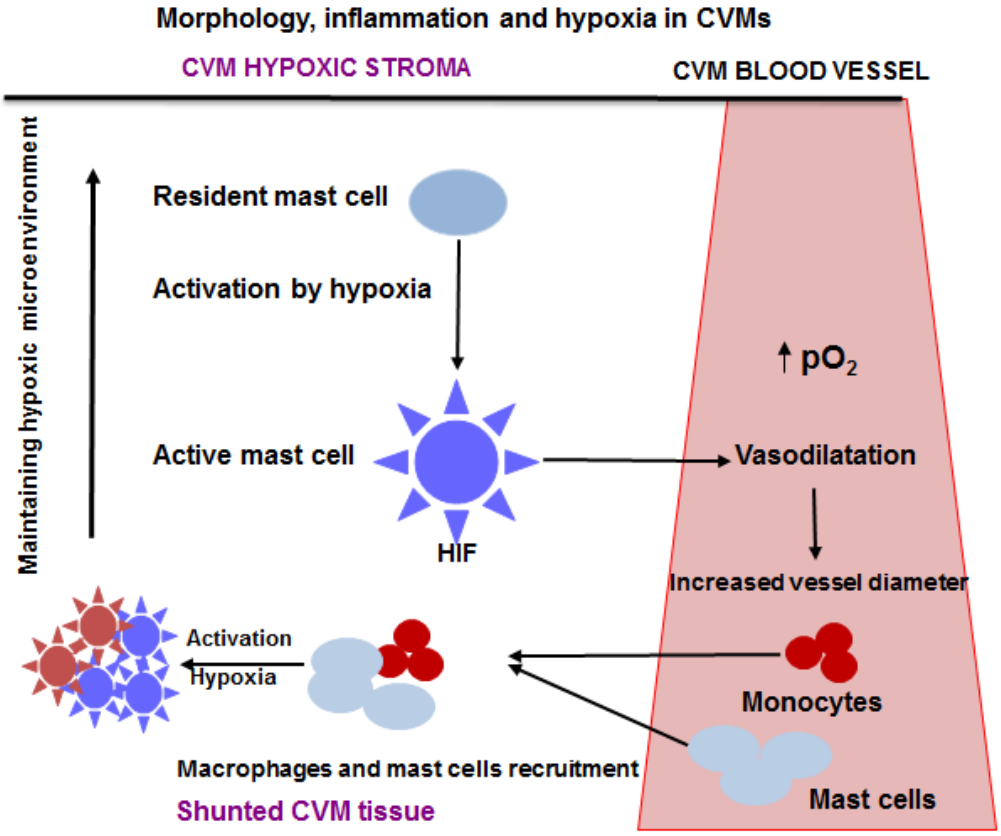


Figure 7. 3. Vessel morphology, inflammation and hypoxia in CVMs

Hypoxia induces expression of numerous angiogenic mediators as a result of stabilisation of HIFs. The distribution of specific angiogenesis markers and their relationship to hypoxia markers HIFs in CVMs and controls were investigated using immunohistochemistry. VEGF-R1, Tie-2 and αVβ3 and αVβ5 were over-expressed by ECs in CVMs. The immuno-expression of all these angiogenic markers was significantly higher in CVMs than in control tissues. All these mediators were localised on the EC of the CVMs.

VEGF-A, one of the most important regulators of angiogenesis, was expressed by the ECs in all categories of CVMs but not in every lesion (see results Chapter 5).

Despite the increased number of vessels in CVMs, the majority of the cases examined,

showed nuclear expression of HIF-1α and more widespread expression of HIF-2α which was observed in the ECs as well as in the stromal macrophages. Normal control tissue showed only very focal expression of HIF in relation to adnexal structures or macrophages. There was a significantly higher immuno-expression of HIF-2α in CVMs comparing to controls (p=0.0004), although there was no significant difference between the expression of HIF-1α in CVMs comparing to controls (p=0.66). HIF-2α appears to play a more important role in CVM pathogenesis than HIF-1α. HIF-2α is related more to a pathological hypoxia and HIF-1α is related more to physiological responses to hypoxia.

These data demonstrate that HIF activation is present in CVMs and hypoxia may be a key feature of CVMs and may regulate important inflammatory and angiogenic cytokines. This statement is supported by the positive correlations found between HIF-2α and VEGF A (p=0.0001), VEGFR-1 (p=0.0043), Tie-2 (p=0.03), αVβ3 (p=0.029) and αVβ5 (p=0.045) immunohistochemical expression in CVMs as well as the positive correlations between the immuno-expression of HIF-2α and the number of stromal macrophages and mast cells within the CVM stroma. The question is whether hypoxia as indicated by expression of HIF is part of the adaptative processes secondary to other changes such as inflammatory cell extravasations in CVMs, or whether hypoxia is a primary feature of CVMs that triggers all other changes. The first hypothesis seems more likely with the current available data. Physiological hypoxia in CVMs could not be demonstrated and high partial oxygen pressures were found within CVM vessels. CVMs contain a higher number of blood vessels per mm^2. As discussed, other mechanisms could be responsible for primary hypoxia/HIFs expression in CVMs such as a defect in the oxygen delivery mechanisms to tissue or even a mutation of VHL, the regulatory gene for HIFs. Experiments to explore these hypotheses were outside the scope of this study.

The expression of NEP was assessed by immunohistochemistry, on CVM tissue sections and controls. NEP was identified using the CD10 marker. NEP is a metallopeptidase known to modulate bioavailability of peptide mediators and growth factors in hypoxic conditions. NEP has been shown to degrade endothelin ET-1, which has important roles in angiogenesis, as well controlling inflammatory responses. We have observed over-expression of CD10 in all CVMs analysed. The expression of CD10 was associated with a prominent stromal inflammatory infiltrate and expression of HIFs, especially of HIF-2α, in CVMs. There was a positive correlation between the immunohistochemical expression of HIF-2α and CD10 in CVMs (p=0.0002).

From these results, hypoxia/NEP/ET-1 emerges as possible signalling axis implicated in pathophysiology of CVMs.

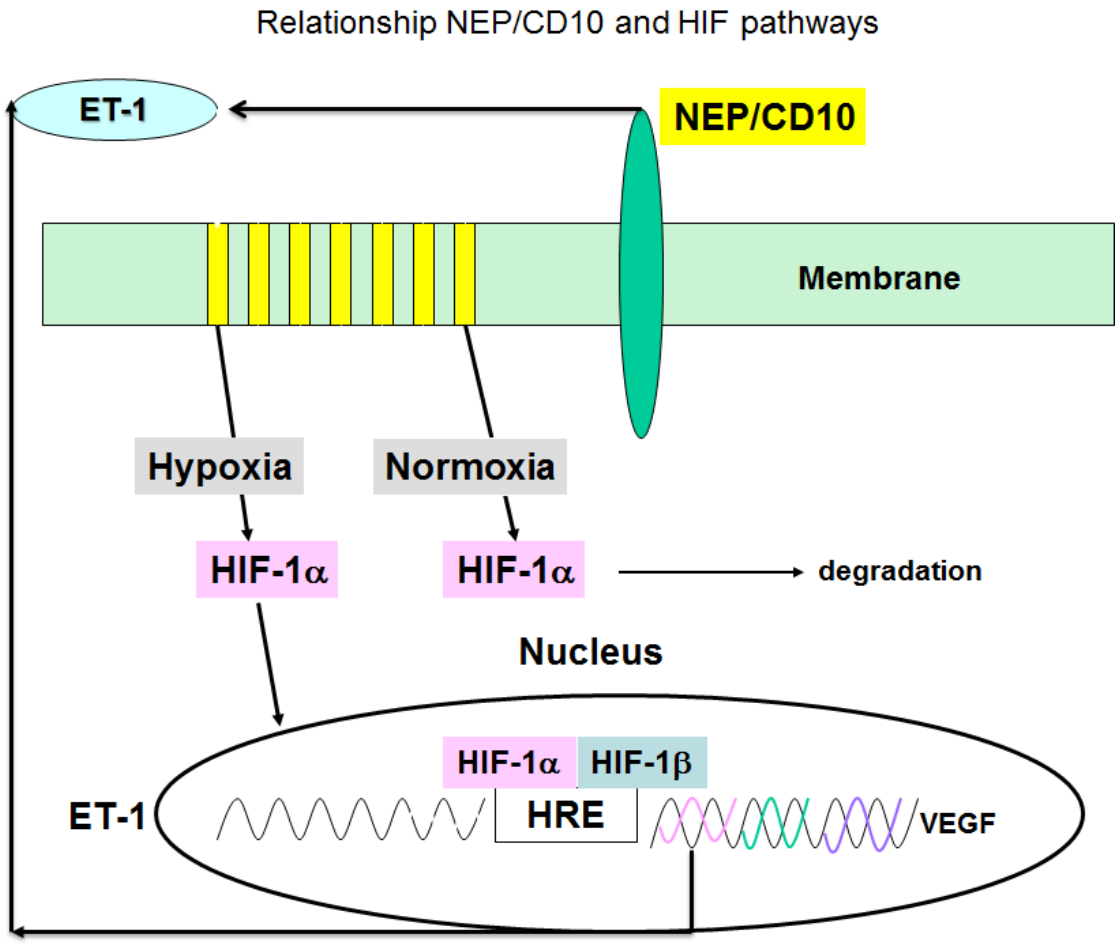


Figure 7. 4. Hypoxia/NEP/ET-1 signalling pathways in CVM pathophysiology

Neutral endopeptidase indirectly interacts with HIF pathways, when HIF is up-regulated. This interaction is known to involve HIF-1α and it is not yet known whether HIF-2α is also implicated. Stabilised HIF-1α leads to the transcription of target genes, including ET-1 and VEGF genes showed in the diagram above. NEP degrades ET-1 and therefore, inhibits its pro-angiogenic pathway.

CD10 expression has been reported as decreased or absent in some poorly differentiated tumours. This would suggest that angiogenesis in CVMs is controlled by specific signalling pathways which are different from those ruling angiogenesis in malignant tumours.

It had been observed clinically that CVM blood even in LF lesions such as VMs demonstrated high oxygen pressures (Prof. Standfield personal communication). Therefore, the pO_2 within the abnormal CVM blood vessels was compared with the pO_2 within veins in close proximity to CVM and, ipsilateral as well as with the pO_2 in veins distal to CVM, usually contra lateral to the CVM. This study confirmed the clinical observation and proved that there was no

statistical difference between the partial oxygen pressure within CVMs vessels and arteries. It was not possible to prove an influence on the nearby venous oxygen pressure because the oxygen pressure within CVM vessels was statistically significantly different to venous blood both on the same side as CVM ($p=0.01$) and on the opposite side to CVM ($p=0.03$). It was not possible to compare pO_2 and HIF-1α and HIF-2α immunohistochemical staining for four patients included in this study. Whilst HIF-2α was positive in all four cases with variable intensity of expression, HIF-1α was weakly positive in one case only. The cases positive for HIF-2α comprised HF and LF lesions from both upper and lower limb.

The data of HIF-1α and HIF-2α expression generated by this study suggests at least two categories of CVMs. The majority of lesions were positive for both HIF-1α and HIF-2α but HIF-2α was more prominent. Other lesions were HIF-2α positive and HIF-1α negative. These categories were confirmed by the results of Western blot analysis in a group of participants. There was one lesion which did not express either marker but this may represent processing artefact as the case came from the archive. Both HIF categories of CVMs showed heterogeneity in the expression of different angiogenic markers, although in the majority of specimens all markers were up-regulated. All specimens expressed Tie-2, although with different intensity. One particular HF lesion located in the upper part of the lower limb was notoriously difficult to manage. This lesion diffusely expressed all the angiogenesis markers tested and expressed HIFs, but αVβ5 was absent. This lesion had multiple embolisations prior to each surgical intervention. A clinically quiescent status was achieved after many years and repetitive interventions. Two other lesions, which clinically were similar, did not express αVβ3. These were intra-muscular CVMs and they both had pre-operative sclerotherapy. One was located in the upper limb and one was in the lower limb. One other lesion that was extremely difficult to manage clinically did not express VEGFR-1 and HIF-2α was over-expressed but there was insufficient tissue available to assess HIF-1α expression. This patient achieved quiescent status after multiple upper limb resections. One patient did not survive surgery and the resected tissue was strongly positive for HIF-2α.

To summarize the clinical outcome of the cohort of patients studied quiescent status was achieved in all patients and 12 (32%) patients (from all 38 patients considered in various parts of this study) were cured. The CVMs that were cured had mostly moderate expression of both HIF-1α and HIF-2α.

The possible relationship between the inflammation, angiogenesis and hypoxia as discussed in this study is shown in the following diagram.

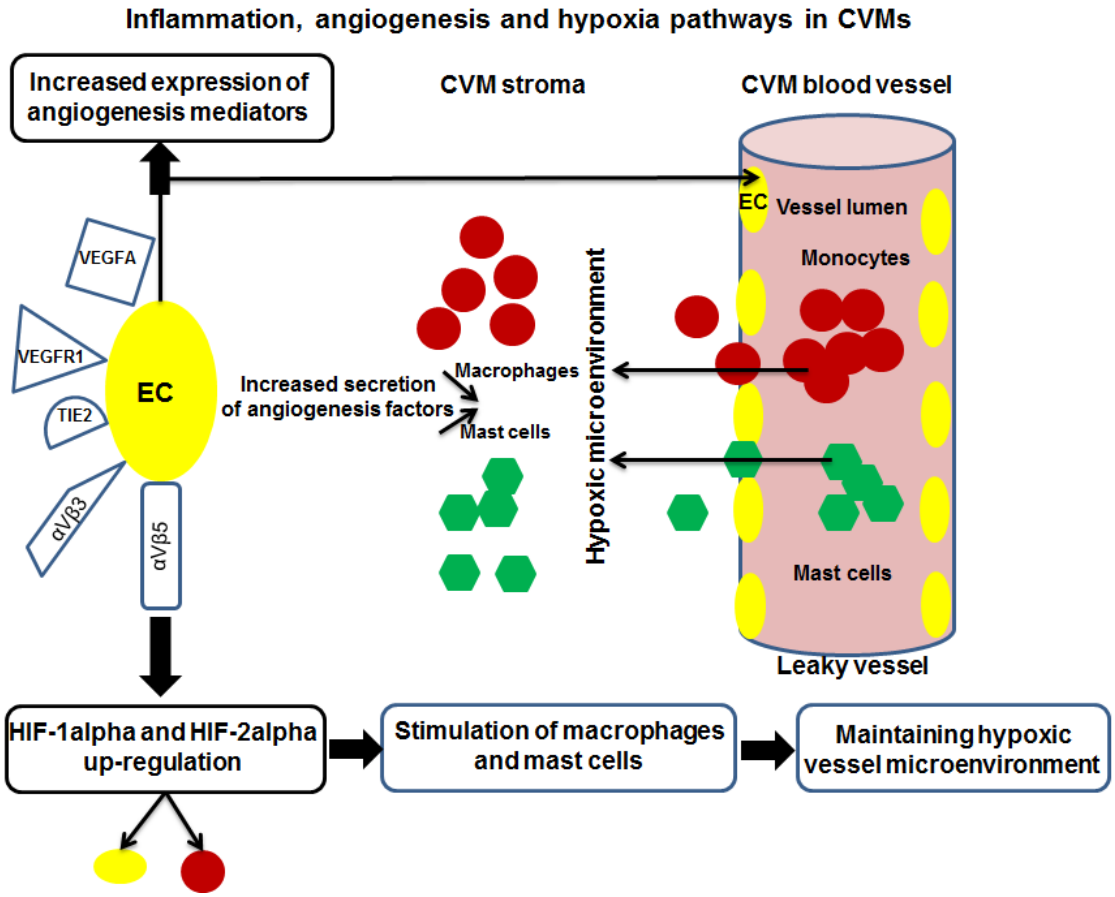


Figure 7. 5. Inflammation, angiogenesis and hypoxia signalling pathways in CVMs

The morphological heterogeneity of the lesions studied suggests that specific molecular patterns may exist within vascular malformations, which may result in further clinical sub-classification. The results point towards possible specific molecular mechanisms and the application of these findings in clinical practice would help to validate these results, as well as to elucidate the molecular mechanisms in CVMs.

This study has helped to improve the understanding of the aetio-pathogenesis of CVMs. As more mechanisms associated with angiogenesis are clarified a new approach to the understanding and subsequent management of vascular malformations will emerge.

7.1 EMERGING NEW MARKERS FOR VASCULAR MALFORMATIONS

There is a need for specific molecular markers not only to differentiate haemangiomas from vascular malformations but also to differentiate different categories of CVMs in order to predict behaviour and outcome. Arguably, this is achievable once the signalling pathways responsible for CVM development and perpetuation are established. The immunohistochemical marker GLUT-1 has been reported to accurately distinguish haemangiomas from vascular malformations. This marker has been used here to confirm the specimens analysed were vascular malformations and not haemangiomas, (vascular malformations do not express GLUT-

1). However, GLUT-1 does not give any other information about the lesion in respect to its behaviour or prognosis.

Consistent positive staining for NEP in the ECs or, in some cases vessel adventitia of CVMs, implies a role for NEP/CD10 in CVM pathophysiology and emerges as a potential new diagnostic marker for CVMs biopsies. Tissue markers are rarely used for diagnostic purposes, they help to determine prognosis, to monitor treatment and detect recurrences. They may be used for diagnosis in conjunction with other parameters. Very few tissue markers are used in clinical practice because of lack of specificity. Some non-specific markers have found a place in monitoring treatment. NEP/CD10 could help monitoring lesional recurrences and assess if a CVM was completely removed at surgery. Those cases of CVM in which NEP stained the vessel adventitia had an excellent outcome from surgery, in two cases patients had ulcers and they healed following surgery and in one case patient had successful serial debulking with no further recurrences.

How CD10 expression can be utilised in clinical practice, still needs to be assessed and additional research work should pe carried out.

7.2 CLINICAL IMPLICATIONS OF RESULTS

7.2.1 Anti-angiogenic therapy – rationale of current use, clinical studies and challenges

This study represents a comprehensive molecular and histological study of CVM tissue. The results are unique and could provide valuable information for other angiogenesis related research projects such as those focusing on cancer angiogenesis, ischemic heart disease or inflammatory conditions such as rheumatoid arthritis.

The molecular alterations that sustain angiogenesis and hypoxia such as VEGF A/VEGFR-1, Tie-2, αVβ3 and αVβ5, HIF, may represent novel targets for CVM treatment protocols. Some anti-angiogenic substances have been found to have potent anti-cancer properties in ‘in vivo’ experimental studies but to date no anti-angiogenic compounds have been tested against CVMs. Since the inhibition of angiogenesis can slow or stop tumor growth or even shrink cancers, similar results arguably can be expected in CVMs.

Avastin (bevacizumab) is amongst the first developed anti-angiogenic substances and in fact is a monoclonal antibody. Avastin targets VEGF and its use was reported in relation to colonic cancer with evidence to support prolonged survival in patients with metastatic disease. Other agents that target VEGF A (sunitinib, sorafenib) are approved drugs but they are reported to cause only transient clinical responses (Huang, 2010). Targeting specifically VEGFR-1 is currently being investigated in clinical trials in relation to metastatic tumours, along with VEGFR-2 receptor as the two receptors have different activities in tumour angiogenesis. New components have been in use, incorporating both receptors and binding VEGF with higher

affinity than previously reported VEGF antagonists. Although VEGF A expression was increased in CVMs (35% of lesions were VEGF A positive), this was not as impressive as the over-expression of the other molecular markers. VEGFR-1 was over-expressed in CVMs (81%), but not in all of them and currently there is no specific target therapy for this receptor only. Should this therapy be considered in CVMs, it would seem logical that only that molecular pattern of CVMs showing an over-expression of VEGFR-1 would benefit. Therefore, anti-VEGF therapy in CVMs is not attractive at present, as the application may be limited and this drug comes with many side-effects, some of them quite serious.

Inhibition of αVβ3 may inhibit angiogenesis and invasion in some cancers but studies on animals in which αVβ3 was 'knocked out' have given conflicting results as in some cases angiogenesis was not compromised (Stupack, et al, 2003). Many vascular disorders are accompanied by alterations in integrin expression. Antagonists of αVβ3 and αVβ5 have been shown to block angiogenesis in some cases, suggesting that these integrins are required. As a result, integrins are therapeutic targets for treating a number of vascular diseases. A few integrin antagonists are being used clinically for cardiovascular and inflammatory diseases. Several other integrin inhibitors are being developed for clinical use (Tucker, 2003, Jin 2004). Amongst those tested or in clinical trials are Tumstatin, S247, m7E3, Vitaxin, Cilengitide. Their use could theoretically be developed in CVMs as αVβ3 appears to be up-regulated and possibly a key mediator in the pathogenesis of CVM.

Targeting Tie-2 is possible as Liu et al (2008) reported the finding of a series of small-molecule Tie-2 inhibitors, which blocked Ang1-induced Tie-2 auto-phosphorylation and downstream signalling, with the aim to treat pathological neovascularisation. The angiopoietin-Tie system is reported to be attractive for therapeutic intervention because it is crucial for angiogenesis and vascular homeostasis and also provides an important link between angiogenic and inflammatory pathways (Huang et al, 2010). Agents targeting this system are currently being developed in clinical trials in cancers and early reports suggest encouraging results. None of the agents are Tie-2 selective at present and their use in CVMs remains just a theoretical possibility.

ET inhibitors have been particularly useful in animal models at preventing hypoxia induced structural remodelling, but little evidence suggests that they are useful in reversing the vascular remodelling process.

HIF-1α was reported to be an exciting target for therapy, mostly in cancers (Rapisarda, 2002). The agents that reduce HIF-1α protein levels do so by decreasing the rate of HIF-1α synthesis, or increasing the rate of HIF-1α degradation or both. There is a multitude of HIF-1α inhibitors but they are not detailed here. YC-1 is a potent HIF-1α inhibitor and it has been

developed for circulatory disorders because it reduces the growth and vascularity of tumor xenografts. This could be developed for cancer patients and also for CVM patients if side effects such as hypotension could be controlled (Yeo, et al, 2003) and if it should be proven that HIF-1α plays a key role in CVM pathogenesis. There are inhibitors of both VEGF and HIF-2α such as Neovastat (AE-941), which has been shown to reduce airway inflammation (Lee, Chung, 2007). It is expected that development of more potent and selective HIF inhibitors will help the treatment of HIF-related diseases (Ban et al, 2011).

Bio reductive drugs are currently being developed as a means of selectively targeting hypoxic cells. Hypoxia directed drug delivery systems might offer the potential for selective treatment in a whole variety of conditions manifesting chronic hypoxia and inflammation. This offers the advantage of tissue specificity with reduced toxicity.

Future anti-angiogenic treatment may become important in the treatment of a variety of conditions. There are a number of issues to be resolved before this can happen. Inhibition of a single target by a specific anti-angiogenic agent may lead to the up-regulation of other angiogenic factors. The approach has to be multi-factorial as there is a need to combine anti-angiogenic agents with agents against other mechanisms of action, targeting several angiogenic molecules and other pro-angiogenic cell types apart from ECs (Carmeliet, 2005).

7.3 FUTURE STUDIES

More research is required to explore angiogenesis, hypoxia and related signalling pathways in relation to CVMs.

The expression of the selected pro-angiogenic and hypoxia markers should be further analysed at the molecular level by extraction and quantitation of RNA to detect angiogenic markers, HIF-1α, HIF-2α and CAIX using PCR and quantitative real-time PCR technology. Primers have already been designed for all these molecules and PCR protocols have been established. RNA from diseased and control tissue should be compared to examine the expression of hypoxia markers and the selected angiogenic markers in the same tissue. This may elucidate the leading control mechanisms in the pathogenesis of CVMs. It may help also to clarify the confusion concerning classification of these lesions by consolidating patterns at molecular level and relating this to specific clinical characteristics, especially to AVMs.

A molecular classification may be the answer to the existent confusion in nomenclature and classification of CVMs. This study provides a hint to a possible classification of CVMs based on CD10 staining but the results need to be further assessed and validated.

A common international terminology would help to precisely predict outcomes. Further analysis of CVMs would indicate which novel or target therapies may be the answer to a cure of CVMs or at least to prevent their clinical progression and reduce morbidity.

The angiogenic or anti-angiogenic mediators studied here represent very few amongst a whole array of such substances. It is known that TGF-β can promote angiogenesis via multiple mechanisms, including up regulation of VEGF expression and recruitment of pericytes. Should TGF-β prove to be an important mediator in CVM angiogenesis, then TGF-β signalling pathway antagonists would be expected to inhibit angiogenesis in CVMs.

Ephrins are molecules with specificity for arteries or vein from embryonic stages, even before the morphology of the vessel is not clear. This area would worth exploring and may bring additional information about CVM pathogenesis. It is well known that CVMs are represented by a network of vessels, often difficult to identify as arteries or veins, especially those subjected to high intra-vascular pressures.

Matrix metalloproteinases (MMPs) are an important group of enzymes associated with invasion and angiogenesis and some of them are related to VEGF A activation. Hypoxia can also potentiate invasion by up-regulating proteases such as MMP-9, leading to a cascade of responses, which can induce angiogenesis. ET-1, which may be expressed in CVMs, may enhance the secretion of ECM-degrading proteinases (Salani et al, 2000). ET-1 activates MMP-2, MMP-9, MMP-3, MMP-7 and MMP-13. In addition ET-1 enhances activation of MT1-MMP and the secretion of TIMP 1 and 2, resulting in the rapid degradation of ECM. These actions have been detected in tumours and it is not known if similar pathways are applicable to CVMs. MMPs inhibitors have been developed but the early findings revealed substances with serious side-effects and they were inappropriately evaluated in late stage disease. More recent MMP inhibitors target both tumour invasion and angiogenesis and they are still being developed.

Immunohistochemical analysis of mediators in agiogenesis on foetal tissue of different gestational ages may clarify the signalling pathways and mediators critical to angiogenesis and implicated in the aetio-pathogenesis of CVMs. This may demonstrate if these mediators are aberrantly expressed or up-regulated. This analysis was beyond the scope of this study.

There is evidence to suggest that angiogenic ECs may be derived from bone marrow precursor cells. These cells have characteristic cell surface antigen expression and have been found in the peripheral blood (Asahara et al, 1997). They have also been found to be active at sites of angiogenesis. It is not known if bone morrow derived ECs play a role in CVMs pathogenesis.

Understanding of the vascular tree development and of disregulated angiogenesis has increased significantly over the last few decades, but there are still many unanswered questions because of the complexity of the vascular system. This work contributes to the growing bank of data about CVMs and sheds light on possible pathways associated with angiogenesis and hypoxia involved in the development of these lesions. It may raise awareness of the thousands

of patients who suffers from these debilitating and disfiguring vascular malformations and impose clinicians and scientists with access to the tissue to expand and continue the research.

7.4 LIMITATIONS OF THIS STUDY

Vascular malformations, the subset of CVMs predominantly studied here are rare conditions.

Therefore, the number of samples analysed in each study is small and no firm conclusions can be drawn. However, no detailed investigations such as we presented here have been previously performed on vascular malformations.

The archive (retrospective) tissues were processed according to local standards but it is unknown whether all the criteria for collection and processing applied to the prospective samples were fulfilled in the archive tissue.

This work involved mainly immunohistochemical techniques and interpretation made use of a scoring system. Although these methods are widely used and accepted, and considered appropriate for the analysis performed, the observations were in part subjective.

APPENDIX

APPENDIX TO INTRODUCTION

A) EVOLUTION OF CLASSIFICATION OF CVMs

Historically, there were debates whether CVMs represent developmental malformations or tumours. They have been named ‘erectile tumours’ by Dupuytren (Jaccoud, 1870). Virchow believed CVMs were vascular tumors and was the first to classify them as angioma simplex, angioma cavernosum and angioma racemosum, based on histological architecture (Virchow, 1863). He and Wegener divided then vascular lesions into angiomas and lymphangiomas in 1880. A further histopathological classification was elaborated (Stout and Lattes, 1967) and this included both congenital and acquired vascular lesions under the term ‘angiomatoses’. According to this classification, the proliferative lesions in childhood were named ‘benign haemoangioendotheliomas’ and the malignant lesions were haemoangioendotheliomas‘.

At a later stage, vascular tumours showing excessive cellularity have been termed both ‘benign (neonatal) haemangioendotheliomas’ (Gonzales-Crussi and Hull, 1978 and Stout, 1943) and ‘infantile haemangiopericytomas’ (Albrecht, 1904 and Stout, 1942). The terms ‘cellular angioma of infancy’ (Taxy and Gray, 1979) or ‘cellular haemangioma’ (Pasyk and Grabb, 1982) have been proposed as most appropriate for vascular tumours of infancy and childhood. They represented the least mature of the haemangiomas and they were not related to what was known as ‘haemangioendotheliomas’. Other authors described vascular tumours in infants and children as presenting variable histological characteristics in different areas of the same tumour, such as haemangioendotheliomas and haemangiopericytomas (Brihaye and Baleriaux, 1975, Eimoto, 1977, Balasz and Denes, 1978, Gonzales-Crussi and Hull, 1978, Taxy and Gray, 1979, Pasyk and Grabb, 1982). Edgerton, in 1976 proposed a clinical classification of ‘angiomas’ based on their natural history. He divided ‘angiomas’ into three categories: those that remained essentially unchanged, those expected to resolve spontaneously and those that progress (Edgerton, 1976, Gampper and Morgan, 2002). This classification however, did not identify the underlying causes accounting for the clinical course of the lesions described. Further classifications of vascular anomalies had been reported and related to their clinical, histopathological features, capacity for dynamic growth and involution, as shown in the table appendix. 1. (Mulliken and Glowacki, 1982; Finn and Glowacki, 1983; Pasyk and Argenta, 1984).

Tabel appendix. 1. Classification of vascular anomalies

Cellularly dynamic	Cellularly adynamic
Haemangiomas	Vascular ectasies
Cellular haemangioma	Salmon patches
Capillary haemangioma	Naevus flammeus (Port wine stain)
Cavernous haemangioma	Naevus araneus
Mixed haemangioma	Multiple teleangiectasies
Verrucous haemangioma	Congenital phlebectasia
Pyogenic granuloma	Angioma serpiginosum
Senile angioma	Venous lake
	Angiokeratomas
	Angiokeratoma Mibelli
	Angiokeratoma Fordyce
	Angiokeratoma circumscriptum
	Solitary and multiple angiokeratomas (Imperial and Helwig type)
	Angiokertaoma corporis diffusum(Fabry's disease)
	Congenital arteriovenous malformations*

*Congenital arteriovenous malformations include rheologic dynamic vascular lesions.

(Adapted from 'Vascular Birthmarks', Pathogenesis and Management, edited by Ryan, JR and Cherry, GW, Oxford University Press, 1987, p4)

A new concept of predominantly arterial, venous and lymphatic lesions or arteriovenous and mixed lesions was introduced and first acknowledged by Malan in 1974. CVMs, have been most commonly, incorrectly and invariably termed haemangiomas ('birthmarks'), until 1980 when Mulliken and Glowacki divided them into haemangiomas and vascular malformations (venous, lymphatic and arteriovenous) based on the cellular (endothelial) proliferative characteristics of these lesions (Mulliken and Glowacki, 1982) (Table appendix.2)

Tabel appendix. 2. Classification of vascular anomalies based on EC characteristics

Haemangioma	Vascular malformation
Superficial	Capillary C
Subcutaneous	Venous (V): low flow
Mixed	Lymphatic (L)
	Arterial (A):high flow

	Arteriovenous (AV) Complex combined (CV, CL, CVL, CAV, CLAV, etc)
Often absent at birth	Present at birth
Appearance during first weeks of life	
Proliferation, stabilisation, spontaneous, gradual involution	No spontaneous regression

Adapted from Plast Reconstr Surg 1982, 69:412-420

The simple distinction of a vascular anomaly as being either a tumour or a vascular malformation was essential from a clinical prospective as the two conditions are different anatomo-pathological entities and have a different natural history and specific management issues.

The word haemangioma has been applied to any vascular anomaly, and the terminology used in the literature to describe haemangiomas has been even more confusing because there was no clinico-histological correlation and several terms have been used for the same lesion. 'Strawberry haemangioma', 'raspberry haemangioma', 'cherry haemangioma', 'juvenile haemangioma' are just few terms used in the past and they do not define histological structure. These were derived from the blame being placed on the mother for eating too much or too little of certain fruits during pregnancy, depending on a local culture. Most physicians identified these lesions in Latin as naevus maternus.

Terms such as 'capillary' or 'cellular' haemangiomas have been used by physicians for lesions predicted to involute. However, there are 'capillary' lesions such as port-wine stains that do not involute.

Terms such as 'capillary or cavernous haemangioma' were erroneously used to describe vascular malformations, especially venous malformations that have been inaccurately named 'cavernous haemangiomas'. It is now recommended to avoid these terms to prevent confusion between haemangioma and vascular malformations and prevent incorrect diagnosis and management (Mulliken, 1993, Hand and Frieden, 2002).

Rosai and Ackerman, 2004 in their Surgical Pathology included haemangiomas within the 'tumors and tumor-like conditions' of blood and lymph vessels and stated they occupy an undefined zone between hamartomatous malformations and true neoplasms. They have been classified based on their clinical appearance and calibre of the vessel involved into: capillary, cavernous, venous, racemose, cirsoid, arteriovenous, intramuscular, spindle cell and hobnail haemagiomas, along with and other benign or malignant vascular lesions. However, both the clinician and the the histopathologist no longer find this classification useful.

The Hamburg classification of CVMs, which has become widely accepted emerged as an important step towards a better understanding of these lesions and it was adopted at the 7th International Workshop on Vascular Malformations in Hamburg, Germany in 1988 (Belov, 1990). This classification takes account of the predominant anatomical (truncular or extratruncular) and patho-physiological type (predominantly arterial, venous, lymphatic, arteriovenous fistulae or combined) of CVMs (Table appendix.3.).

Tabel appendix. 3. Hamburg classification (Belov, 1989)

Type	Form	
	Truncular	Extratruncular
Predominantly arterial malformations	Aplasia Obstruction Dilatation	Infiltrating Limited
Predominatly venous malformations	Aplasia Obstruction Dilatation	Infiltrating Limited
Predominantly lymphatic malformations	Aplasia Obstruction Dilatation	Infiltrating Limited
Predominantly as a-v fistulae characterized malformations	Deep a-v fistulas Superficial a-v fistulas	Infiltrating Limited
Combined vascular malformations	Arterial and venous without shunt Haemolymphatic with or without shunt	Infiltrating haemolymphatic Limited haemolymphatic

This classification emphasises that the CVM can sometimes involve more than one vascular system and can present clinically as different forms of malformation, not only as arterial, venous, lymphatic, and capillary malformations but also as combined arteriovenous or haemolymphatic malformations (Belov, 1990, Belov, 1993). Each lesion is sub-classified according to the embryonal stage at which the developmental arrest occurred. The

extratruncular form occurs at an earlier stage of embryonal life than the truncular form (Belov, 1993, Woolard, 1922, Bastide and Lefebvre, 1989, Lee, 2004).

The International Society for the Study of Vascular Anomalies (ISSVA) founded in 1992 aims to advance clinical and scientific knowledge in all aspects of vascular anomalies, proposed a classification for these lesions based on cellular features, flow characteristics and clinical behaviour in 1992, as shown in Table appendix.4 (Enjolras, 1997).

Tabel appendix. 4. ISSVA* Classification of Vascular Anomalies 1992

Vascular Tumour	Vascular Malformations	
	Simple	Combined
Haemangioma proliferative phase	Capillary malformation	Arteriovenous fistula, arteriovenous malformation, capillary-venous malformation, capillary-lymphatic-venous malformation (Klippel-Trenaunay syndrome)
Involutive phase	Lymphatic malformation (macrocystic, microcystic, mixed)	Lymphatic-venous malformation, capillary-arteriovenous malformation (Parkes-Weber syndrome), capillary-lymphatic-arteriovenous malformation
Other tumours	Venous malformation	

Adapted from http://radiographics.rsnajnls.org

*ISSVA = International Society for the Study of Vascular Anomalies

This classification was further amended and in 1996 and was adopted as official.

Tabel appendix. 5. Amended classification system for CVMs by the ISSVA, 1996

Tumours
Haemangioma of infancy
Lobular capillary haemangioma (pyogenic granuloma)
Rapidly involuting congenital haemangioma (RICH)
Non-involuting congenital haemangioma (NICH)
Tufted angioma (angioblastomaof Nakagawa)
Kaposiform haemangioendothelioma
Congenital eccrine angiomatous hamartoma
Spindle-cell haemagioendothelioma

Malformations
Capillary malformation (port-wine stain)
Venous malformation, including blue rubber bleb nevus (Bean) syndrome
Lymphatic malformation (lymphangioma; cystic hygroma)
Arteriovenous malformation (including Bonnet-Dechaume-Blanc, Wyburn-Mason, and Cobb syndromes)
Capillary-lymphatic-venous malformation (most common type seen in limbs with Klippel-Trenaunay syndrome)
Parkes-Weber syndrome (combined malformation with arteriovenous fistulae)
Cutis marmorata teleangiectatica congenital
Glomuvenous malformation (glomangioma-venous malformation, including glomus tumour)

(Adapted from ISSVA classification)

It has been stated that using this classification system, at least 90% of vascular anomalies seen in infants and children can be categorised without the need of additional investigations or histological examination (Hand and Frieden, 2002).

The consideration of haemodynamic status of the CVM was first introduced by Mulliken in 1993 and subsequently another important classification was defined, dividing CVMs into high (fast) flow such as arteriovenous malformations, arterial malformations, and arteriovenous fistulas and low (slow) flow lesions such as capillary malformations, venous malformations, and lymphatic malformations (Jackson, Carreno et al, 1993). This is a radiological classification based on the vascular dynamics of the lesion and presence of arteriovenous shunting and refers to the speed of flow through the lesion and the rate of shunting between the arterial and venous components. This classification is important as a guide for clinicians to choose the appropriate form of treatment. It is important to note that various eponymous syndromes fall into these categories of vascular anomalies depending on their anatomical location. This emphasise the importance of describing a complex-combined vascular lesion based on the underlying vascular defect (Enjorlas, Chapot et al, 2004).

Kawanabe et al proposed another classification system of vascular lesions in 1996, in which the treatment was selected based on the flow characteristics of the lesion, which in fact represents a fusion of previous classifications (Table appendix.6.).

Tabel appendix. 6. Classification of surface vascular lesions

Traditional classification
Capillary haemangioma
Strawberry haemangioma
Strawberry nevus
Port wine stain
Flame nevus
Cavernous haemangioma
Venous angioma
Lymphangioma
Arteriovenous malformation
Classification of Jackson et al, 1993
Haemangioma
Vascular malformation
Low-flow lesion
High-flow lesion
Lymphatic malformation
Flow-related classification and recommended treatment
Slow-flow lesion: sclerotherapy
Intermediate-flow lesion: sclerotherapy (plus embolisation)
High-flow lesion: embolisation (plus scelotherapy)

Adapted from http://radiographics.rsnajnls.org

Subsequently, it has been reported that the erythrocyte glucose transporter protein-1 (GLUT-1) is strongly and diffusely expressed by the ECs of haemangiomas during all stages of their natural history (Blei, 2005, North and Waner, 2000), but it is not expressed in other CVMs. This new finding was included into the ISSVA classification (Table appendix.7).

Tabel appendix. 7. Current classification of the ISSVA

Vascular tumors	Vascular malformations
Haemangioma of infancy (GLUT-1 positive)	Simple malformation
Superficial	Capillary (port-wine stain)
Deep	Venous
Mixed	Lymphatic
Congenital haemangioma	Microcystic (e.g., lymphangioma)
Rapidly involuting congenital haemangioma	Macrocystic (e.g., cystic hygroma)

(RICH) Non-involuting congenital haemangioma (NICH) Kaposiform haemangioendothelioma Tufted angioma Pyogenic granuloma (lobular capillary haemangioma) Haemangiopericytoma	Arteriovenous malformation (AVM) Combined malformation Capillary-lymphatic-venous (includes most cases of Klippel-Trenaunay) Capillary-venous (includes mild cases of Klippel-Trenaunay) Capillary-venous with arteriovenous shunting and/or fistulae (Parkes-Weber syndrome) Cutis marmorata telangiectatic congenita

(Adapted from Curr Opin Pediatr 17:501-509. 2005 Lippincott Williams & Wilkins)

Although this classification has been extremely useful for many specialists there are rare situations in which clinical and histologic overlap between vascular tumors and malformations have been identified (Garzon, 2000, Hand and Frieden, 2002).

A useful clinical sub-classification was issued for AVMs – the ISSVA-Schobinger staging (Kohout, Hansen et al, 1998). According to this, AVMs can be classified into four clinical stages: 1. dormancy, 2. expansion, 3. destruction and 4. destruction and congestive cardiac failure (Table appendix.8.).

Tabel appendix. 8. ISSVA-Schobinger staging

I (quiescence)	Pink bluish stain, warmth, and Arteriovascular shunting
II (expansion)	Same as stage I, plus enlargement, pulsations, thrill, bruit, and tortous/tense veins
III (destruction)	Same as stage II, plus dystrophic skin changes, ulceration, bleeding, persistent pain, or tissue necrosis
IV (decompensation)	Same as stage III, plus cardiac failure

Houdart et al, 1993, have proposed a classification of intracranial arteriovenous malformations based on angiographic appearances of these lesions and this is currently applied and used to plan treatment of peripheral AVMs. This classification applies only to HF lesions and complements Mulliken's classification. Based on this classification, there are three types of fistulas within AVMs: a. Arteriovenous in which maximum three separate arteries are supplying a single venous component; b. Arteriolovenous in which multiple arteries communicate with a single central dilated venous component; c. Arteriolovenulous in which there are many

communications between arterioles and venules (Figure appendix.1.). This classification helps establish the access route in the process of embolisation of the vascular anomaly, as a method of treatment of these lesions, which will be dicussed later.

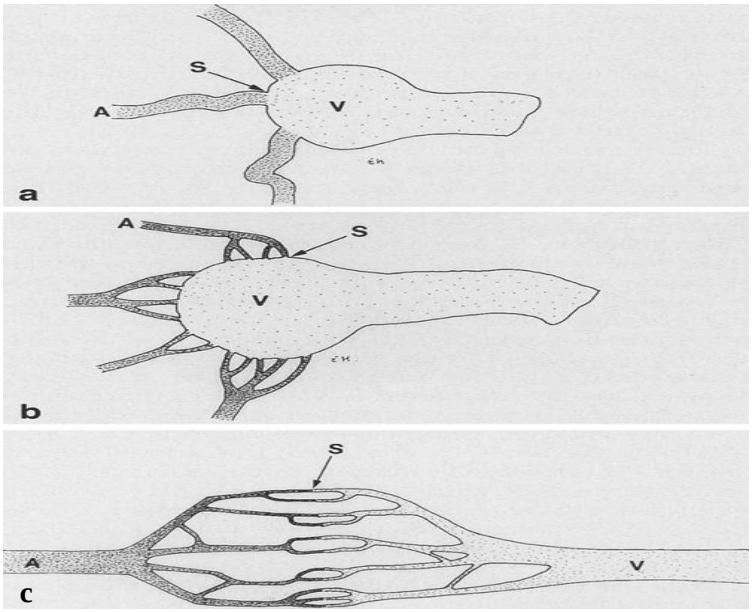


Figure appendix. 1. Angio-architectural classification of AVMs

Angio-architectural classification of AVMs, adapted from Interventional Neuroradiology (1993) 35: 381-385 – a proposed angiographic classification of intracranial arteriovenous fistulae and malformations by Houdart et al.

Histology textbooks present additional extensive classifications of CVMs but they have little clinical applicability as features described are overlapping or non-specific (Weisse and Goldbloom, 2001).

The term ‘angiodysplasia’ has been used in the past to describe CVMs but is currently considered inappropriate as it has the propensity to add to already existent confusion (Lee, Laredo et al, 2007).

In describing various vascular anomalies the terms ‘congenital’ and ‘acquired’ have been aknowledged. Experts (Mulliken, Fishman and Burrows, 2000) have recently stated that the term ‘congenital’ should probably be restricted to a vascular lesion that is present and fully developed at birth and the term ‘acquired’ should be applied to lesions appearing after the first year of life, in spite of the fact that this late lesions could still be present at birth, as in vascular malformations, although not clinically apparent.

Haemangiomas as the most common lesions of the CVMs have been debated more. However, the majority of haemangiomas are self-limiting and only few of them (10-20%) require aggressive treatment (Mueller and Mulliken, 1999). Vascular malformations represent the real challenge for any specialist and little research has addressed these lesions, mainly due to

lack of tissue as operative treatments were used sparingly due to their high risk of major haemorrhage associated with surgery. Reference will be mostly made to AVMs and haemangiomas, lesions which can be difficult to distinguish on occasions.

B) INVESTIGATIONS OF CVMs

Diagnosis of all vascular anomalies is based on detailed and accurate clinical history and thorough physical examination. Several other means to further investigate and assess the lesion are available, ranging from non-invasive to less invasive and invasive methods. There is no single test that would indicate best management for a particular lesion but choosing the appropriate imaging techniques are critical in establishing the diagnosis, assessment, treatment and prognostic. Emphasis is placed on non-invasive techniques wherever possible, but especially in children. The objective in the diagnosis of vascular anomalies is the precise identification of each component of the vascular defects. It is known that several different defects can coexist in the same region or affecting different areas and the truncular and extratruncular forms developed from different stages of embryogenesis, can also coexist (Lee, 2004).

Below is a brief description of the standard radiological investigations used for vascular anomalies, but also details of these studies tailored to the main categories of vascular anomalies considered here, including when are they employed and how can they differentiate between the known sub-categories of vascular anomalies. Selective invasive studies are reserved to plan treatment.

Common radiological investigations

Plain X-rays, although not routinely performed, may be useful to assess bone and joint deformities associated to some categories of vascular anomalies as previously discussed.

Colour Duplex Doppler ultrasonography is a convenient, rapid and non-invasive method of assessing most vascular anomalies. The ultrasound probe emits a continuous beam of ultrasound waves that deflects from red cells moving inside the blood vessels comprising the anomaly. The frequency of signals reflected shifts in proportion to the velocity of the cells being presented as acoustic signals. Duplex scanning combines image with Doppler signals such as red colour indicates flow away and blue towards the ultrasound probe. This helps to differentiate high-flow from low-flow lesions and to detect size of vessels, and extent of lesion.

Computer tomography (CT) is still used but has been mostly replaced by magnetic resonance imaging (MRI), which is a better method for evaluation of vascular anomalies. Spiral CT with three-dimensional reconstructions is used to assess bony abnormalities prior to surgery or if MRI is contraindicated. Phleboliths may be demonstrated on CT.

MRI offers the best imaging for vascular anomalies is non-invasive and is the most useful test for initial assessment, planning management and follow-up. Rak et al (1992) described the MR appearance of symptomatic vascular anomalies and indicated that MRI was essential in separating vascular lesions into high flow and low flow types. There are specific examination protocols for these lesions, using both T1-weighted and T2-weighted imaging. The distinction between venous malformations and arteriovenous malformations and fistulae is on T2-weighted MR images. These demonstrate venous malformations 'bright', of a very high signal intensity and arteriovenous malformations and fistulae have little or no signal due to the phenomenon known as 'flow void'.

Angiography is an invasive study, usually considered for lesions selected for treatment if clinical examination and ultrasonography indicated a high-flow lesion. Angiography on low-flow lesions is inaccurate and not useful. Angiography on high-flow lesions allow identification of the arterio-venous communications, which helps planning best approach for treatment that may be performed at the same time (embolisation, further discussed in the next sub-chapter).

Haemangiomas

In over 93% of cases the diagnosis of haemangioma is based on clinical features (Gampper et al, 2002). Deep haemangiomas in particular may be difficult to differentiate from other soft tissue tumours.

Ultrasonography can differentiate a haemangioma in a proliferative phase, which exhibit fast-flow. It can also differentiate a deep haemangioma from a venous malformation but it may be difficult to differentiate a proliferating haemangioma from an arterio-venous malformation as both may have fast-flow. This study is often useful to follow–up response to therapy but provides limited information.

MRI is the gold standard and superseded the other techniques in assessing such lesions.This may require sedation or general anaesthesia in children less than 6 years of age (Mulliken et al, 2000). Heamangiomas appear on MRI as fast-flow lesions with flow voids and tissue predominance and they may be difficult to differentiate from AVMs (Esterly, 1996). They are iso-intense or hypo-intense to muscle on T1-weighted imaging and hyper-intense on T2-weighted imaging. They enhance homogenously with intravenous gadolinium (Gampper et al, 2002).

Nuclear scanning with technetium Tc 99m-tagged red blood cells may be used when multiple deep haemangiomas or visceral lesions are suspected (Barton et al, 1992).

Angiography has value when the presentation of the haemangioma is atypical, or if embolisation or surgery is considered (Esterly, 1996).

Biopsy of haemangiomas is rarely necessary and there is a risk of un-controllable bleeding. Biopsy may be useful to differentiate atypical cases from other soft-tissue tumours, such as myofibromatosis, kaposiform haemangioendothelioma (a low grade sarcoma), and rhabdomyosarcoma (Frieden et al, 1997).

Venous malformations

The presence of a venous malformation is confirmed using non-invasive or less invasive tests including MRI T2-weighted image, duplex ultrasonography, whole body blood pool scintigraphy (WBBPS), transarterial lung perfusion scintigraphy (TLPS), air plethysmography, MR venography (MRV) and/or MR arteriography (MRA), CT with contrast and tri-dimensional reconstruction, radioisotope (RI) lymphoscintigraphy, ultrasound lymphangiography, MR lymphangiography (Lee at al, 2007). These studies help in diagnosing the lesion and assessing the extent of the malformation before treatment.

Ultrasonography should be the initial imaging modality used and it demonstrates absence of flow or low-velocity venous flow (Dubois et al, 2001). The absence of flow may suggest thrombosis.

On CT scan, venous malformations appear as hypo-attenuated, heterogenous lesions that enhance slowly at the periphery after contrast injection. (Dubois et al, 2001).

MRI scan provides the most information. Venous malformations are hypo-intense on T1-weighted MR images and hyper-intense on T2-weighted images. MRV is useful for assessment of extensive lesions affecting extremities.

Invasive studies including standard or selective angiography, phlebography, ascending, descending, and/or segmental venography, or percutaneous lymphangiography are usually performed on symptomatic lesions, with the aim of establishing the best therapeutic strategy (Lee et al, 2007).These techniques also help identify mixed lesions such as a venous malformation with arteriovenous components. Direct phlebography may be performed to obtain a more detailed analysis of the lesion or as part of sclerotherapy treatment. It involves the injection of contrast into the venous system, which may be a foot vein, (ascending venography), on the femoral vein (descending venography) or into a specific venous segment (segmental venography). Venography may or may not demonstrate communications between deep veins and a venous malformation. Hypoplasia or aplasia of the deep venous system may be found in almost 50% of venous malformations (Eifert et al, 2000) and this may represent a contraindication to removing large superficial veins (Rosen et al, 2000).

Transarterial lung perfusion scintigraphy (TLPS) was developed as a test less invasive than angiography, for the physiological assessment of the arteriovenous shunting status of the

vascular lesions located in the lower extremities. This can provide accurate information on the reduction of the shunting volume post-treatment (Lee et al, 2005).

Arteriovenous malformations (AVMs)

If treatment and especially surgical treatment is contemplated, these selected lesions would have been assessed by ultrasonography, MRI and/or magnetic resonance angiography (MRA). The superselective angiography is performed close to the planned intervention. Direct opacification of the nidus (the confluence of abnormal vessels, where arteriovenous shunting occurs without a capillary bed), helps diagnosis and treatment (Hyodoh et al, 2005).

Foetal diagnosis of vascular anomalies

Diagnosis based on ultrasound evaluation of the developing foetus is increasingly possible. Marler et al, 2005 published a review with their experience with 29 patients having pre-natally diagnosed vascular anomalies. They concluded that vascular anomalies can be diagnosed in the second and third trimesters of prenatal development but the technique is unreliable. This has psychosocial and ethical implications and currently postnatal evaluation and diagnosis remains the accepted standard (Arneja et al, 2008).

C) TREATMENT OF CVMs

The appropriate management of vascular anomalies is dependent on an accurate diagnosis. The decision to treat a vascular anomaly is still are one of the most difficult clinical decisions due to their extremely variable clinical presentation ranging from asymptomatic lesions to life threatening conditions.

The concepts of management of vascular anomalies have changed over the last few years and a slightly more robust and logical approach has been introduced based on the general acceptance of the Hamburg classification and, on the introduction of a multidisciplinary team approach to their management (Lee, 2002).The multidisciplinary team approach involves the participation and coordination of many specialists. This provides full integration of all therapies available, adapted to each individual case. (Lee et al, 2007). This approach is not evidenced based and published outcomes of various surgical or interventional techniques recommended by the multidisciplinary teams are rather obscure.

The treatment strategy is usually focused on the ‘primary’ lesion, followed by treatment of ‘secondary’ disorders associated with the vascular lesion. Treatment methods are based on the exact characterisation of the haemodynamic nature of the lesion. Treatment is usually reserved for symptomatic lesions but large, cosmetically un-acceptable lesions, or those in critical locations may also be considered for treatment even though the lesion is asymptomatic.

Guidelines for treatment indication for CVMs have been published by Lee in 2004 and they are given in the table below.

Tabel appendix. 9. Treatment indications for congenital vascular malformations

Treatment indications for congenital vascular malformations
1. Absolute indication
Haemorrhage: caused mostly by VM or AVM
Increasing or progressing risk of high-output heart failure: AVM
Secondary complication of chronic venous hypertension: AVM or VM
Lesions located at a life- and/or limb-threatening region (e.g. airway): VM or AVM
Lesions threatening vital functions: various CVMs
2. Relative indications
Disabling pain and/or discomfort of progressive nature
Functional disability or impairment affecting daily activity and quality of life
Cosmetically severe deformity accompanied by physical and/or psychological disability with severe negative impact on quality of life
Vascular-bone syndrome with rapid progress and long-bone growth discrepancy accompanied by significant pelvic tilt and/or compensatory scoliosis
Lesions located in a region associated with a potentially high risk of complication (e.g., haemarthrosis and/or trauma-prone condition; deep vein thrombosis)
Lesions with recurrent infection (local and/or systemic sepsis)
Lesions with persistent lymph leakage

Adapted from Ann Vasc Surg 2004; 18:380-392, Critical Issues in Management of Congenital Vascular Malformation, By Lee BB

Similar treatment indications were published for AVMs by Zhou et al in 2005. Appropriate time of treatment depends on the type of vascular anomaly (Lee et al, 2002). Life or leg-threatening malformations require urgent treatment.

General concepts of treatment of vascular anomalies

C.1 Conservative

Conservative methods in the management of vascular anomalies range from simply observing the lesions at regular intervals, educating the patients or parents, application of elasticated compression garments, to local, intra-lesional or systemic pharmacotherapy.

C.2 Endovascular techniques

Intentional occlusion of the blood flow within the vascular lesion is used by interventional radiologists who employ image guided procedures known as endovascular techniques, mainly embolisation and sclerotherapy. The vascular lesions can be closed by advancing a small plastic tube into the feeding artery of the lesion. This is used to inject small particles of foreign materials such as medical glue, alcohol, small beads or coils, which are

floated into the vascular lesion until this is full and the flow has ceased. Sclerotherapy means delivery of an ablative or irritant liquid directly into the abnormal blood vessels, usually via a percutaneously placed cannula. Embolisation is reserved for high-flow vascular anomalies and sclerotherapy may be effective in high or low flow lesions. These therapies aim to cause a combination of vascular thrombosis and endothelial ablation resulting in fibrosis.

Endovascular techniques were introduced in the early 1970s and have been increasingly employed as they developed during the last decade. Recent advances in endovascular equipment and imaging allow safe supra-selective catheterization and embolisation of feeding vessels of high flow lesions, aiming to induce localised vascular thrombosis (Svendsen, 1994). Embolising or sclerosing agents such as absolute ethanol, polidocanol (Aethoxysklerol; Kaigen, Osaka, Japan), ethanolamine oleate (Oldamin for injection; Grelan Pharmaceutical, Tokyo, Japan), n-butyl cyanoacrylate (Histoacryl; B. Braun, Melsungen, Germany), various types of coils, polyvinyl alcohol foam powder (Ivalon; Cook, Bloomington, Ind), superabsorbent polymer microspheres, detachable balloons, foam pledgets have been used in various combinations, with different protocols, depending on the location, severity and extent of the lesion (Hyodoh et al, 2005).

The flow of AVMs can be reduced by embolic therapy but such treatment may be short-lived or palliative in long term (Hyodoh et al, 2005, Enjolras, 1997, Marchiano, 1996, White, 2000).These techniques are often used in the treatment of CVMs, when feasible. They are especially important when the risk of bleeding associated with surgery is too high, the location of the lesion or the infiltration of important or vital structures prevents a surgical excision or debulking. Percutaneous sclerotherapy is accepted as independent therapy for vascular malformations (Hyodoh et al, 2005). Embolisation of venous malformations is known to have disappointing results but percutaneous sclerotherapy may be effective in these lesions (Lee, 2001). Repeated courses of sclerotherapy can lead to clinical improvement of venous malformations (Suh, 1997). This technique can also be used as an adjuvant therapy to surgery. Pre-operative embolisation is known to facilitate surgery and decrease operative blood loss (Zhou et al, 2005, Lee, 2007, Rosen et al, 2000). Post-operative supplemental endovascular therapy has also been reported to improve outcome of surgical therapy such as surgical excess is avoided. (Lee et al, 2007).

New strategies of combining embolisation materials such as coils, particles or N-buthyl cyano-acrylate with absolute alcohol have been introduced, aiming to gain synergistic effects and to reduce complications reported with absolute alcohol (Lee, 2004). Re-canalisation and recurrence of the lesion are the main limitations of these therapies as well as: skin necrosis, nerve damage, inadvertent embolisation of normal tissues, thrombo-embolism.

The last decade has seen a major change to the strategy for CVM management resulting from the realisation of the harm caused by the embolisation of feeding vessels. This is now condemned since all the extratruncular CVM lesions may be stimulated by the embolisation of the feeding arteries. Endovascular therapies requires specialist skills as they are potentially dangerous and can lead to complications such as full-thickness skin necrosis, nerve injury, bleeding, muscle atrophy and contracture, deep vein thrombosis, pulmonary embolus, haemolysis, renal toxicity and even cardiac arrest. This is why these techniques are usually employed on symptomatic lesions only, used alone or in association with surgery.

C.3 Surgery

Surgical therapy for various vascular anomalies is initially aimed at the primary lesion and has the aim to decrease haemodynamic activity and the resulting impact on the body. This consists of non-radical operations, usually debulking performed in stages. Subsequently, surgery aims at improving function, quality of life and cosmetics (Lee, 2004).

Surgical excision remains the only solution for 'cure' of any vascular lesion but usually requires radical resection, which has high associated morbidity, mainly major haemorrhage (Lee et al, 2007).

The predominantly conservative treatment approach should be considered a thing of the past (Loose, 2007). In order to undertake this complex surgery, special skills and experience are required.

Ligation of the feeding vessels was performed in the past on AVMs but this technique led to a rapid development of collateral vessels which made future treatments rather difficult (Coleman, 1973, Coursley, 1956, Reid, 1925, Svendsen, 1994, Zhou, 2005).

Previous studies on AVMs have suggested that a cure can only be expected when the whole lesion is removed (Zhou, 2005, Hyodoh, 2005) or the confluence point of the abnormal vessels in AVMs, which is called a 'nidus' is destroyed (Hyodoh, 2005, Lee, 2007). Surgery should involve the ligation of all feeding vessels and the entire malformation. Well-demarcated lesions, which are confined to limited anatomical regions, may be removed by performing a surgical excision. For patients with life/limb threatening complications of AVMs or with severe symptomatology where surgical excision is not technically feasible, debulking surgery (serial partial removal) may have significant benefits and does not necessarily lead to recurrences (Phillips et al, 2005). Debulking surgery is a controversial procedure but has potential to correct severe symptoms (Belov et al, 1990, Loose, 1990, Loose, 2001, Loose, 2007). However, debulking is major surgery with significant risks of bleeding and associated complications. Published data (Belov, 1990, Belov, 1993, Bastide, 1990, Lee, 2007) have suggested that debulking is not conceptually good unless it is warranted to control serious complications. Some

authors believe that simple debulking will only stimulate the diffuse infiltrating lesion to grow back, as 'extratruncular' lesion in Hamburg classification (Belov, 1990). The explanation behind this rationale is that these lesions may have characteristics of or represent remnant embryonic tissue originating from an early stage of embryogenesis, so that it possesses mesenchymal cell characteristics and grow back when stimulated by debulking. To date there is no scientific data to support these claims and there is no evidence demonstrating the embryonic origin of CVM tissue. Partial excisions do not imply worsening symptoms or recurrences (Phillips et al, 2005). Excision may be performed at an early stage for well-localised stage 1 AVMs (see Schobinger stages).

Compartmentalization using non-absorbable sutures is another surgical option in selected cases (Jackson, 2005). This involves 'over sewing' the AVM vasculature and inducing thrombosis. In theory, this is a mechanism of feeding artery ligation and may indicate its liability to recurrence.

Specific treatment methods

Haemangiomas

It is estimated that 10-20% of haemangiomas require intervention (Mueller et al, 1999) and it is universally accepted that the majority of haemangiomas do not need treatment as they undergo spontaneous involution (Fishman et al, 1993). Rudimentary knowledge of the pathogenesis of haemangioma led to empirical and unsatisfactory therapies (Tan et al, 2000, Mulliken et al, 1995, Hasan et al, 2000).

Treatment decisions in respect to infantile haemangioma take into account the size and location of the lesion, the age of the patient, the growth phase of the haemangioma, associated features and the potential for psychosocial distress for parents and patient.

Observation is all that is required for small, harmless lesions but parents of an affected child need detailed explanation of the condition and reassurance. Edgerton (1976) named this approach 'masterful neglect' and Mulliken et al (1988) emphasised the principle primum non nocere. A useful principle in the treatment of these lesions is that of 'active non-intervention' (Garzon et al, 2000).

Large lesions, especially those affecting the face can cause serious distress to the parents and sometimes to the child. Haemangiomas leading to complications require treatment. There is a sub-category of haemangiomas between the harmless and the complicated lesions treatment of which is still debated by specialists. Although usually harmless, some specialists would intervene in such cases, to prevent psychological problems developing in early childhood in an affected child.

Local therapies are available for ulcerated or bleeding lesions.These include: daily application of a topical antibiotic, non-stick wound dressings, hydrocolloid dressings, hydrated petrolatum, viscous lignocaine, corticosteroids.

Pain management may be indicated. Pulsed-dye laser has been reported to help healing and pain (Achauer et al, 1991, Morelli et al, 1994). Excision can be performed on an ulcerated lesion of the scalp, torso or extremity but rarely is performed for a facial lesion. Haemangiomas of the eyelid may be troublesome and those not responding to medical therapy require excision or debulking. Prominent haemangiomas in involuting phase may be considered for excision in the pre-school period. Skin changes resulting in the involuted phase can be dealt with by excision and only rarely are reconstructive techniques required. Bleeding is dealt with by compression and only occasionally requires suturing of the bleeding points or excision. Diffuse, large infiltrative haemangiomas can lead to life-threatening haemorrhage. These lesions are usually in-operable and they are treated with blood transfusions, parenteral nutrition and anti-angiogenic drug therapy to stimulate their involution (Mulliken et al, 2000).

Topical corticosteroids have been reported to be effective in small, superficial haemangiomas. Injection of corticosteroids may be considered in small, localised cutaneous haemangiomas. Since the 1960s systemic corticosteroids have represented the first-line treatment in complicated, life-threatening haemangiomas. Recombinant Interferon alpha is considered a second-line drug for complicated or life-threatening haemangiomas but some clinicians recommend its use as a first line drug (Mulliket et al, 2000). There is no evidence of any benefit by administering corticosteroids and Interferon alpha together. OK-432 is a denaturated streptococcal protein which is reported to stimulate the immune response and accelerate the resolution of a haemangioma (Kim et al, 2004). Bleomycin injected directly into a lesion has been reported to be effective, but its mode of action in haemangiomas is still to be established (Sarihan et al, 1997). Chemotherapeutic agents such as vincristine and cyclophosphamide have been used in exceptional cases (Frieden et al, 1997). Vincristine is effective in the setting of vascular lesions associated with Kasabach-Merrit phenomenon but is gaining increased recognition as a potential agent for infants with steroid-resistant haemangiomas or those who cannot tolerate steroids.

Embolisation is indicated in fast-flow haemangiomas causing congestive heart failure, airway or ocular obstruction, life-threatening coagulopathy, and not-responding to other therapies. Endovascular therapies are not used for routine management of haemangiomas.

Photocoagulation using laser therapy is debated. Several types of lasers have been used in the treatment of haemangiomas including flash lamp-pumped pulsed-dye, carbon dioxide, neodymium: yttrium-aluminum-garnet, potassium-titanyl-phosphate or argon lasers (Gampper

et al, 2001). The pulsed-dye laser penetrates up to the dermis and only the superficial surface of the haemangioma is affected. The lesions this treated are usually amongst those that are clinically insignificant. Continuous-wave carbon dioxide laser (Sie et al, 1994) is an accepted laser therapy applied in the proliferative phase of subglottic haemangioma but tracheostomy is often required to maintain the airway. Another current laser therapy is the use of a bare fiber (Nd: YAG) inserted through multiple needle passages into the haemangioma (Berlien et al, 1990).

Radiation therapy use has been reported in severe, life-threatening haemangiomas, not responding to other treatments (Caldwell et al, 1995). This method was popular in the 1940s and 1950s but subsequently abandoned as outcomes were worse than leaving the lesion un-treated. Dondon et al, 2004, presented data documenting the increase in cancer mortality in adults after radiotherapy for cutaneous haemangiomas during childhood.

Cryotherapy, in the form of topical liquid nitrogen and carbon dioxide have been used in the treatment of superficial haemangiomas but has limited acceptance due to subsequent scarring and hypopigmentation (Cremer, 1998, Edgerton, 1976).

Supportive measures are implemented, such as diuretics and digoxin in cases of high-output cardiac failure, oral antibiotics for infected lesions, depending on the specific complication.

Compression therapy has been tried for haemangiomas but with no evidence that this would accelerate the involution of the lesion.

The prospect of new inhibitors of angiogenesis and their use in this field is a current matter of debate.

Venous malformations

Loose published in 2007 the principles of therapeutic strategy for these lesions: 1. active causal treatment, abolishing the haemodynamic dysfunction, 2. harmonised individual treatment, 3. early operation in childhood, 4. radical operation without loss of function, 5. operative treatment by stages, 6. combined treatment.

Most venous malformations are treated conservatively as many authors suggested that asymptomatic lesions can be safely observed.

Elastic compression garments may help by reducing the swelling, pain and risk of coagulopathy in an affected limb. Prophylactic aspirine (80mg daily) has been recommended to prevent painful thrombotic episodes and phlebolith formation (Marler et al, 2005). Intravascular coagulopathy may need control with heparin. Infected ulcerated lesions are treated with antibiotics with guidance from the wound swabs taken prior to commencement of the antibiotic treatment.

Aggressive intervention in young patients is usually pursued in lesions causing impairment of vital functions or those causing the vascular bone syndrome resulting in leg length discrepancy (Lee et al, 2005). An affected lower extremity can be complicated by pelvic tilting and scoliosis. To avoid such complications, stapling epiphysiodesis of the knee cartilages is often performed but this intervention may exacerbate the lesion (Enjolras et al, 2004).

Surgical treatment alone or in combination with pre-operative endovascular techniques is considered optimal management for the truncular form of these lesions with no risk of recurrence (Loose, 1989, Mattasi, 1989). These treatment methods are also used for extratruncular lesions with an accepted risk of recurrence. The removal of extratruncular lesions together with muscles or bone segments may be inevitable. This approach supports Malan's opinion that the criteria applied to surgical treatment in vascular malformations are not different from those applied in 'cancer surgery', meaning extreme and radical resections may be required, even when this leads to considerable disfigurement (Malan, 1974). The surgical tactics are adjusted to the pathological form of malformation (Loose, 2007) and may represent: 1. an operation to reduce the haemodynamic activity of the malformation (venous hypertension); 2. an operation to eliminate the malformation; 3. reconstructive surgery.

Severe venous malformations resulting in a non-functional limb require early amputation followed by early rehabilitation with the use of prosthesis. (Lee et al, 2007).

Supra-selective catheterisation of feeding vessels and the trans-catheter administration of embolic agents have revolutionised the treatment of venous malformations.

Vein stripping for superficial veins causing discomfort may be performed once the deep veins have been assessed and proven adequate.

Some venous malformations such as those containing a localised slow-flow nidus can be successfully treated with sclerotherapy (Hyodoh et al, 2005). Multiple sessions of sclerotherapy are usually necessary and they have a palliative effect as venous malformations have the potential to re-canalise and recur (Mulliken et al, 2000). Lee et al (2001) reported that absolute ethanol sclerotherapy can lead to excellent results in complex forms of venous malformations with considerable but acceptable morbidity and with no recurrence or deterioration of symptoms at 10 months.

Foam sclerotherapy has been reported to have excellent outcomes and is favoured over liquid therapy (Pascarella et al, 2005).

Endovenous thermal ablation such as laser or radiofrequency ablation, have been used to treat venous malformations with acceptable results (Sidhu et al, 2005).

Arteriovenous malformations

These are high-flow (HF) lesions and they are the most difficult vascular lesions to treat. Complete destruction of the nidus of an AVM is the only way to cure these lesions but this is often difficult to achieve. Small, superficial AVMs can be excised but most AVMs are large, diffuse and infiltrating important adjacent structures, therefore very difficult or impossible to excise (Jackson et al, 1996). Early stage (I and II) lesions can be resected and reconstructed easily.

Embolisation and surgery are the main therapeutic options for these lesions. It is advised that the intervention should be delayed until serious complications occur. Emergency embolisation may be necessary in cases of congestive heart failure. In some cases, embolisation worsens the patients' symptoms, due to aggravation of adjacent tissue ischaemia (Burrows, 2006). The usual strategy in AVMs is arterial embolisation of the nidus, 24-72 hours in advance of surgical intervention, in attempt to decrease intra-operative haemorrhage. This can be performed by an arterial or venous approach, depending on the angiographic classification of the AVM, as described by Houdart et al. in 1993. Pre-operative embolisation does not minimise the extent of the resection. The surgeon must decide on the extent of the section. Inappropriate surgical intervention may stimulate 'dormant' vascular malformations to become active and symptomatic (Hyodoh et al, 2005). Extensive AVMs infiltrating vital anatomical structures may be amenable to palliative embolisation only and debulking may have a limited role. When wide excisions are performed and primary closure is not possible, the resulting defect may require reconstruction with skin grafts, local flaps or free tissue transfer. Surgery on very large AVMs may require the use of cardiopulmonary bypass and hypothermic cardiac arrest in order to limit blood loss (Mulliken et al, 1978). Surgery on extensive AVMs is controversial because is difficult to predict the outcome. Further research into these lesions in an attempt to design new ablative therapies may benefit patients with inoperable disease.

Treatment of AVMs with laser, steroids, or irradiation has not been effective.

D) THEORIES AROUND AETIO-PATHOGENESIS OF CVMs

D.1 Angiosomes theory

The arteries supplying CVM tissue with blood are responsible for the supply of the skin and the underlying structures. The anatomical territory of a source artery in the skin and deep tissues was found to correspond in most cases and this gave rise to the angiosome concept. It was found that arteries follow closely the connective tissue framework of the body. The primary supply to the skin is by direct cutaneous arteries which vary in calibre, length and density in different areas. This primary supply is reinforced by numerous small indirect vessels, which are terminal branches of arteries supplying the deep tissues. The angiosome territories

have been established for most anatomical regions and this information provides data for the surgeon to help plan safer incisions and better reconstructive flap procedures. It also provides information that may help explain the development of some vascular anomalies and help in planning their management (Houseman et al, 2000). Vascular studies with in vivo angiogram studies of vascular malformations in the head and neck showed that the site of these malformations coincides with the so called 'choke anastomotic zones' that linked adjacent angiosomes. Houseman found that in the head and neck areas, the angiosomes anastomose with one another either by large 'true anastomoses' without change in calibre or in some areas by reduced calibre 'choke' anastomoses. The anastomoses between angiosomes usually occur within tissues- skin, muscle, fat, gland or bone- not between them. In some sites the connection is almost non-existent and is formed by minute vessels. The choke anastomoses link adjacent vascular territories and the malformations are always fed by two or more arteries. Authors emphasised that it is likely that choke vessels regulate flow between angiosomes under normal circumstances. They also indicated that it seems possible that the factors that regulate the development and function of these choke vessels are either deficient or abnormal and that this has led to the development of vascular anomalies. In 1794 John Hunter stipulated that a foetus had a fixed number and pattern of blood vessels at some stage of embryonic development and that subsequent change in their anatomy was a response to pre-natal and post-natal growth and development.

D.2 Genetic theories

It is recognised that haemangiomas occur sporadically but more recently data accumulating suggests that is likely that haemangiogenesis involves a genetic alteration. Blei et al, 1998, reported six families in whom haemangiomas or vascular malformations were present in multiple generations, suggestive of an autosomal dominant inheritance. Also, several of the patients with haemangiomas had relatives with vascular malformations, suggesting a possible common underlying genetic linkage. In three of these families genetic mutations were linked to the long arm of chromosome 5 (5q) (Walter et al, 1998). Ritter et al, 2002, published for the first time data demonstrating dynamic expression of a variety of genes functioning as growth factors, signal transduction and immune response in haemangiomas. They identified T cells in haemangiomas and indicated that the high expression of 2, 3 dioxygenase, a T cell-related enzyme, may modulate the haemagioma life-cycle (Ritter et al, 2003).

Another theory is that vascular malformations follow the somatic mosaic hypothesis of Happle, who stipulates that the presence of the mutation in the zygote is embryonic lethal, but there may be a mosaic distribution with cells bearing the mutation surviving in proximity to normal cells.

Genetic studies are beginning to identify changes in the cascade of development and processes that caused vascular malformations (Vikkula et al, 1998). A mutation in the gene encoding for receptor kinase Tie-2 causes familial cutaneous-mucosal venous malformations (Vikkula et al, 1996, Boon et al, 1994). This receptor is expressed specifically by ECs. Another venous malformation subtype called glomuvenous malformation is often familial and caused by the gene glomulin found on the short arm of chromosome 1(1p) (Brouillard et al, 2002). Over-expression of VEGF-C leads to lymphatic EC proliferation and vessel enlargement (Dumont et al, 1998, Jeltsch et al, 1997). Angiopoietin-1 deficiency causes defects in the vascular wall and over-expression leads to increased vascularisation (Suri et al, 1996, Suri et al, 1998). Multiple cerebral 'cavernous' venous malformations are inheritable and are caused by mutations in KRIT-1 (Laberge-le Couteulx et al, 1999).

Some mutations associated with vascular anomalies have been localised to known genes or mapped to specific chromosomal regions but others remain obscure. Known genes include AGGF1 for Klippel-Trenaunay syndrome, RASA1 for capillary malformations, KRIT-1, MGC4607, PDCD10 for cerebral cavernous malformations, VEGFR-3, FOXC2, NEMO, SOX18 for lymphoedema, ENG, ACVRLK1, MADH4 for HHT, NDP for Coats'disease, Notch3 for CDASIL, PTEN for Proteus syndrome.

Sato et al (1995) speculated that arteriovenous malformations also result from a somatic mutation in endothelial or perivascular cells.

One hypothesis invokes a somatic mutation, the local loss or gain of genetic material during foetal development that produces an altered cell line that is stimulated by autocrine or paracrine signalling. Therefore, one of the most accepted theories explaining the development of haemangiomas concerns the primary defect intrinsic to ECs (Tille et al, 2004, Boye et al, 2001). Boye et al, 2001, observed that ECs isolated from haemangiomas in nine infants exhibit enhanced proliferation and migration but this cell migration in the presence of the angiogenic inhibitor endostatin, was not inhibited but rather stimulated, suggesting a an altered cellular phenotype. These results indicate that haemangiomas represent clonal expansions of abnormal ECs and that they may be caused by a somatic mutation in a gene that regulates ECs proliferation (Walter et al, 2002 and Boye et al, 2001). This would mean that all ECs composing a haemangioma originate from the same progenitor cell that carries the somatic mutation and causes the abnormal proliferation.

The peripheral, sporadic, vascular malformations with no proven genetic mutations to date represent the focus of this study.

D.3 Miscellaneous theories

There is some speculation that haemangiomas may arise from ectopic placenta or a shift of the endothelium to the placental phenotype, due to the increased risk of haemangiomas in children of mothers who had chorionic villus sampling and due to shared immunoexpression between placental tissue and haemangiomas (GLUT-1, FCβRII, merosin, etc) (Burton et al, 1995, Bree et al, 2001, North et al, 2000). Embolic placental ECs could reach foetal tissue from chorionic villi through right-to-left shunts characteristic of the normal foetal circulation. The placental origin theory is attractive because it would explain the exclusively perinatal or congenital presentation of the haemangiomas. The increased incidence of haemangiomas associated with chorionic villus sampling, which on occasions disrupt vascular structures, suggests that errors in vasculogenesis may provide a fertile field in which haemangiomas later proliferate (Drolet et al, 1999).

A viral cause has been suspected but the angiogenic human herpes virus 8, the common agent in Kaposi sarcoma, has not been found in human haemangioma (Dupin et al, 1998). Parapox virus infection causes proliferation of ECs and dilatation of cutaneous blood vessels in humans (Lyttle et al, 1994). The avian haeamangioma virus has been identified in spontaneous 'haemangioma-like' lesions in hens (Soffer et al, 1990).

D.4 Endothelial cells in CVMs

In an adult the average EC divides only twice in a lifetime. However, EC proliferation can be very rapid when needed, such as in a wound healing process. Therefore, the EC proliferation must be kept under tight control. EC activation status is determined by a balance between positive and negative regulators of EC proliferation, migration and proteinase production (Pepper et al, 1996).

Dosanjh et al, 2000 described that the ECs in haemangiomas converted to a spindle-shaped morphology similar to that of foetal ECs and concluded that both cell morphology and protein expression of neonatal haemangioma ECs were more characteristic of embryonic microvascular ECs, suggestive of a dysfunction in the normal growth and maturation of ECs in haemangioma.

Pasyk et al, 1982 conducted an ultrastructural study on haemangiomas and confirmed the existence of two types of immature cells, ECs and pericytes in the same lesion. The presence of the crystalloid inclusions in the ECs and absence of the Weibel-Palade bodies, deficiency of factor VIII and absence of tissue fibrinolytic activity, all suggested that ECs in these lesions are immature.

Kleinman et al, 2003, demonstrated increased levels of circulating EC precursors in children with haemangiomas. Yu et al, 2004, showed the presence of EC precursor cells in

proliferating haemangioma and speculated that they may contribute to the growth of haemangioma.

There may be aberrant pericyte-endothelial interactions and signalling in the growth of the thin walled and ectatic vessels of the vascular malformations. Stabilisation of the ECs and their signalling pathways has been identified as important in controlling these malformations. Therefore, the receptors involved in the cellular interactions between endothelium, pericytes, smooth muscles, may become targets of future therapies.

The cells cultured from AVMs in skin are different from normal ECs in culture. They have a higher rate of proliferation and a lack of inhibition by known angiogenic inhibitors, such as interleukin 1β, tumor necrosis factor α, interferon-γ, TGFβ, and a lack of expression of leucocyte adhesion molecules (Wautier et al, 1999).

Recent studies imply EC are directly involved in the pathology of haemangiomas and vascular malformations through different mechanisms. This is why further characterisation of the ECs in vascular malformations is essential in understanding the key role and mechanism by which these cells trigger the pathways leading to the progression and involution of these lesions.

E) OTHER MOLECULES ASSOCIATED WITH CVM RELATED ANGIOGENESIS

Basic fibroblast growth factor (bFGF) induces cell proliferation in a variety of cell types of mesodermal and neuro-ectodermal origin. In vivo, bFGF may play a role in embryonic development (Kimelman et al, 1987) and induces neovascularisation in different angiogenesis assays (Folkman et al, 1987). Purified bFGF induces migration and proliferation of ECs in tissue culture (Shing et al, 1984). Also, anti-bFGF has been shown to inhibit angiogenesis in normal wound repair, indicating that bFGF is intrinsically involved in normal neo-vascularisation (Broadley et al, 1989).

Basic FGF is up-regulated during proliferation in haemangiomas. Urinary bFGF levels are usually high in infants with proliferating haemangiomas and diminish to normal levels during regression (Mulliken et al, 2000).

TGF-β and its receptors TGF-βR1 and TGF-βR2 may regulate some very early events in EC differentiation and may contribute to endothelial maturation (Risau et al, 2000). Humans with mutations in TGF-β signalling pathway genes show a range of vascular defects including dilated, fragile and haemorrhagic vessels, defective re-modelling, severe vascular malformations including AVMs, disrupted vascular smooth muscle cell recruitment and maintenance (Vargesson et al, 2003).

TGF-β is a multifunctional polypeptide that regulates the proliferation and differentiation of various cells with an angiogenic effect in vivo but inhibits the growth of ECs in vitro. It may induce differentiation of mesenchymal cells into pericytes and smooth muscle

cells (Rohovsky et al, 1996) inhibit ECs proliferation and stimulate matrix deposition (Hirschi et al, 1996). Decreased TGF-β may lead to increased proliferation of ECs.

Pepper et al (1993) demonstrated that TGF-βR1 at relatively high concentrations (5-10ng/l) inhibited EC invasion and capillary lumen formation, while lower concentrations (100pg/ml-1ng/ml) of TGF-βR1 potentiated the effect of bFGF- and VEGF-induced angiogenesis in vitro. These results support the belief that the nature of the angiogenic response elicited by a specific cytokine is contextual, depending on the presence and concentration of other cytokines in the pericellular environment of the responding EC.

Eph receptor tyrosine kinases and their ligands (ephrins) play a key role during the development of the embryonic vasculature but their role and regulation in adult angiogenesis is still to be defined. Ephrins help establish clear molecular differences in arterial and venulous ECs at very early stages of development, when there is no clear morphological distinction between presumptive arterial and venous domain.

The differences between arteries and veins are imprinted early in embryogenesis such that arterial ECs express the transmembrane ligand ephrin-B2 and veins express the receptor Eph-B4 (Wang et al, 1998). However, it remains unclear as to where and how the critical interactions occur between cells expressing these markers (Wang et al, 1998). The widespread expression of other B-class family members indicates these molecules play a critical role in vascular development (Adams et al, 1999).

Vihanto et al, 2005 implied that hypoxia up-regulated Ephs and ephrins in the skin. Ephrins cannot induce potent mitogenic responses from target cells and have been mostly implicated in the process of neural cell guidance. B-ephrins can promote attachement of ECs to extracellular matrix components by activating integrin function (Huynh-Do et al, 1999) and it has been speculated that integrin regulation may be important for Eph function. Like the Angiopoietins, the ephrins seem to act primarily in later stages of vascular development, although they may contribute to the initial formation of vessels.

F) PHYSIOLOGY RELATED TO STUDY OF HYPOXIA

F.1 Measurement of blood gases

This measurement evaluates how effectively the lungs are delivering oxygen to the blood and how efficiently they are eliminating carbon dioxide from it. This test also indicates how well the lungs and kidneys are interacting to maintain normal blood acidity. The measurement of blood gases is usually done to assess respiratory disease and other conditions that may affect lungs, to manage patients receiving oxygen. At the same time the test gives information about the kidney function. This analysis is often performed during general anaesthesia, especially for complex, lengthy cases. Measurement of blood gases is traditionally

performed on blood from an artery but venous blood could also be used. The test measures the partial pressure of oxygen and carbon dioxide in the blood, the oxygen content, oxygen saturation, bicarbonate content and blood pH. Oxygen in the lungs is carried to the tissues through the bloodstream, but only a small amout of this oxygen can dissolve in arterial blood depending on pO_2, which is the pressure that the gas exerts on the walls of the arteries. Measuring the partial pressure of oxygen is in fact measuring how much oxygen the lungs are delivering to the blood. The oxygen that is not dissolved in the blood combines with haemoglobin. The oxygen measurement in blood gases analysis indicates how much oxygen is combined with the haemoglobin. For this measurement a heparinised, freshly withdrawn, bubble-free arterial or venous blood sample is necessary. Prompt analysis of the sample is required as any delay reduces the pH and pO_2 and increases pCO_2. Air bubbles lead to a false increase in pO_2. This measurement was used on a group of CVM patients in this study and details of the methods and bood gas analyser used are given later.

F.2 Blood-tissue gas exchange

This term represents the movement of oxygen from the blood stream into the tissues. Oxygen moves between the systemic capillary blood and the tissues by simple diffusion. It is known that a gas diffuses from an area of higher partial pressure to an area of lower partial pressure. There are two sites of exchange of oxygen and carbon dioxide: the lungs and the tissues. The exchange of gases between the air in the pulmonary alveoli and the blood in the pulmonary capillaries is called external respiration. Internal respiration is the exchange of gases between the blood in the systemic capillaries and the tissues. The rate of transfer of O_2 through tissue is proportional to the tissue area, with the difference in oxygen partial pressure between the two sides inversely proportional to the thickness (West, 2004).

Oxygen diffused in blood at the lung level is bound to haemoglobin to form the oxyhaemoglobin complex. 98.5% of all oxygen in blood is carried bound to haemoglobin. Because of this, the % of haemoglobin molecules carrying oxygen is important. Oxyhaemoglobin can be caused to release oxygen by the addition of H^+ ions. The difference in pH of arterial (7.44) and venous blood (7.35) is sufficient to cause the release of O_2 to the tissues, where the partial pressure of oxygen at rest is about 40mmHg. Therefore, a lower pH in tissue can determin the oxygen to be delivered by haemoglobin as well as increased temperature, increased levels of 2,3-diphosphoglycerate and CO_2. The quantitative analysis of oxygen/carbon dioxide exchange in tissues is rather difficult due to experimental challenges in accurately determining pO_2 and pCO_2 in tissues. The increase in tissue blood flow (given in CVMs by an increase of vessel number or blood vessel diameter) is an effective way of increasing tissue O_2 supply. High blood flow means further increase in blood flow becomes

ineffective because the O_2 supply by diffusion is limited. There are two physiological ways of improving diffusion conditions of O_2 in tissues. One is an increase in the capillary diameter thus increasing the surface available for diffusion. The other is the opening of closed, unperfused capillaries increasing the capillary density and reducing the diffusion distance (Piper, 1982). These are theoretical models and in real tissues oxygen diffusion mechanisms are more complicated. In CVM, and especially in AVMs capillary shunting has been described, therefore there is a high chance that the corresponding tissue will be hypoxic even in spite of a high pO_2 within the CVM vessels.

F.3 Account of ventilation-perfusion inequality

In an ideal lung the ventilation/perfusion (V/Q) ratio is 1. A real lung is not ideal and there are always differences in V/Q ratio in different parts of the lung. One factor influencing the V/Q ratio is gravity. When an area of lung receiving blood is not ventilated (for example due to a blockage in the airways), then, that blood passes through the lung without receiving any oxygen. This blood flow represents a pulmonary shunt. Shunted blood with its very low oxygen content mixes with the rest of the pulmonary blood and lowers the overall oxygen content of the mixture. The pulmonary shunt represents a form of V/Q inequality. An area with no perfusion is termed dead space. To interpret a blood gas analysis of the blood within the CVM vessels, arteries and veins, it is important to understand V/Q inequality and underlying clinical pathology that can lead to V/Q inequality, and exclude patients with conditions that may alter the results. Therefore, all participants with respiratory disorders, musculo-skeletal diseases and depression of the brain's respiratory center should be excluded.

F.4 Oxygen delivery to tissues and oxygen consumption

An abnormally low pO_2 in tissues is called tissue hypoxia. This is frequently caused by low oxygen delivery. An adult at rest uses about 250 ml of oxygen every minute and this means that about 25% of the arterial oxygen is used every minute. Generally, there is more oxygen delivered to the tissues than the amount used by tissues. When oxygen consumption is high, the increased oxygen requirement is provided by an increase in cardiac output. Low cardiac output, low haemoglobin, will lead to an inadequate oxygen delivery to tissues. If the oxygen delivery to tissues falls in respect to oxygen consumption, tissues will extract more oxygen from the haemoglobin. This can happen up to a threshold and what follows is anaerobic metabolism and lactic acidosis (Law, Bukwirwa et al, 1999). Tissue hypoxia can be due to a low pO_2 in arterial blood caused for example by pulmonary disease ('hypoxic hypoxia'), or a reduced ability of blood to carry O2 as in anemia or carbon monoxide poisoning ('anaemic hypoxia'), or due to a reduction in blood flow, either generalised, as in shock, or localised because an obstruction ('circulatory hypoxia').

To accurately assess oxygenation in CVMs patient studied ideally should have haemoglobin within normal range and patients with cardiac diseases known to affect the cardiac output should be excluded.

APPENDIX TO MATERIAL AND METHODS

A) HISTOCHEMICAL STAINS PROTOCOLS

A.1 Deparaffinising the slides

To melt the wax away from the fixed tissue, before staining slides were heated on a hot plate to 60°. Slides were then immersed twice into separate jars of xylene (Genta Environmental Limited, York, UK), which is a wax solvent, each time for 20 minutes. Slides were then drained of xylene and dipped into 100% alcohol to remove the wax solvent. This procedure was repeated three times to allow alcohol to rinse the slides thoroughly. The slides were then dipped in 100% alcohol (IMS 99%) (Genta Environmental Limited, York, UK) for two minutes. At the end the slides were rinsed in tap water and then in distilled water.

A.2 Haematoxylin and eosin staining (H&E)

H&E is a basic histo-chemical stain and helps to differentiate nuclei from other tissue components. Haematoxylin is not a dye, and develops colouring properties on oxidation to haematin. Haematoxylin has very little tissue affinity and requires the help of an inorganic molecule to attach to tissue. Eosin is an acid dye, which is negatively charged and attaches to the tissue of opposite charge.

De-waxed paraffin sections are immersed in Harris' haematoxylin (Surgipath Europe Limited) between 30 seconds to a few minutes, rinsed in running tap water and then differentiated by immersing into 70% acid alcohol (70% alcohol with 100% HCl to make a 0.5% solution) for 30 seconds. They are rinsed again in running water, blued in ammoniated water (approximately 30 seconds), washed in tap water again and immersed in 1% aqueous eosin (Surgipath Europe Limited) (time of staining varies), which stains cytoplasm. They are then rinsed in running tap water, dehydrated through 3 changes of ascending alcohols (starting with 70% and finishing with 99%) for about 4 minutes, followed by 3 changes of xylene (2 minutes). Once slides are cleared, they are mounted using automatic slide mountant (Leica CV5000). Some of the slides analysed were stained on an automatic H&E stainer, which used the protocol described above (Autostainer, Vision Biosystem, UK). Nuclei stained blue and other tissue components appeared in shades of red and pink.

A.3 Masson's Trichrome

This histochemical technique aims to differentially stain connective tissue. Trichrome is so named because it selectively colours the tissue in three colours and helps the differentiation between muscle and collagen. Connective tissue and smooth muscle cells stain differently

because the staining affinity of the tissue is affected by molecular size, density of tissue, pH and use of colourless dyes such as phosphomolybdic and phosphotungstic acid, which help stainig the connective tissue. This stain will demarcate muscle and red blood cells (red), collagen (green) and nuclei (blue/black). Apart from showing a clear definition between the collagen and smooth muscle, Masson's Trichrome helps define the different layers of smooth muscles such as circular and longitudinal.

The reagents used in the Charing Crosss Hospital protocol used here were 5% chromic acid, 1% chromazone red in 1% acetic acid, 1% phosphomolybdic acid and 1% light green in 1% acetic acid. By sequentially applying acid dyes in ascending order of size, differential staining is achieved due to varying pore sizes in connective tissue components. Wide spread connective tissue in a section usually provides an internal control but a freshly fixed piece of heart or intestine can be used as control for this stain.

One % Chromazone red (Surgipath Europe Limited) in 1% acetic acid is applied to de-waxed tissue sections for 5 minutes. Sections are rinsed again in distilled water and differentiation is achieved with phosphomolybdic acid (Surgipath Europe Limited) applied for 5-10 minutes. Sections are rinsed with distilled water and the trichrome mixture (2 parts 1% chromazone red in 1% acid acetic, 4 parts 1% phosphomolybdic acid, and 6 parts 1% light green in 1% acetic acid) is applied for 10 minutes. They were then rinsed in phosphomolybdic acid, treated with acetic acid for 2 minutes, dehydrated, cleared and mounted.

A.4 Elastic Van Gieson

This technique is used to demonstrate elastic fibres. Elastic stains have an affinity for sulphur compounds which may be found in elastic fibres. Control sections may not always be required but lung, skin and artery may be used. There are two components to this staining: Van Gieson stain differentiates between muscles and connective tissue and Verhoeff's elastic stain is a haematoxylin stain containing ferric chloride and Wright's iodine solution.

Tissue sections to be analysed were treated with 70% alcohol and Millers stain was applied for 2 hours followed by differentiation in 70% alcohol. Millers stain is harmful and flammable. Concentrated acid was prepared in fume cupboard and always handled with rubber gloves.Van Gieson stain (5-10 ml 1% acid fuchsine in distilled water, in 100 ml saturated picric acid) was applied for 5 minutes. Picric acid has a molecular weight of 229, penetrates into tissue and is held by hydrogen bonds. Acid fuchsin has a molecular weight of 585, has difficulty penetrating some structures and stays mostly on the surface, and it is therefore easily washed off. These solutions give the differential staining between connective tissue and muscles. Sections were then rinsed in distilled water, blotted, dehydrated in absolute alcohol, cleared and mounted. Elastic fibres show in black, collagen in red and muscle in yellow.

A.5 Toluidine blue

This stain is performed to demonstrate meta-chromatic substances. Mast cells are widely distributed in the connective tissue. Their cytoplasm contains granules composed of heparin and histamine, which are meta-chromatic. Acid mucins react with certain cationic dyes and 'polymerise' them to a form which has a different absorbtion characteristic to the normal dye. Tissue appropriate to the meta-chromatic substance to be demonstrated such as cartilage for chondroitin sulphate, intestine containing mast cells for heparine sulphate may be used as control material.

Reagents used were 0.3% potassium permanganate, 0.2% Toluidine Blue and 0.2% uranyl nitrate. Uranyl nitrate is radioactive but of low radiotoxicity; dry powder is therefore kept in a metal container. De-waxed slides were then treated with permanganate for 5 minutes, rinsed in water, bleached with oxalic acid and Toluidine Blue was applied for 2 minutes. After rinsing in distilled water, uranyl nitrate was applied for 10 seconds or until the differentiation is complete as visualised microscopically. Sections were rinsed once more in distilled water, blotted, dehydrated, cleared and mounted. Mast cell granules and acid mucins show in red/violet. Metachromasia, tissue elements staining a different colour from the dye solution, is due to the pH, dye concentration and temperature of the basic dye.

B) COMPUTERISED IMAGE ANALYSIS-ASSESSMENT OF COLLAGEN PROCENTAGE

Defining the region of interest (ROI)

This defines the area of interest on any given slide for particle detection. This was important if there were areas on slides which were not uniformly stained or should not be included. By choosing the option of drawing the area of interest on the projected field, it was possible to exclude such areas. For this, image was selected, 'Analysis' option was chosen and 'Define ROI' used. Using 'Draw ROI' option the area of interest was defined.

Setting colour threshold

This command was used to set the logical thresholds for binarisation of true colour images in stained slides used in this study. From each of the projected field at 40 x magnification, several pixels were sampled. RGB colour model was used. Using this lower and higher limit of red, green and blue parts of the hue, saturation and intensity (HSI) was defined. All values were between 0 and 255, except hue. As the exact thresholds for the stained areas were not known, they were defined by selecting a set of points in the image, whose pixel values then defined the limits. From the 'Image' menu 'Set colour threshold' was chosen. The option 'New' enabled the cursor to be moved to the area of interest and hotspots defined. By clicking on more than one hotspot after pressing 'Add', the threshold can be widened to include both image limits and image thresholds. To check the accuracy of defined threshold, image was

opened again, and ‘Set colour threshold’ option was chosen, and ‘Preview’ gave the defined threshold. Using the preview function, this range of threshold can be projected on the field repeatedly for comparison with another field. This was repeated until the chosen set of threshold values agreed with most of stained areas of interest. To reduce the chance of excluding potentially low uptake areas, at least 5-10 different slides were studied in this way before selecting a particular threshold for detection of stained areas. This threshold was saved for that particular stain.

Binarising of the image

Once the colour threshold for the field of interest was chosen, the image was converted to a binary format. This defined the thresholds on the image, resulting in a black and white picture as every pixel within the defined colour range turns white while the rest turn black. Thus, the stained areas of interest will be white.

Morphological filter

The main task in IA is the detection of objects in binary images. Sometimes objects, which were close together, but not touching, have to be treated as one single object and other times as separate. This problem was solved based on mathematical morphological theories. The central techniques were erosion (shrinking of an object), dilation (expansion of object), or combination. To execute this, ‘Morphological filter’ option was chosen on ‘Operate’ menu. This compares the value of central pixel with the values of all pixel set. This was followed by application of filter ‘Erosion’, which separated the single objects that touch each other, by decreasing the size of individual objects which do not touch each other and increase the size of objects. Small holes in the objects were closed and small irregularities at the periphery of objects were equalised.

Define detection

This was used to parameterise the detection. As the region of interest in individual fields was chosen, ‘ROI’ option was used and for border particle definition, ‘Truncate’ was used to cut off the particles outside the ROI.

Detection of particles

The command ‘Detect’ in the ‘Analysis’ menu was used to analyse the chosen image in the display buffer for particles, based on the settings of the threshold values. If the particle falls outside the set threshold, it was neglected.

ROI results

This gave the results of measurements. The average percentage expression of positive stain on the different fields was analysed for each slide and represented the collagen percentage.

IA resulted in a set of values that indicated the stain percentage in the area of interest (ROI). These values were averaged to give a mean percentage stain for one CVM or control specimen.

C) IMMUNOHISTOCHEMICAL STAINING PROTOCOL

The following steps were taken in the process of immunohistochemical staining: 1) tissue processing, 2) antigen retrieval, 3) background blocking, 4) primary antibody application, 5) secondary antibody application, 6) substrate preparation, 7) development, 8) counter-staining and 9) image analysis

C.1 Tissue processing for IHC

Immunohistochemical stains were performed on tissue sections cut at 3μm from paraffin-embedded blocks on a microtome. The sections were floated in water and picked up on APES-coated (3-amino-propyl-triethoxysilane), formalin dipped slides. Tissues have negative charge and by treating the slides with an agent with positive charge such as APES, the tissue sections adhered better. Sections were allowed to dry overnight at room temperature and heated to 40-60°C at least an hour prior to staining. The tissues on the slides were treated with 2% solution of 30% H_2O_2 in Methanol for 3 minutes, which blocked the endogenous peroxidases present in erythrocytes, leucocytes and other cells, which otherwise may lead to high non-specific background staining. Aqueous H_2O_2 is diluted with Methanol to prevent damage to peroxidase rich tissue. The slides were rehydrated by washing them in running water before proceeding to antigen retrieval.

C.2 Antigen retrieval

Formalin, like other Aldehyde-based fixatives can mask certain tissue antigens. This effect is thought to be the result of formation of methylene bridges, which occur between reactive sites like amines, thiols, aromatic rings, on different portions of the same molecule or of adjacent proteins. Time, temperature and fixative concentration are all variables related to the extent of masking. This antigenic masking can be reversed, and the visualisation of antigens can be significantly improved by using antigen retrieval techniques. These techniques represent the application of heat for varying lengths of time to formalin-fixed paraffin embedded tissue sections in aqueous solution, a procedure named heat induced epitope retrieval (HIER) or using proteolytic enzymes, a procedure named proteolytic enzyme induced epitope retrieval (PIER). Microwave oven, pressure cookers and steamers are commonly used as heating methods for HIER.

The following methods were used in our research work: heating by pressure cooking, microwaving and enzymatic digestion as shown in the following table.

Tabel appendix. 10. Antibodies and antigen retrieval methods used

Antigen	Retrieval method
CD3	Pressure cooking
CD20	Pressure cooking
Factor VIII	Protease enzyme digestion for 10min at 37°C
CD68	Protease enzyme digestion for 5min at 37°C
Ki67	Pressure cooked for 90 seconds
Alpha SMA-1	None
S100	None
VEGF A	None
VEGFR-1	None
Tie-2	Pressure cooked for 90 seconds
αVβ3	Protease enzyme digestion for 15min
αVβ5	Protease enzyme digestion for 15min
TGFβ	Pressure cooked for 90 seconds
HIF-1α	Pressure cooked for 90 seconds
HIF-2α	Pressure cooked for 90 seconds
ET-1	Pressure cooked for 90 seconds
ETA	Pressure cooked for 90 seconds
ETB	Pressure cooked for 90 seconds
CD10	Pressure cooking
GLUT-1	Pressure cooked for 90 seconds
CAIX	Protease enzyme digestion for 15min at 95°C
ERβ	None
Progesteron receptor (PR)	Pressure cooked for 90 seconds

The methods were optimised following a preliminary study in which tissue slides were subjected to different methods of antigen retrieval, then immunostained and assessed for the quality of tissue preservation and consistency of staining. Section of CVMs and control tissue were processed through microwave heating, pressure cooking and enzymatic digestion and then immunostained with selected antibodies at different dilutions. The immunostained slides were analysed together with a consultant pathologist and the best antigen retrieval method and antibody dilution was chosen for each antigen.

Citrate buffer was used in this study for HIER. This was obtained from 21g citric acid and 10 litres distilled water. The pH was adjusted to 6.0 using 265ml of 1M sodium hydroxide (NaOH).

Pressure cooking was applied to tissue sections on APES coated slides, which were placed in a metal rack with at least one space between each slide. This allowed bubbles to flow freely between the slides without disrupting the tissue sections. The citrate buffer (1400ml) was brought to boil in the pressure cooker on the hotplate with the lid open. The slides were lowered into the boiling buffer and lid locked. The pressure cooker took about 5 minutes to reach pressure with all the weights on. The cooker was allowed to continue for 90 seconds from the point when steam was escaping from under the weights at a steady rate. It was then removed from hot plate and kept under cold running water until valves released. The lid was opened and the slides were placed in a tap water bath. This antigen retrieval technique was used for CD3, CD20, CD10, Ki67, HIF-1α and HIF-2α antibodies.

The enzymatic technique was used for antigen retrieval for Factor VIII and CD68 immunostaining, using protease XXIV. 0.01% Protease XXIV was mixed with TBS (pH 7.6) at 37°C. Sections of tissue on APES slides were placed in distilled water at 37°C. A bath of 100ml of TBS was pre-warmed to 37°C and 0.01g of protease was added just before introducing the slides. The protease dissolved immediately. The slides were left in the bath for 5 minutes and then removed and washed in tap water.

C.3 Blocking of non-specific binding

This was carried out using normal serum from the host species in which the secondary antibody to be used was raised. This binds to protein-binding sites by either non-specific absorption or by binding to specific but un-wanted antigens in tissue. The secondary antibodies used were human anti-mouse and anti-rabbit antibodies, at 1:10 concentration in TBS (6.4g Tris, 44ml in HCl, 80g Sodium Chloride, and 10ml distilled water). The tissue sections were ringed with PAP pen and blocked with chosen serum. This was left to react for 10 minutes to achieve antigen blocking. The blocking buffer was then drained off the tissue which was not washed.

C.4 Primary antibodies used for IHC

The primary antibodies and dilutions used in this research are listed below (Table appendix. 11.).They were separetly applied to tissue sections and incubated at room temperature for an hour. The slides were then washed twice with TBS. Primary antibodies were omitted for negative control sections.

Tabel appendix. 11. Primary antibodies for immunohistochemistry and dilutions used

Antigen	Antibody	Antibody dilution
CD3	Monoclonal anti-human, Vector Laboratories, VP-C316	1:100
CD20	Monoclonal anti-human, Dako, M0755	1:600

Factor VIII	Polyclonal anti-human, Dako, A0082	1:800
CD68	Mouse monoclonal antihuman, DAKO, Clone 2B11+PD7/26	1:200
Ki67	Monoclonal anti-human, Vector Laboratories, VP-K542	1:200
SMA 1-α	Monoclonal anti-human, Sigma, A2547	1:6000
S100	Polyclonal, anti-human, Dako, Z0311	1:1000
VEGF A	Monoclonal antihuman, Labvision	1:2000
VEGFR-1	Mouse monoclonal anti-human, R&D Systems, Clone 49560	1:100
Tie-2	Mouse monoclonal anti-human, R&D Systems, Clone 83715	1:100
αVβ3	Mouse monoclonal anti-human, Chemicon, Clone LM609	1:200
αVβ5	Mouse monoclonal anti-human, Chemicon, Clone P1F6	1:200
TGFβ	Mouse monoclonal anti-human TGF Beta, Serotec, Clone TB21	1:50
HIF-1α	Mouse monoclonal anti-human, Labvision	1:100
HIF-2α	Mouse monoclonal anti-human, Novus Biologicals	1:150
ET-1	Mouse monoclonal anti-human, Abcam, Clone TR.ET.48.5	1:20
ETA	Rabbit polyclonal anti-human, Abcam	1:50
ETB	Rabbit polyclonal anti-human, Abcam	1:50
CD10	Novocastra	1:30
GLUT-1	Mouse monoclonal anti-human, Abcam, Clone SPM498	1:100
CA IX	R&D Systems	
ERβ	Rabbit monoclonal anti-human	1:200
Progesteron	Monoclonal anti-human	1:150

A section from all CVM cases studied was immunostained for GLUT-1, since vascular malformations do not express this marker but haemangiomas are positive.

C.5 Application of secondary antibodies

The primary antibodies used in this study were raised in mouse or rabbit, therefore anti-mouse and anti-rabbit antibodies were used as secondary antibodies. A biotin label was used in order to generate amplification, as is required in the ABC technique for IHC. Biotin has a high affinity for avidin, which in turn conjugate to an enzyme to amplify the signal. This is why biotinylated human anti-mouse and anti-rabbit antibodies were used as secondary antibodies for this study. The antibody was applied at dilution 1:400 in TBS and sections were incubated at room temperature for 35 minutes.

C.6 ABC peroxidase technique

The ABC (Avidin-Biotin complex) Peroxidase technique was used for IHC staining and is the standard technique in surgical pathology at Charing Cross Pathology Department, where this work was performed. This represented a variation of the indirect technique of IHC staining, where an unlabelled primary antibody (first layer) reacts with tissue antigen and a labelled secondary antibody (second layer) reacts with the primary antibody. This method is different from what is called the direct method, where a labelled antibody reacts directly with an antigen in the tissue.

The ABC technique involves three layers. The first layer is the un-labbeled primary antibody; the second layer is the biotinylated secondary antibody and the third layer, the complex of avidin-biotin peroxidase. The peroxidase is then developed by the DAB to exhibit a colorimetric effect.

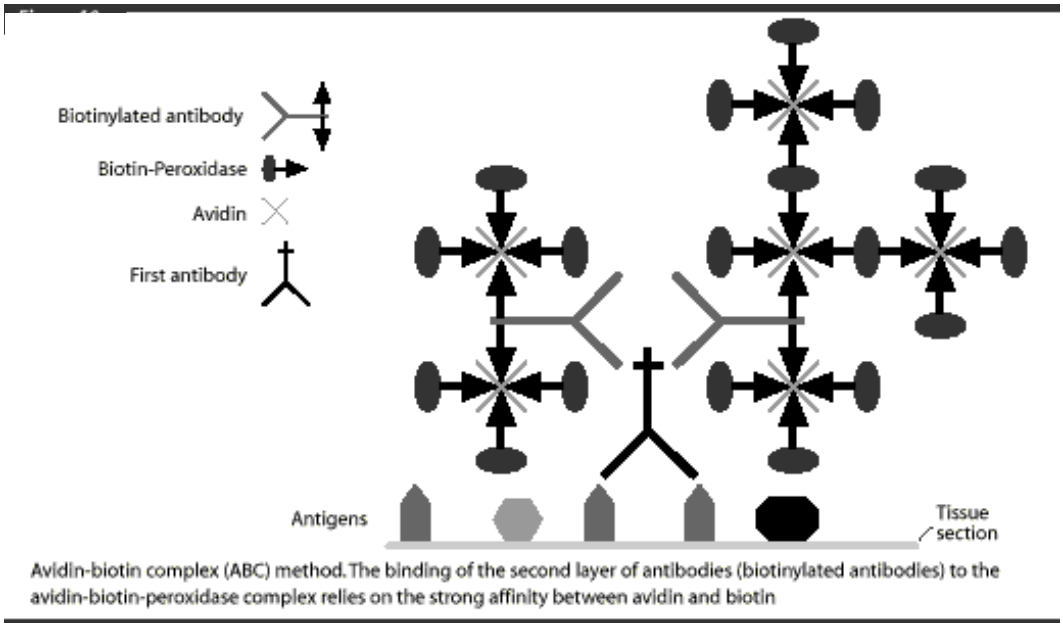


Figure appendix. 2. The ABC peroxidase technique for immunohistochemistry

(The principle of ABC peroxidase 3 layer technique, adapted from www.aasp.org/shap/issues)

Avidin is a large glycoprotein with a molecular weight of 68 Kilodaltons, found in egg white and has a very high affinity for the small molecule vitamin, biotin with a molecular weight of 24 Kilodaltons. The affinity of avidin to biotin is over one million times higher than antibodies have for most antigens and this binding is irreversible. Avidin has 4 binding sites for biotin and most proteins including enzymes like peroxidases can be conjugated with several molecules of biotin. These properties allow macromolecular complexes to be formed between avidin and biotinylated enzyme. Biotin can be conjugated to a variety of biological molecules such as antibodies. Avidin has the disadvantage that it has a high isoelectric point of about 10 and therefore they are positively charged at neutral pH. This is why it may bind to negatively charged structures such as a cell nucleus. Another disadvantage is that avidin reacts with molecules like lectins via the carbohydrate moiety. To avoid these problems, avidin can be replaced with streptavidin. Streptavidin is a protein with a molecular weight of 60 Kilodaltons isolated from bacterium Streptomyces avidinii, and similar to avidin, has four high affinity binding sites for biotin. The physical properties of streptavidin make it more suitable for IHC because it has an isoelectric point close to neutral pH and has fewer strongly charged groups at pH used for IHC. It also does not bind to lectins.

The ABC technique used in this study utilised streptavidin biotin complex in pre-formed complexes. Streptavidin and horse radish peroxidase (HRP) were mixed for at least 30 minutes at room temperature to prepare the complexes, which were then attached to the biotinylated antibody. These complexes were diluted 1:100 using TBS and were left for 35 minutes on the slides after which they were reduced with TBS.

C.7 DAB visualisation

DAB (3, 3 – di aminobenzidine) produces a brown precipitate which is insoluble in alcohol. In the presence of H_2O_2, DAB was converted to this coloured product and water by peroxidase. As the HRP is attached to the antigen via the avidin-biotin complex, adding DAB will produce an enzymatic reaction which will lead to an insoluble visible DAB precipitate. A combination of 10ml of distilled water, 4 drops of buffer stock, 8 drops of DAB stock and 4 drops of hydrogen peroxidase were added to the antibody treated slides for 3-5 minutes. They were then washed with running tap water and counter stained with Haematoxylin. The antigenic sites were stained dark brown from DAB precipitate and the nuclei blue from Haematoxylin. The slides were then mounted with aqueous mounting medium, sealed with wax and marked.

D) HISTOLOGICAL MORPHOLOGY ASSESSMENTS

D.1 Assessment of blood vessel number

The number of vessel present per microscopic field was considered an index for comparison of morphological and angiogenic change in this study and it was assessed using

AnalySIS software. Factor VIII (known as the von Willebrand factor) was used to delineate blood vessels in CVM and control tissue as this marker was considered to best visualise ECs lining the blood vessels. Factor VIII is a glycoprotein present in EC and shows a granular pattern of reactivity. Factor VIII stain was positive in ECs which delineated the blood vessels and it was used to count vessel numbers.

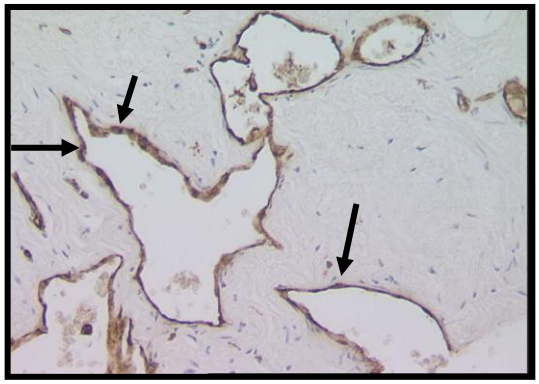


Figure appendix. 3. Positive expression of Factor VIII immune-staining CVM tissue

The number of vessels was quantified by counting the number present in one field at x40 magnification. All vessels with a visible lumen present per field were counted, including those showing as incomplete vessels in the studied section. AnalySIS software allowed to point count the cells. The slide above shows a typical field with blood vessels delineated by the brown-stained ECs. Immunostaining for Factor VIII followed the protocol described earlier. On the projected image on the AnalySIS software, the touch count option allowed interactive counting of number of objects of interest. To ensure that each object was counted only once, it was marked with an overlaying dot. The number of dots was recorded on an Excel spreadsheet. The CVM or control tissue was scanned along the areas of interest for 5 different fields. Each of the fields at x 40 magnification was found to cover 40,000μm^2 of the slide. The average of the blood vessel counts was calculated and considered as the vessel count per microscopic field.

D.2 Assessment of blood vessel diameter

Blood vessel diameter was measured to assess morphological changes and angiogenesis in CVMs. It has been suggested that abnormal local concentrations of angiogenesis mediators such as VEGF, regulates vessel diameter and are responsible for increased blood vessel diameter, but this has not been proven for CVMs.

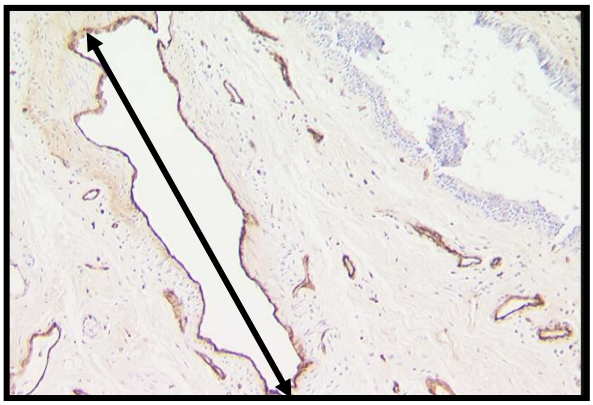


Figure appendix. 4. Line representing measurement vessel diameter using Factor VIII stain

The blood vessel diameter was measured at x40 magnification using the images captured from the Factor VIII stained slides as these showed the blood vessel contour clearly. The largest apparent length of the blood vessel was chosen as the maximum blood vessel diameter and this was measured for each vessel seen on a given field provided the vessel had a lumen. The diameter included the outer border of the dark brown stain of the ECs. Incomplete vessels seen on each field were also included and the measurement was carried out in the same manner. The software AnalySIS allowed the measurement of the distance between two points on a straight line. The command 'arbitrary distance' on the AnalySIS software aided the measurement of the distance between two points at any position or direction on the projected image. The measurement was marked with a line as an overlay. The software allowed multiple measurements. Five different fields of interest for each analysed slide were considered and for each field an average diameter was obtained as a result of measuring all vessel diameters present per field. The average diameter for the five fields analysed, was the specimen average blood vessel diameter.

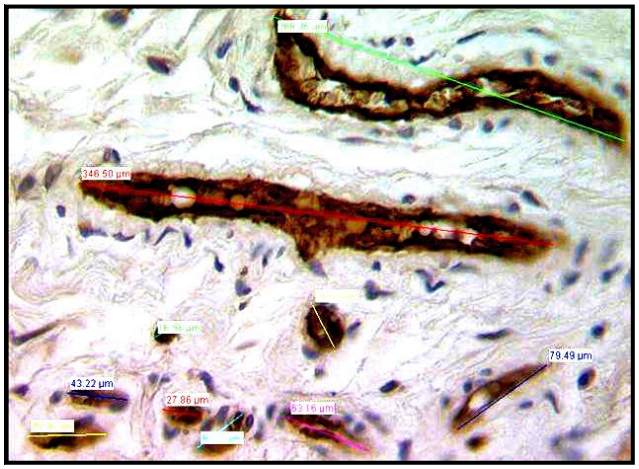


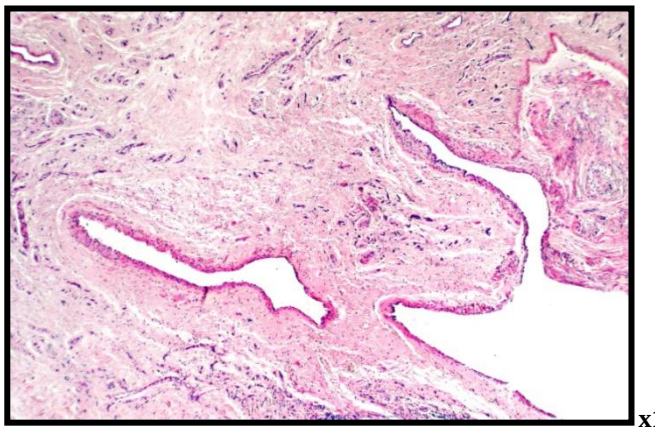
Figure appendix. 5. Use if IA to calculate average blood vessels diameter

The average diameter for the above field was obtained by adding measured diameters of all blood vessels seen on the field and dividing the sum by the number of vessel seen.

D.3 Assessment of cell count

The inflammatory cell infiltrate of the CVM or control tissues was analysed using the immunohistochemical techniques described earlier with CD68 for macrophages, CD3 for lymphocytes T cells, CD20 for lymphocytes B cells and ki67 for proliferative cells. All these cells were quantified by counting the number of cells per field with x40 magnification, which represented 1mm^2. Toluidine blue histochemical stain was also used to identify mast cells.

The CVMs as well as the control tissue were initially screened using the H&E stained slides to identify any technical faults, tissue abnormalities, granulomas and to select the fields of interest represented by the abnormal blood vessels seen in CVMs and their vicinity, as well as the vascular parts of the control tissue, rather than the surrounding fat or stromal tissue. The H&E screening also helped to confirm the distribution of the positive reaction when this was analysed on immunostained slides.



x10

Figure appendix. 6. H&E stain of a CVM, showing a representative field for analysis

The brown-stained inflammatory cells (as seen in figures 4.5 and 4.6) were counted using AnalySIS software, which was used to point count the cells. The touch count option of the AnalySIS software allowed interactive counting of cells of interest and each cell was marked with an overlying dot so that it was counted once only. Cells with clear demarcation of cytoplasm and nucleus were selected to avoid including stain artefacts. The number of cells was counted on 5 different relevant fields for each section and the result recorded on an Excel spreadsheet. The average of the 5 fields was calculated and taken as the cell count per field. Similar techniques were used for counting mast cells on Toluidine stain and cells positive for CD3, CD20 and ki67 immunostains.

Light microscopy was also used to count macrophages and mast cells in the preliminary study (17 CVM specimens and 10 control specimens). A 1 mm^2 microscopic grid

under light microscopy was used by two independent observers to count the cells observed, over 5 different fields. The quality of data collected was assessed by analysing the inter-observer agreement.

D.4 Assessment of nerve fibres number

Nerve fibres have been seen in close association with abnormal vascular channels seen in CVMs. Nerve fibre count was performed as part of the morphological analysis of CVM since vessel and nerve development share some common biochemical mediators such as ephrins. Nerve fibres count was analysed using S100 immunostained CVM or control tissue. The slides stained with S100 showed typical brown staining of the nerve fibre (as shown in the figure 3.5), which were counted using x40 objective, on five different fields, as previously described. The average number of nerve fibres considered the nerve fibres number per field.

D.5 Assessment of % area of collagen

Masson's Trichrome stain was used to analyse the area of collagen present in CVMs and control tissue as this stain differentially stains the connective tissue. Collagen stained green and the muscle and red blood cells stain red. Nuclei stain blue/black as showed in figures 3.6, 3.9, 3.10 and 3.11.

The measurement of collagen area in one field was performed using IA, which calculated the average procentage area that stained green and corresponded to the collagen distribution. The quality of the Masson's Trichrome staining was assessed using positive controls to ensure uniformity of analysis. IA of 5 different fields was performed for each of the tissue sections at 40 x magnification. From each field of interest, the collagen areas were sampled. Their red, green, blue and hue, saturation and intensity values were stored. Once the field was chosen and projected on the computer screen, IA involved a number of stages, which are detailed elsewhere.

D.6 Qantitative analysis of immunostains

Quantitative assessment of immunostaining was not performed using IA since there was more variation between batches of staining which made interpretation very difficult. Instead, a conventional three-tier grading system was used.

The staining localisation and intensity for each of the markers were independently assessed by two observers (NM and AF). These two independent observers examined all sections randomly and the inter-observer agreement was analysed at the end using kappa coefficient analysis. The two observers met to agree the number of CVMs and control specimens to be analysed, to clarify for each marker the criteria for assessment with respect to localisation of the stain, to differentiate between diffuse and focal staining, and technical artefact. It was agreed that negative staining would score 0, focal areas with sparse staining or

occasional isolated positive staining would score 1, weakly positive staining or,at least one focus with intense positive staining or diffuse weak to moderate staining would score 2 and strongly positive, intense staining would score 3. Diffuse, sparse immunoreactivity observed throughout tissue sections was considered to represent background or non-specific staining and thus was graded negative. For each specimen the immunostaining was assessed in five different microscopic fields by each observer, one using light microscope and the other using IA.

D.7 General description of CVM morphology

Image analysis was used to scan all sections from 17 specimens included within the pilot study. These included: H&E, Elastic Van Gieson, Masson's Trichrome, SMA 1-α, ki-67, CD68, Factor VIII, S100 and Toluidine Blue. A comprehensive description of the presence of small, medium or large vessels, thick o thin vessels, precence of stromal cellular infiltrate, type and location of the infiltrate in relation to the blood vessels, presence of proliferative cells, disruption of internal or external elastic lamina, presence of collagen or fat infiltrate, location of the nerve fibres in relation to the abnormal channels comprising the CVM, presence of macrophage or any other features were recorded. This descriptive analysis was used to establish if these were morphological features that correlated with clinical behaviour of the CVM and outcome.

D.8 IHC controls

Positive controls tissues were collected in accordance with recommandations on the datasheet for each primary antibody and under the guidance of an experienced pathologist.

Tabel appendix. 12. Positive control tissue for immunohistochemical studies

Antigen	Control tissue
CD3	Tonsil
CD20	Tonsil
Factor VIII	Vessels
CD68	Tonsil
Ki67	Tonsil
SMA1- α	Muscle
S100	Melanoma
VEGF A	Vessels
VEGFR-1	Skin
Tie-2	HUVEC
αVβ3	Skin
αVβ5	Skin

TGFβ	Prostate
HIF-1α	Synovium
HIF-2α	Synovium
ET-1	Human bowel
ETA	Placenta, Liver
ETB	Liver
CD10	Tonsil
GLUT-1	Oesophagus
CAIX	RCC, gastric mucosa
ERβ	Breast
Progesteron	Breast

Negative controls were used to test the specificity of the antibody used. This was done by omitting the primary antibody in the process of tissue staining. CVM and control vessel tissue were used for this purpose.

E) WESTERN BLOT PROTOCOL FOR HIFs PROTEIN ANALYSIS

Western blot analysis is an immunoblotting technique which provides information about presence, molecular weight, and/or quantity of an antigen by combining protein separation via gel electrophoresis with specific recognition of antigens by antibodies. This method is designed for separating a complex mix of protein on a gel, and then for transferring the proteins to a nitrocellulose membrane. Membrane bound proteins can then be probed with various antibodies to determine expression of proteins of interest. Immunoblotting is useful when the antigen of interest is insoluble or readily degraded and cannot be easily immunoprecipitated. Since most gel electrophoresis procedures result in denaturation of the antigen, only polyclonal and monoclonal antibodies that recognise the denaturated form of the antigen can be utilized in immunoblotting.

The Western blot technique is described in detail below.

Buffers are solutions which reduce the change of pH on addition of small amounts of acid or base, or on dilution. A number of different buffers are usually necessary for Western blot analysis.

To prepare samples for running on a gel, tissues need to be lysed to release the HIF proteins. This solubilises the proteins and they can migrate individually through a separating gel. The lysis buffer used was an 8M urea in Milipore H20 (1/10 vol Glycerol, 1/20 vol 20%SDS, 1/1000 vol 1M DTT, 1/100 vol 1M Tris pH 6.8). Phenylmethylsulphonyl fluoride

(PMSF) was added just before use (1/100 vol from a 50mM stock). A protease inhibitor tablet (Roche) was added to tissue immediately before use (one tablet for every 10mls of buffer).

The frozen tissue sample to be analysed is cut on dry ice to prevent thawing and tissue degradation. The cut section is further divided into very small pieces so that it can be homogenised properly. Tissue is placed into a tube, urea buffer (as detailed above) is added and both are homogenised immediately. The tube is kept on ice at all times. Between samples the homogeniser is wiped with paper towel and then briefly run into water. The tissue samples are left on ice for ten minutes and then the tubes are centrifuge at 1000 rpm, 4°C for 5 min. Protein extract is then removed to an Eppendorf tube. Samples are stored at -20°C or -80°C (the later being preferable). The protein extract is quantified using the Pierce BCA protein assay.

Antibodies usually recognise a small portion of the protein of interest and to enable access of the antibody to this portion is necessary to unfold the protein by denaturing the protein. A loading buffer with an anionic denaturing detergent is used for this purpose (25ml 4x TrisHCL/SDS, pH 6.8, 20 ml glycerol (Merck AnalaR, Germany), 4g SDS, 3.1g DTT (Sigma Aldrich, UK), 10 mg bromophenol blue, add H_2O to 100ml). This is usually kept frozen and defrosted just before use. The protein extract is mixed with the loading buffer up to 100µl final volume. The gel required a load of at least 30µg of protein per lane. Samples are then heated for 5 min at 95°C, spin down and left at room temperature but kept on ice, for loading. The next stage is the gel electrophoresis, meaning separating the proteins according to their molecular weight. The protein extract is run on standard 6% SDS gel, with stacking gel. Gels are placed in the electrophoresis tank as instructed by the manufacturer and bathed in the running buffer (2L of running buffer - 1g/L SDS (Merck AnalaR, Germany, no 444464T), 3g/L Tris base (Merck AnalaR, Germany, no 103156X), 14.4g/L Glycine (Merck AnalaR, Germany, no 101196X) or, if using new gels (Tris- acetate), 0.5 liter of 20X running buffer is made: 50mM TRIS base (60.6g), 50 mM Tricine (89.5g), SDS 0.1% (10g), which again is kept frozen at 20°C and defrosted just before use. A positive control lysate is used to demonstrate that the protocol is efficient and correct and that the antibody recognises the target protein, which may not be present in tissue samples. The Rainbow molecular weight marker is used to determine the protein size and to monitor the progress of the electrophoretic run. Approximate 10µl is loaded on the electrophoresis gel. The samples are then loaded and run at 80V until proteins have run into gel, then the gel is turned up to 150V. When the dye molecule (the ‘migration front’) reaches the bottom of the gel, the power is turned off. The gel is not stored and the transfer stage is commenced immediately. A wet transfer was carried out and 3L of transfer buffer was made up (3g/L of Tris base, 14.4 g/L Glycine, 200mls of Methanol/L). The transfer membrane, a Nitrocellulose membrane, (Immobilon-P, Millipore) is placed next to the gel and the gel and

membrane are sandwiched between sponge and paper as follows. The gel is transferred into transfer buffer in a small container and place on filter paper. The transfer membrane is cut to the exact size of the gel. Six pieces of paper are cut to exact size of gel. The transfer membrane is activated by placing it in methanol for 20 seconds then in water for 2 minutes. Transfer membrane is then put into transfer buffer for 10 minutes.

Two sponge layers are put into the transfer buffer - 3L solution. The transfer perforated cassette is placed flat on the bench – black side to the back. The 1st layer of sponge is placed on the bottom. Three sheets of filter paper soaked in transfer buffer are then layered over this – ensuring there are no air bubbles between the layers. The gel is layered on, again ensuring there are no air bubbles. The activated membrane is layered onto the gel. Three more pieces of filter paper soaked in transfer buffer are placed on the top and then the 2nd sponge over all. The cassette is closed, sealed and placed into the transfer tank. The cooler element is placed inside tank or the tank is transfered to cold room. The 3L of transfer buffer is poured in – the tank is placed on the stirrer and the flea is added to stir gently. The lid is put on connect to potentiostat and run for one and a half hours at 100V, or overnight at 20V and then in the morning 30 minutes at 60V. The membrane is then blocked to prevent non-specific background binding of the primary or secondary antibodies to the membrane. For this purpose, the membrane is placed in blocking buffer (minigel to make up 200mls - PBS (Merck AnalaR, Germany), 10g 5% fat-free milk powder (Premier Bands UK LTD), 2g 1% BSA (Sigma Aldrich, UK), 0.2mls 0.1% Tween (Sigma Aldrich, UK)) for 1 hour gently tipping on platform at room temperature. The membrane is incubated first with the primary antibody and then with the secondary antibody. The primary antibody is added to the blocking buffer at a concentration of: 1:250 for HIF-1α – transduction labs monoclonal clone 54, 1:1000 for HIF-2α (Novus Biologicals catalogue number NB-100-132) and then incubated with the membrane for 1 hour. One ml per blot was used at room temperature, in small closed boxes, to prevent drying. The membrane was then washed 4 times for 5 minutes in PBS/0.01%Tween, shaking vigurously. The secondary goat anti-mouse antibody was added at a concentration of 1:5000 (Dako Cytomation P0447) in blocking solution and left for 1 hour. The membrane was washed again 4 times for 5 minutes in PBS/0.01%Tween. For visualisation of the protein bands the developing stage is performed. The ECL kit (Amersham Biosciences RPN2106), 'Protex' metal box in which to expose film, transparency (to place between film and membrane), film (Kodak MXB Film 100 NIF, 18x24cm, autoradiography film blue sensitive), pippetor with tips, scissors, Falcon tube, are brought to dark room. The chemiluminescent reagent is prepared: 1 ml of ECL solution 1 and 1 ml of solution 2 are mixed after removing them from 4°C and allowing them to warm at room temperature. ECL solution is pipetted over membrane and is allowed to spread by capillary

action over the membrane. The solution is left for 1 minute on a flat surface without moving it. Excess ECL is dabbed off using blue towel or the membrane was picked up with forceps and the excess of chemiluminescent reagent was allowed to run off. The membrane was placed protein side down onto cling film (to ensure a flat air bubble free contact) and wrapped up. Then this is exposed to X-Rays in a 'Protex' box for several different exposure times, 5 minutes, 10 minutes, or 30 minutes depending on speed of development and the films are then developed.

F) PROTOCOL FOR BLOOD GASES ANALYSIS STUDY

Prospectively, blood samples were obtained from research participants with CVMs from within the vascular malformation and from systemic venous and arterial blood during planned surgery for their vascular lesion.

Four blood samples were obtained from each participant, from arterial and venous vessels distal to the malformation; venous vessels from the contralateral limb to the malformation and the malformation itself, each sample consisting of approximately 2 mls blood. All patients had their samples taken during the induction of anaesthesia for the operative management of their CVM or during the procedure itself. This way patient discomfort was reduced to minimum. The patient laid semi recumbent on a bed with the chosen, non-dominant arm supported on a pillow. The required equipment was assembled in a clean tray. Under aseptic conditions the artery required was identified in the desired area which is cleaned appropriately. To withdraw blood from the malformation, artery or venous vessels a needle was inserted, bevel facing upwards, towards the vessel at an angle of 20-30˚ to the horizontal of the blood vessel. A syringe was used and gentle aspiration applied. Once the required amount of blood is obtained (1-2 mls) the needle was withdrawn from the patient and safely disposed of in a sharps' bin. Cotton wool balls or gauze squares were applied to the puncture site for a minimum of 5 minutes before inspecting the area for swelling or bleeding. If they occured, pressure was re-applied for a further 5 minutes. Similar procedures were used to obtain an equivalent blood sample from the abnormal blood vessels comprising the vascular malformation affecting the same participant. The CVM samples in this study were obtained by the consultant surgeon doing the operation. Venous samples were obtained in a similar manner, mostly from patient's arms, unless the CVM lesion prevented the sampling. In this case the venous sample was taken from lower limbs. Venous samples had to be taken away from the CVM lesion.

All samples were taken at the same time. For analysis blood samples obtained as described were transferred into an arterial blood gas syringe, clearly labelled and coded and immediately analysed on a blood gas machine for the partial pO_2 and pH using standard methods with the Rapid point 405 analyser system available on site. This is operated using a touch sensitive visual display unit.

Principle of analysis

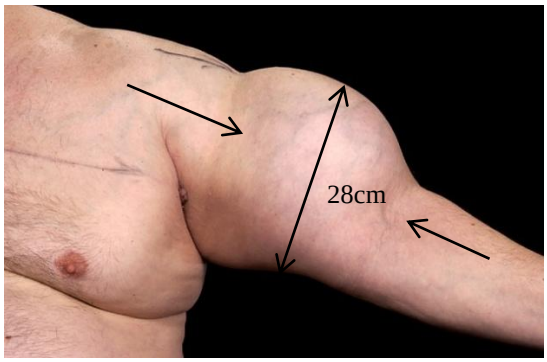
The pO2 sensor is a complete electrochemical cell that incorporates amperometric technology. The sensor consists of a platinum cathode and a silver anode, an electrolyte solution and a gas permeable membrane. A constant (polarizing) voltage is maintained between the anode and the cathode. As dissolved oxygen passes through the membrane into the electrolyte solution it is reduced at the cathode. The circuit is completed at the anode when the silver is oxidized. The amount of reduced oxygen is directly proportional to the number of electrons gained at the cathode. Reference range for pO_2: 10.7-14.0 kPa (arterial blood), 5.3-6.7 kPa (venous blood).

This study involved blood sampling from subjects affected by CVMs and undergoing surgery for excision or debulking of vascular malformations. The subjects were identified in vascular clinic at Hammersmith Hospital or as inpatients at Charing Cross Hospital or Hammersmith Hospital, prior to their operation. All patients with respiratory diseases, acute or chronic, patients with musculoskeletal diseases, malignancy, inflammatory diseases, conditions causing depression of brain's respiratory center or other conditions where angiogenesis or hypoxia may be altered, were excluded (see 2.1), so that the results of this study are not skewed.

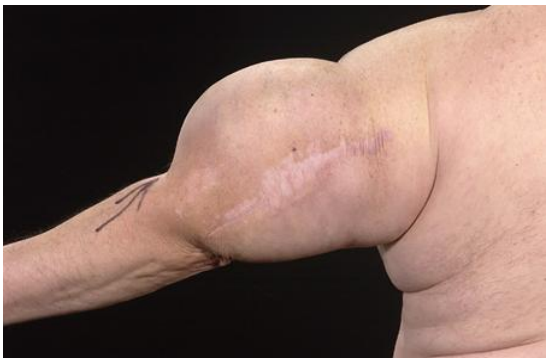
All patients received in advance an invitation letter to participate in this study and an information sheet about the study. Written informed consent was obtained from each participant after explanation of the procedure is given and verbal consent obtained before the patient is put to sleep for his/her planned operation.

APPENDIX – CLINICAL PICTURES

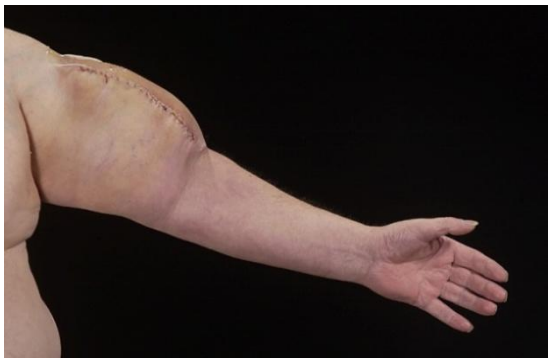
This addition shows pictures of patients with CVM, from whom tissue was prelevated for this study under the conditions described in Chapter 2.



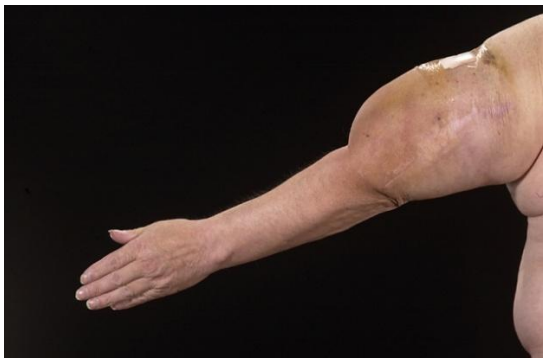
a)



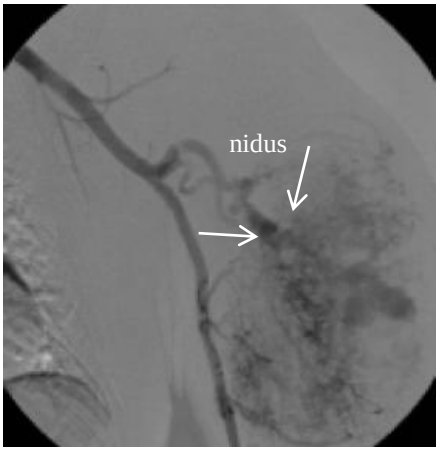
b)



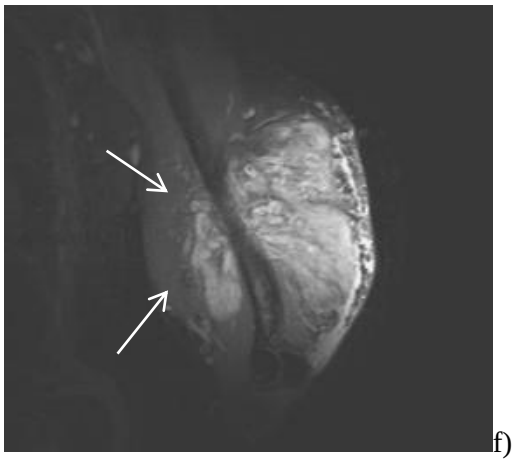
c)



d)



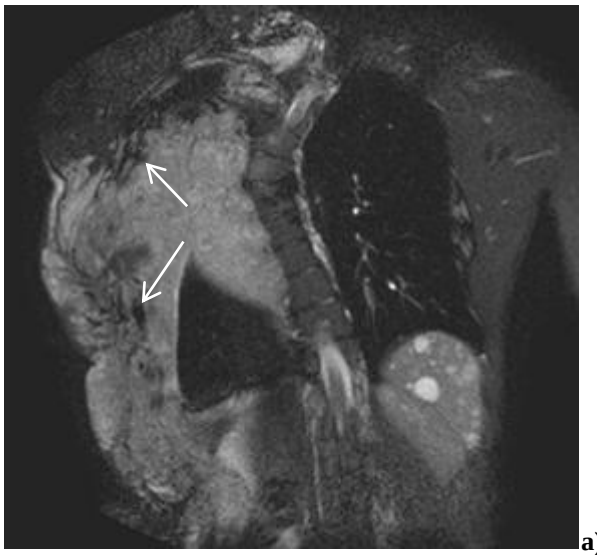
e)



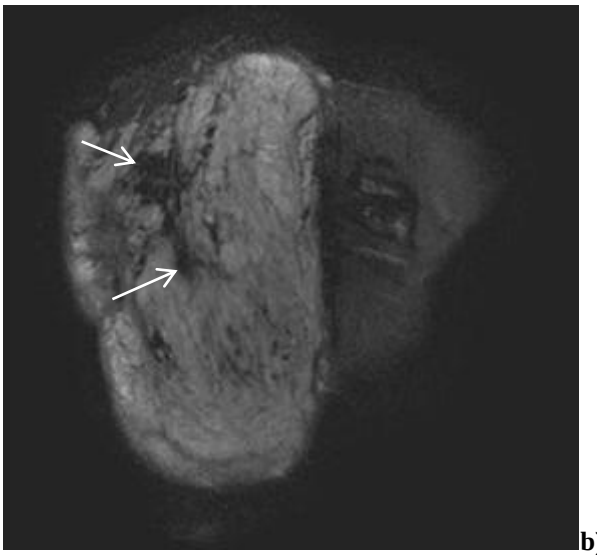
f)

Figure appendix. 7. CVM image 1

1a)-d) 64-year-old male with a large borderline HF AVM involving the left upper arm, at different stages post-surgery; e) Selective angiography from left profunda brachii showing marked vascular staining within the mass and some early venous return. The performing radiologist comment was that the rate of the a-v shunting was not especially rapid; c) Un-enhanced coronal STIR (Phillips 1.5T) images demonstrates the extent of the lesion, which involves all the muscles of the upper arm, with some sparing of the most medial aspect of these muscles (arrows).



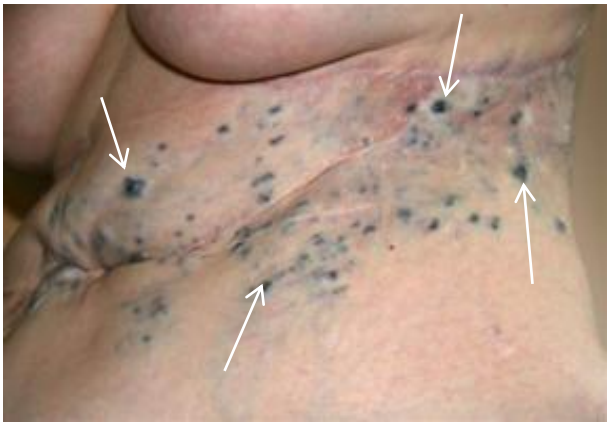
a)



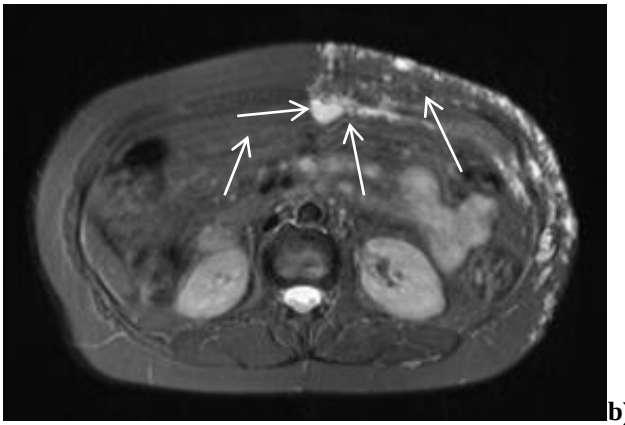
b)

Figure appendix. 8. CVM image 2

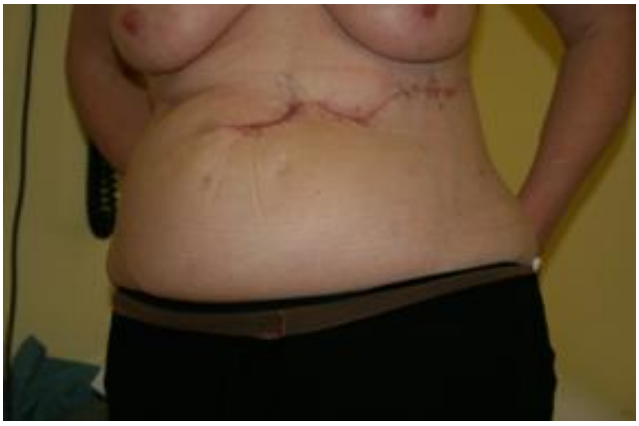
MRI chest (Philips 1.5T) of a 22-year-old male showing the extent of a complex AVM, which involves the right thoracic wall and right loin, causing compressive effect. The fat suppressed coronal breath-hold (FFE/M2D) images (a) and (b) demonstrate multiple tortuous vessels (see flow voids-arrows) within in chest wall, extending sub-diaphragmatically and their association with kypho-scoliosis.



a)



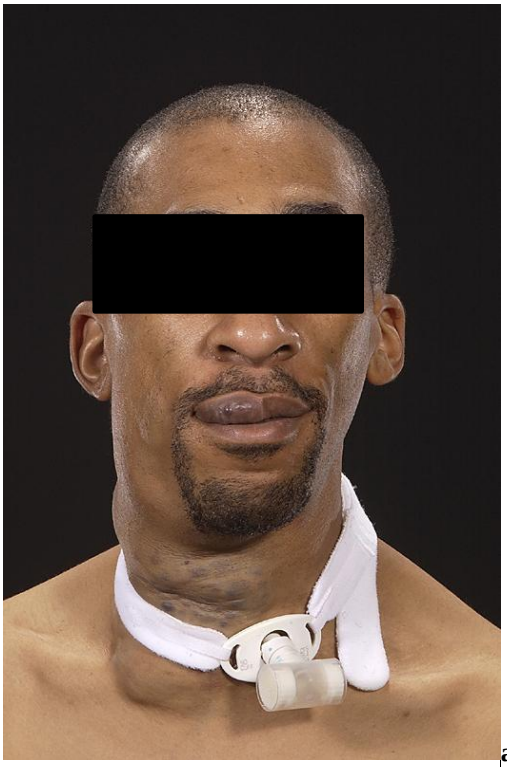
b)



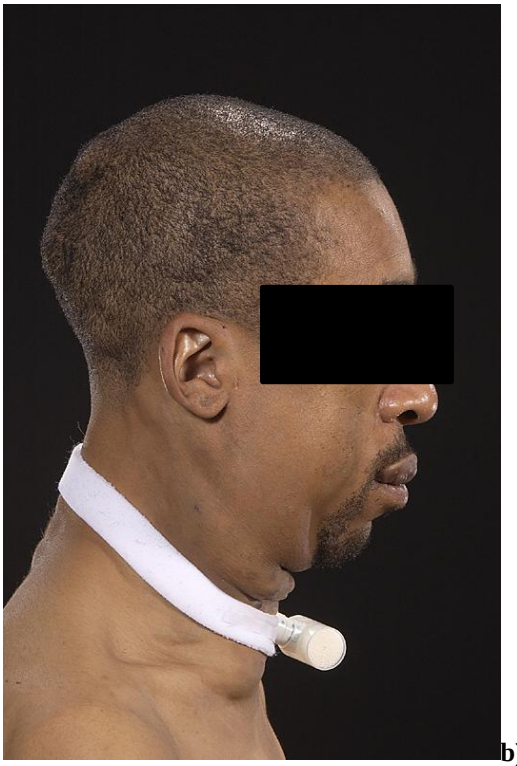
c)

Figure appendix. 9. CVM image 3

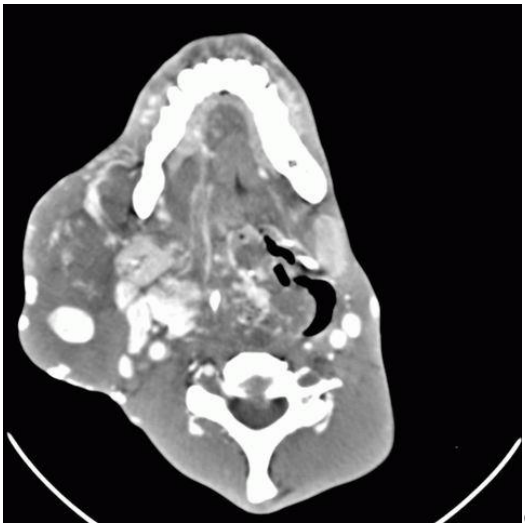
a) 58-year-old female with a thoraco-abdominal LF vascular malformation of the left chest/abdominal wall with evidence of previous surgery and remnant vascular lesion with changes within the skin and subcutaneous fat (arrows); b) Axial STIR through the abdomen (Philips 1.5T) demonstrating the extension of the vascular malformation to the abdominal wall with nodular protrusions into the abdominal cavity (arrow); c) Clinical picture of the same patient demonstrating the final operative result after multiple debulking interventions.



a)



b)



c)

Figure appendix. 10. CVM image 4

a), b) - 35 year old male suffering end-stage complications of a LF VM, with airway and upper gastro-intestinal obstruction symptoms. Tracheostomy was inserted. Salvage procedure was performed to relieve compression; c) the right cranio-facial LF VM is extending behind the nasopharynx and is causing marked deviation of the oropharynx and larynx. Enhanced axial CT image at the level of C2, shows the VM crossing the midline and deviating the oropharynx to the left, with multiple dilated small vessels extending throughout the whole of the pharynx.

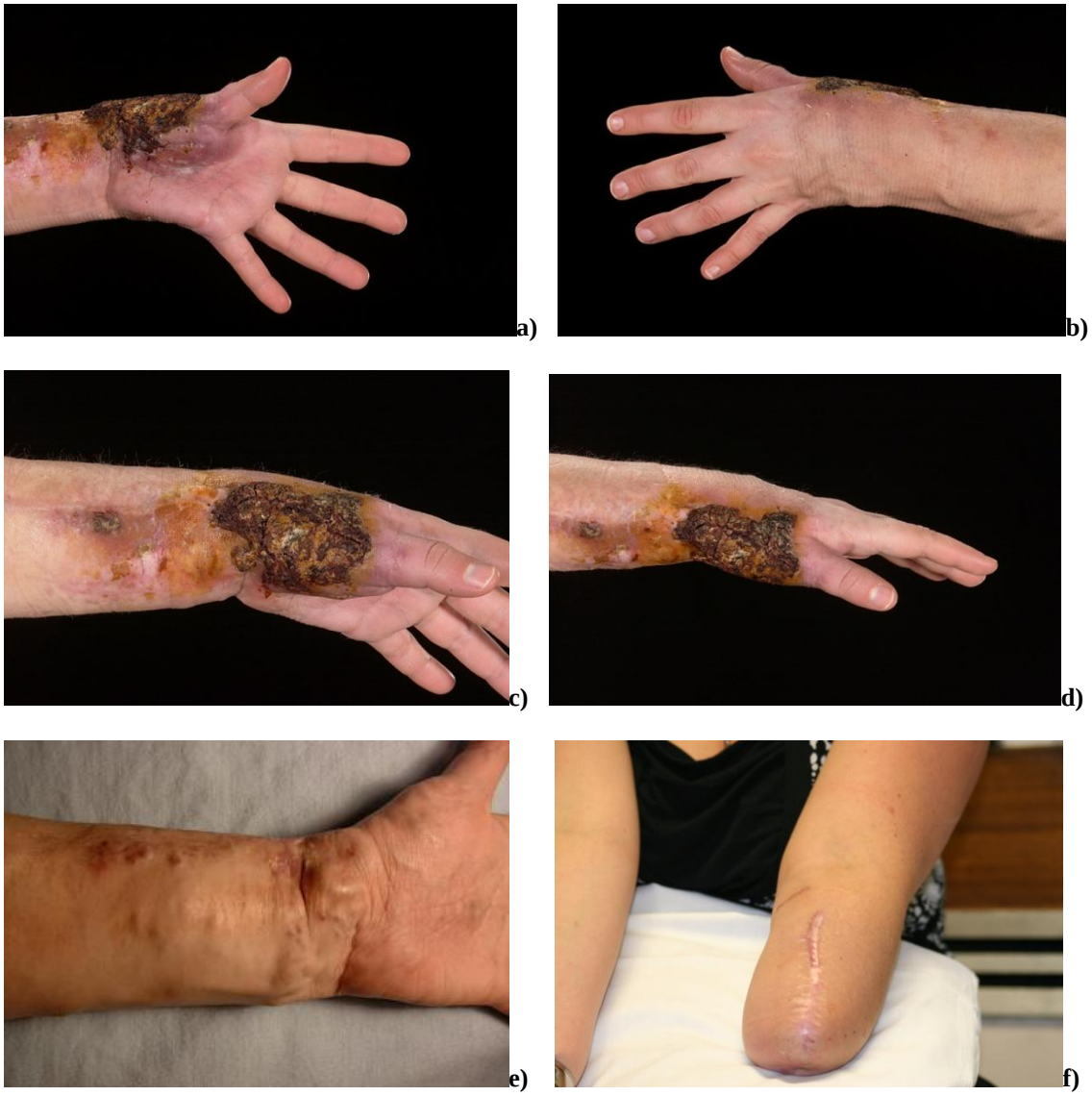
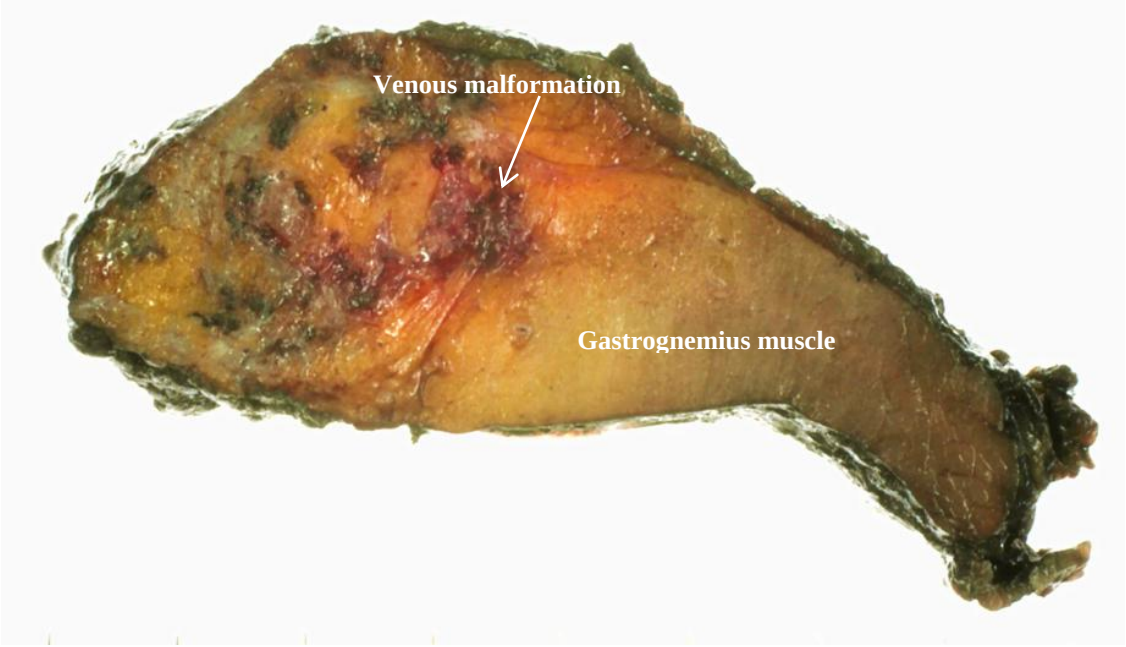
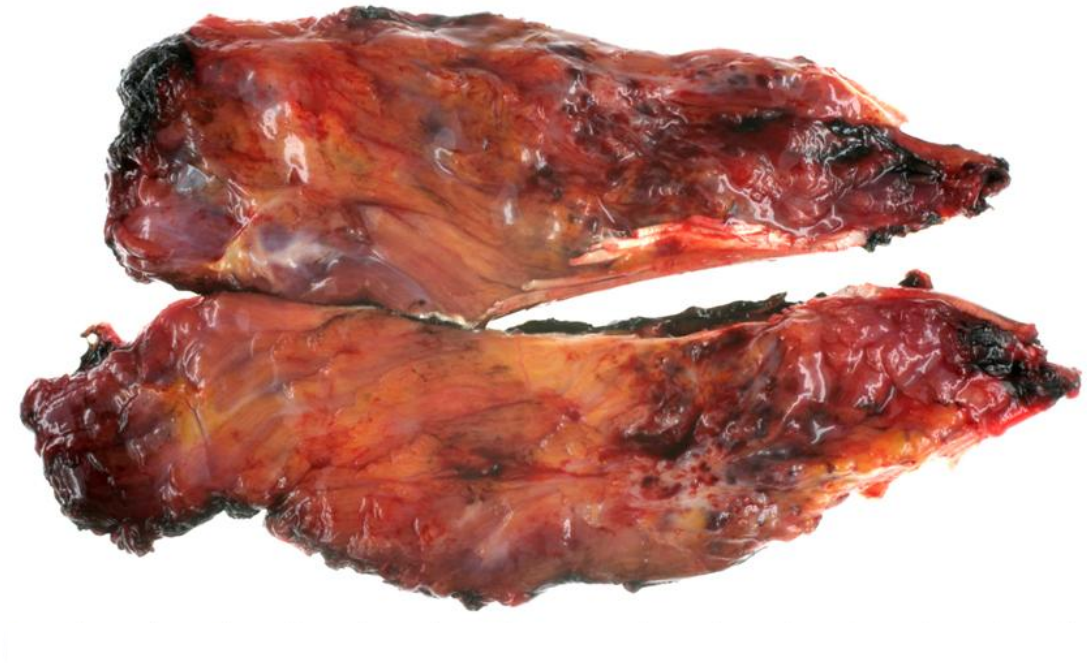


Figure appendix. 11. CVM image 5

a)-f) - 29 year old female with a HF AVM over left forearm. She developed a bleeding ulcer following a session of embolization and required serial debulking and ultimately an amputation of her forearm due to bleeding complications, infection and severe pain.



a)



b)

Figure appendix. 12. CVM image 6

a) Macroscopic image of a resection specimen of a LF VM infiltrating gastrognemius muscle; b) LF VM specimen removed from forearm

APPENDIX-FINAL COMMENTS

COMMENTS ON STATISTICS USED IN THIS RESEARCH WORK AND DIFFICULTIES WITH STUDY DESIGN

When this study was commenced, it was well known that we are many years away from a cure for CVMs as there was very little information in the literature about pathogenesis of CVMs. The current treatment of CVMs is not satisfactory as very few lesions achieve a cure, usually by surgical means. Therefore, we had and still have to invest time and work to understand these difficult and complex vascular malformations and to identify medical means such as drugs or a combination of drugs and surgery in order to induce regression of these lesions.

Although it is well known that there is no ideal study, the author of this study would like emphasize few issues and flaws in relation to the statistics used in this research work, as well as some of the difficulties encountered when this study was designed and performed.

In order to assess sample size required for this study it was found that there were no data available for estimation of power calculations in assessing the expression of angiogenesis and hypoxia markers in CVMs or control tissue in humans using immunohistochemistry. The calculation of the sample size was based solely on values found in literature in healthy controls for serum VEGF. It was hypothesized that serum levels of VEGF are directly proportional to their tissue expression and it was found that to achieve 90% power at 1% significance level, considering multiple testing, the study would require 60 subjects, 30 in CVM group and 30 in the control group. More than 30 subjects were considered for most variables tested in this study but in some instances the author was limited by the fact that some tests could only be performed on frozen tissue; therefore a smaller number of subjects were tested for those variables. The reason for this is that frozen tissue was only available prospectively and when sufficient tissue was redundant from surgery. In these cases, archived tissue which was formalin fixed and used to tests other variables could not be used.

All analyses in this study were performed on 10 control subjects instead of 30 as was initially planned. Given the uniformity of results on the 10 samples tested the author concluded it was unnecessary to extrapolate a significant amount of work to another 20 extra subjects.

One of the most important flaws in this study is the use of multiple analyses on the same small dataset, which could potentially lead to the increase in type I error. A Bonferroni correction was applied to the histological morphology and immunohistochemical dataset analyses, such that $p<0.005$ indicates significance. Although this method is acceptable for a small number of simultaneous tests, it may be over-conservative, and perhaps other tests such as the least significant difference test, Scheffe's test or Newman-Keuls tests should have been considered to enable the reader to better understand significant findings in this study.

Following the results obtained, especially in the morphological part of the study, the author should have considered analysing whether data obtained was parametric or not, such as parametric tests should have been used in any appropriate circumstances as they are more powerful, rather than just simply assuming data were non-parametric.

In the attempt to establish links and pathways between the stromal infiltrate in CVMs, angiogenesis and hypoxia markers, multiple correlations were performed in this study using Spearman rho correlation coefficient. One could also argue why this method was used to establish correlations between variables since other methods are also available. Various approaches are possible for each type of data and here data could have been summarised by means of a regression line. The choice is partly a matter of personal preference, hoping to present data in the simplest and clearest way possible.

Although multiple correlations were performed on a small dataset, and a correlation coefficient was calculated, this was the way the author decided to emphasize results, being aware of the multiple testing and possibility that the resulted statistically significant results may not be as significant as quoted in figures. On the other hand, the validity of the results can be observed in the histological and immunohistochemical images presented in this study and

perhaps one would say that so extensive statistical analyses were not necessary for the type of study proposed.

The results of these statistical calculations do not determine on their own further actions but they are interpreted in a clinical context. The author has not drawn any firm conclusions on the basis of the statistically significant results presented in this study but they only limited to their sensible interpretation.

In the immunohistochemical part of this study results were read by two independent observers and were compared for their agreement using kappa coefficient test. The Bland-Altman plot is another method used to compare two measurement techniques and quantify inter-observer agreement. This is a simple approach because it focuses exclusively on the differences. In this study a statistician was consulted for this purpose, who suggested that kappa coefficient method was more appropriate for dataset obtained. Even so, the kappa coefficient obtained (0.32), although satisfactory, this is not great and perhaps the author should have considered re-analysing data again at a later stage, given the subjectivism and human error factor involved in the scoring method used for interpreting immunohistochemical results. Image analysis could have decreased the human error in this study. This was not used due to difficulties resulted from staining tissues in different batches. The immunohistochemical stains for a given variable had different background of staining and made impossible a uniform reading of staining results. . In retrospect, the author would have waited to obtain all specimens required for study before performing any staining such as all staining are performed at once.

In the chapters 2 and 3, the majority of results are presented in tables. However, graphs are often better than tables when there is a need to provide a summary message, although tables usually give more precise information. Some of graphs and tables in this work should have been better labelled, additional legends would have been useful in some cases and error bars should have been depicted in graphs.

Another significant flow in this study is concerning the tissue used for analyses in this study. This is a mix-up of archived and prospectively collected tissue. Although the archived

tissue was collected and preserved using the Departmental standard techniques, this was carefully checked against the histopathological reports to ensure vascular malformations were considered and also checked with patient's medical records, still the author had no control on this part of study. In addition, CVM analysed were not classified by any of the currently used classifications. The author ensured these lesions were not haemangiomas, therefore all were GLUT-1 negative and the fact that they were unclassified vascular malformations was part of the study design, given the persistent confusion in the literature in respect to CVM classification. All CVMs could retrospectively be matched according to the current accepted classifications.

Controls were carefully chosen in this study such as they represent a close match to the tissue analysed. Skin vessels were deemed best controls as they were represented by blood vessels embedded in stromal tissue and located peripheral, similar to CVMs. In the pilot part of this study, individual veins, redundant following varicose veins surgery or remnant arteries and veins following plastic surgery interventions were considered but they lacked the surrounding stromal tissue and were abandoned. Skin controls used in this study originated all in female patients and for a better comparison male subjects should have been considered. Also, the skin used had different origin (abdomen, chest, limbs) and this in itself could represent a source of variability, although this was not observed in the samples analysed.

CVMs are present at birth and therefore it would have been interesting to analyse foetal tissue for the histochemical and immunohistochemical expression of analysed markers. Although ethical approval was obtained for analysis of foetal tissue in this respect, this would have been in itself a whole study and beyond the initial proposal for this study.

The method used to measure blood vessel diameter in this study is not a standardised method but the author preference as described in the Methods chapter. There are other alternatives to measure blood vessel diameters and author accepts that as well as the fact that measuring diameters bi-dimensionally on stained slides, this is somehow 'artefact' as it represents a snap shot of a section through the CVM tissue and not true diameters in totality.

Specific chemical mediators were chosen to be analysed using immunohistochemical methods in this study and the decision in this respect lies with the author. Although it was desirable to know the expression of even more markers of angiogenesis and hypoxia in CVM, one has to confine his work as it is impossible to analyse everything. With the increased understanding of angiogenesis pathways, the markers were chosen from those involved in the few likely pathways possible to be dis-regulated in CVMs, knowing that CVMs result from faulty angiogenesis.

The results from the investigation of hypoxia in CVMs in this study would have been more relevant should the author have found means of measuring tissue hypoxia in CVMs. This route was explored and discussion with scientists with experience in investigating tissue hypoxia took place. The outcome was that even trying to measure tissue hypoxia using microelectrodes placed in CVM tissue, this will add a factor of error caused by the tissue necrosis induced by the placement of microelectrodes into tissue and therefore leading to mis-reading of tissue hypoxia.

Although this study has limited clinical application at present, the results obtained add to the literature and scientific knowledge and represent a thorough basic analysis and an excellent background for pursuing this research work further, especially in the current context when technology moves at high speed. With the advances in gene analysis technology, gene arrays availability, rapid expansion of genomics and proteomics technologies, significant amount of information can be obtained from a very limited amount of tissue, which may be obtained from biopsies or, great advantage may be taken from the CVM tissue bank available in Histopathology Department at Charing Cross Hospital. Worldwide there is a lack of CVM tissue and this is the reason that data described here is missing in the literature and practically there is very little scientific data on vascular malformations.

The author takes responsibility for the fact that this study is far from perfect but this basic study is a novelty and should be pursued, especially HIF work which appears promising in relation to its clinical applications.

BIBLIOGRPHY

1. Practice Guidelines for Blood Component Therapy: A Report by the American Society of Anesthesiologists Task Force on Blood Component Therapy. Anesthesiology 84, 732 (1996).
2. Abdool-Carrim, A. T. Management of congenital arteriovenous malformations. Journal of the Royal College of Surgeons of Edinburgh 35, 39 (1990).
3. Achauer, B. M. Ulcerated anogenital hemangioma of infancy. Plastic and reconstructive surgery 87, 861 (1991).
4. Adams, R. H. Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes & development 13, 295 (1999).
5. Adegboyega, P. A. Hemangioma versus vascular malformation: presence of nerve bundle is a diagnostic clue for vascular malformation. Archives of pathology & laboratory medicine 129, 772 (2005).
6. Albrecht, E. Ueber hamartome. Verhoff's Deutsche pathologische Gesellschaft, 153-7 (1904).
7. Amir, J. Strawberry hemangioma in preterm infants. Pediatric dermatology 3, 331 (1986).
8. Angelisova, P. Association of CD10/neutral endopeptidase 24.11 with membrane microdomains rich in glycosylphosphatidylinositol-anchored proteins and Lyn kinase. Blood 93, 1437 (1999).
9. Arneja, J. S. Vascular malformations. Plastic and reconstructive surgery 121, 195e (2008).
10. Asahara, T. Isolation of putative progenitor endothelial cells for angiogenesis. Science 275, 964 (1997).
11. Askn, S. et al. Expression of integrins in cerebral arteriovenous and cavernous malformations. Neurosurgery 58, 159-168 (2006).
12. Astner, S. Treating vascular lesions. Dermatologic therapy 18, 267 (2005).
13. Attina, T. Endothelin antagonism in pulmonary hypertension, heart failure, and beyond. Heart 91, 825 (2005).
14. Azizkhan, R. G. Mast cell heparin stimulates migration of capillary endothelial cells in vitro. The Journal of experimental medicine 152, 931 (1980).
15. Bader, B. I. Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. Cell 95, 507 (1998).

16. Bagnato, A. Expression of endothelin 1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. Cancer research 59, 720 (1999).
17. Bagnato, A. Emerging role of endothelin-1 in tumor angiogenesis. Trends in endocrinology and metabolism 14, 44 (2003).
18. Bagnato, A. Targeting endothelin axis in cancer. Cancer treatment and research 119, 293 (2004).
19. Bakker, E. N. Blood flow-dependent arterial remodelling is facilitated by inflammation but directed by vascular tone. Cardiovascular research 78, 341 (2008).
20. Balazs, M. Fine structure of multiple neonatal haemangioendothelioma of the liver. Virchows Archiv. A, Pathological anatomy and histology 379, 157 (1978).
21. Ban, H. S. Hypoxia-inducible factor inhibitors: a survey of recent patented compounds (2004 - 2010). Expert opinion on therapeutic patents 21, 131.
22. Barleon, B. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood 87, 3336 (1996).
23. Baroni, C. On the relationship of mast cells to various soft tissues tumours. The British journal of cancer 18, 686 (1964).
24. Barrio, V. R. Treatment of hemangiomas of infancy. Dermatologic therapy 18, 151 (2005).
25. Bastide, G. The organogenesis and anatomy of vascular malformation. International angiology 9, 137 (1990).
26. Bastide, G. Bases anatomiques des malformations vasculaires. Phlébologie 43, 553 (1990).
27. Bastide, G. & Lefebvre, D. Anatomy and organogenesis and vascular malformations (eds. Belov, S., Loose, D. A. & Weber, J.) (Einhor-Presse Verlag, Reinbek, Germany, 1989).
28. Battistini, B. Endothelins: circulating plasma levels and presence in other biologic fluids. Laboratory Investigation 68, 600 (1993).
29. Bazzoni, G., Dejana, E. & Lampugnani, M. G. Adhesion molecules in Endothelial Cells and their role in vascular morphogenesis (eds. Risau, W. & Rubanyi, G. M.) (Harwood Academic Publishers, California, 2000).
30. Beasley, N. J. Carbonic anhydrase IX, an endogenous hypoxia marker, expression in head and neck squamous cell carcinoma and its relationship to hypoxia, necrosis, and microvessel density. Cancer research 61, 5262 (2001).
31. Beasley, N. J. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected

patients. Cancer research 62, 2493 (2002).

32. Belov, S. Classification, terminology, and nosology of congenital vascular defects (eds. Belov, S., Loose, D. & Weber, J.) (Einhorn-Presse, Reinbek, Germany, 1989).
33. Belov, S. Classification of congenital vascular defects. International angiology 9, 141 (1990).
34. Belov, S. Anatomopathological classification of congenital vascular defects. Seminars in vascular surgery 6, 219 (1993).
35. Belov, S. & Loose, D. A. Surgical treatment of congenital vascular defects. International angiology 9, 175 (1990).
36. Ben-Av, P. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. FEBS letters 372, 83 (1995).
37. Berenguer, B. Rapidly involuting congenital hemangioma: clinical and histopathologic features. Pediatric and developmental pathology 6, 495 (2003).
38. Berg, J. N. Evidence for loss of heterozygosity of 5q in sporadic haemangiomas: are somatic mutations involved in haemangioma formation? Journal of clinical pathology 54, 249 (2001).
39. Berlien, H. P. Lasers in pediatric surgery. Progress in pediatric surgery 25, 5 (1990).
40. Berman, A. E. Integrins: structure and signaling. Biochemistry 68, 1284 (2003).
41. Bicknell, R. Introduction to the endothelial cell (ed. Bicknell, R.) (Cambridge University Press, Oxford, 1996).
42. Bicknell, R. Novel angiogenic signaling pathways and vascular targets. Annual Review of Pharmacology and Toxicology 44, 219 (2004).
43. Blair, R. G., Meng, H. & Marchese, M. J. Human mast cells stimulate vascular tube formation: tryptase is a novel, potent angiogenic factor. J Clin Invest, 99:2691 (1997).
44. Blei, F. Familial segregation of hemangiomas and vascular malformations as an autosomal dominant trait. Archives of dermatology 134, 718 (1998).
45. Blei, F. Basic science and clinical aspects of vascular anomalies. Current opinion in pediatrics 17, 501 (2005).
46. Boesiger, J. Mast cells can secrete vascular permeability factor/ vascular endothelial cell growth factor and exhibit enhanced release after immunoglobulin E-dependent upregulation of fc epsilon receptor I expression. The Journal of experimental medicine 188, 1135 (1998).
47. Boon, L. M. Assignment of a locus for dominantly inherited venous malformations to chromosome 9p. Human Molecular Genetics 3, 1583 (1994).

48. Boon, L. M. Congenital hemangioma: evidence of accelerated involution. The journal of pediatrics 128, 329 (1996).
49. Boudreau, N. Induction of the angiogenic phenotype by Hox D3. The journal of cell biology 139, 257 (1997).
50. Boye, E. Clonality and altered behavior of endothelial cells from hemangiomas. The journal of clinical investigation 107, 745 (2001).
51. Brakebusch, C. Integrins in invasive growth. The Journal of clinical investigation 109, 999 (2002).
52. Bree, A. F. Infantile hemangiomas: speculation on placental trophoblastic origin. Archives of dermatology 137, 573 (2001).
53. Breier, G. Angiogenesis in embryonic development--a review. Placenta 21, S11 (2000).
54. Bremnes, T. Regulation and intracellular trafficking pathways of the endothelin receptors. Journal of biological chemistry 275, 17596 (2000).
55. Breugem, C. C. Use of magnetic resonance imaging for the evaluation of vascular malformations of the lower extremity. Plastic and reconstructive surgery 108, 870 (2001).
56. Brihaye, M. Angiomatous tumours of the orbit. Modern problems in ophthalmology 14, 368 (1975).
57. Broadley, K. N. Monospecific antibodies implicate basic fibroblast growth factor in normal wound repair. Laboratory Investigation 61, 571 (1989).
58. Brogi, E. Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. Circulation 90, 649 (1994).
59. Brooks, P. C. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79, 1157 (1994).
60. Brooks, P. C. Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science 264, 569 (1994).
61. Brooks, P. C. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell 85, 683 (1996).
62. Brouillard, P., Boon, L. M. & Mulliken, J. B. Mutations in a novel factor, glomulin, are responsible for glomuvenous malformations 9'glomangiomas'). Am J Hum Genet, 70:866 (2002).
63. Brown, L. F. Vascular permeability factor mRNA and protein expression in human kidney. Kidney International 42, 1457 (1992).
64. Brown, R. L. Pediatric head and neck lesions. The Pediatric clinics of North America

45, 889 (1998).

65. Burke, B. Expression of HIF-1alpha by human macrophages: implications for the use of macrophages in hypoxia-regulated cancer gene therapy. The Journal of pathology 196, 204 (2002).
66. Burkhardt, M. Receptor- and non-receptor-mediated clearance of big-endothelin and endothelin-1: differential effects of acute and chronic ETA receptor blockade. Journal of hypertension 18, 273 (2000).
67. Burrows, P. E. Diagnostic imaging in the evaluation of vascular birthmarks. Dermatologic clinics 16, 455 (1998).
68. Burrows, P. E. Endovascular treatment of vascular anomalies (ed. Rutherford, R. B.) (WB Saunders, Philadelphia, 2006).
69. Burton, B. K. An increased incidence of haemangiomas in infants born following chorionic villus sampling (CVS). Prenatal diagnosis 15, 209 (1995).
70. Byzova, T. V. A mechanism for modulation of cellular responses to VEGF: activation of the integrins. Molecular cell 6, 851 (2000).
71. Caldwell, J. B. Cutaneous angiosarcoma arising in the radiation site of a congenital hemangioma. Journal of the American Academy of Dermatology 33, 865 (1995).
72. Carlson, T. R. Direct cell adhesion to the angiopoietins mediated by integrins. The Journal of biological chemistry 276, 26516 (2001).
73. Carmeliet, P. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380, 435 (1996).
74. Carmeliet, P. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394, 485 (1998).
75. Carr, M. M. Extremity arteriovenous malformations: review of a series. Canadian journal of surgery 37, 293 (1994).
76. Carreau, A. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. Journal of Cellular and Molecular Medicine 15, 1239.
77. Carroll, V. A. Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway. Cancer research 66, 6264 (2006).
78. Chang, J. Proliferative hemangiomas: analysis of cytokine gene expression and angiogenesis. Plastic and reconstructive surgery 103, 1 (1999).
79. Chang, M. W. Updated classification of hemangiomas and other vascular anomalies. Lymphatic research and biology 1, 259 (2003).

80. Chen, F. Apoptosis and angiogenesis are induced in the unstable coronary atherosclerotic plaque. Coronary artery disease 16, 191 (2005).
81. Chen, M. T. Treatment of high-flow vascular malformations in the head and neck with arterial ligation followed by sclerotherapy. Annals of plastic surgery 36, 147 (1996).
82. Cheung, D. S. Hemangioma in twins. Annals of plastic surgery 38, 269 (1997).
83. Chiller, K. G. Hemangiomas of infancy: clinical characteristics, morphologic subtypes, and their relationship to race, ethnicity, and sex. Archives of dermatology 138, 1567 (2002).
84. Chiller, K. G. Molecular pathogenesis of vascular anomalies: classification into three categories based upon clinical and biochemical characteristics. Lymphatic research and biology 1, 267 (2003).
85. Chiller, K. G., Arbiser, J. & Frieden, I. J. Vasculogenesis and angiogenesis in the development of cutaneous vascular birthmarks. (2001).
86. Church, M. K. The human mast cell. The journal of allergy and clinical immunology 99, 155 (1997).
87. Cines, D. B. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 91, 3527 (1998).
88. Claffey, K. P. Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways. The Journal of biological chemistry 267, 16317 (1992).
89. Claffey, K. P. Regulation of VEGF/VPF expression in tumor cells: consequences for tumor growth and metastasis. Cancer and Metastasis Reviews 15, 165 (1996).
90. Clauss, M. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. The Journal of experimental medicine 172, 1535 (1990).
91. Cohen, T. Interleukin 6 induces the expression of vascular endothelial growth factor. The Journal of biological chemistry 271, 736 (1996).
92. Coleman, C. C., Jr. Diagnosis and treatment of congenital arteriovenous fistulas of the head and neck. The American journal of surgery 126, 557 (1973).
93. Comati, A. Upregulation of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha in leptomeningeal vascular malformations of Sturge-Weber syndrome. Journal of neuropathology and experimental neurology 66, 86 (2007).
94. Conforti, G. Human endothelial cells express integrin receptors on the luminal aspect of their membrane. Blood 80, 437 (1992).
95. Connors, J. P. & Mulliken, J. B. Vascular Tumors and malformations in childhood (ed.

Rutherford, R. B.) (Saunders, USA, 2005).

96. Coursley, G. Congenital arteriovenous fistulas in the extremities. Angiology 7, 201 (1956).
97. Cremer, H. Cryosurgery for hemangiomas. Pediatric dermatology 15, 410 (1998).
98. Crowther, M. Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. Journal of leukocyte biology 70, 478 (2001).
99. Cutrona, G. Expression of CD10 by human T cells that undergo apoptosis both in vitro and in vivo. Blood 94, 3067 (1999).
100. Dadras, S. S. Infantile hemangiomas are arrested in an early developmental vascular differentiation state. Modern pathology 17, 1068 (2004).
101. Dalion, J. Mastocytes et tumeurs vasculaires. Journal des sciences médicales de Lille 83, 683 (1965).
102. Danen, E. H. Integrins in regulation of tissue development and function. The Journal of pathology 200, 471 (2003).
103. Defilippi, P. Alpha 6.beta 1 integrin (laminin receptor) is down-regulated by tumor necrosis factor alpha and interleukin-1 beta in human endothelial cells. The Journal of biological chemistry 267, 18303 (1992).
104. Dejana, E. Fibrinogen induces endothelial cell adhesion and spreading via the release of endogenous matrix proteins and the recruitment of more than one integrin receptor. Blood 75, 1509 (1990).
105. Dejana, E. Endothelial integrins and their role in maintaining the integrity of the vessel wall. Kidney International 43, 61 (1993).
106. Dejana, E. Endothelial adherens junctions: implications in the control of vascular permeability and angiogenesis. The journal of clinical investigation 100, S7 (1997).
107. Denekamp, J. Vascular endothelium as the vulnerable element in tumours. Acta radiologica. Oncology 23, 217 (1984).
108. Dethlefsen, S. M. An ultrastructural study of mast cell interactions in hemangiomas. Ultrastructural pathology 10, 175 (1986).
109. Detmar, M. Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. The Journal of Investigative Dermatology 105, 44 (1995).
110. Detmar, M. Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. The Journal of Investigative Dermatology 111, 1 (1998).
111. Detoraki, A. Vascular endothelial growth factors synthesized by human lung mast cells

exert angiogenic effects. The journal of allergy and clinical immunology 123, 1142 (2009).

112. Dickey, K. W. Management of large high-flow arteriovenous malformations of the shoulder and upper extremity with transcatheter embolotherapy. Journal of vascular and interventional radiology 6, 765 (1995).

113. Dj, D. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes & development 8, 1897 (1994).

114. Dondon, M. G. Cancer mortality after radiotherapy for a skin hemangioma during childhood. Radiotherapy and Oncology 72, 87 (2004).

115. Dosanjh, A. In vitro characteristics of neonatal hemangioma endothelial cells: similarities and differences between normal neonatal and fetal endothelial cells. Journal of cutaneous pathology 27, 441 (2000).

116. Drake, C. J. An antagonist of integrin alpha v beta 3 prevents maturation of blood vessels during embryonic neovascularization. Journal of cell science 108, 2655 (1995).

117. Drolet, B. A. Hemangiomas in children. New England Journal of Medicine, The 341, 173 (1999).

118. Dubois, J. Soft-tissue venous malformations in adult patients: imaging and therapeutic issues. Radiographics 21, 1519 (2001).

119. Dumont, D. J. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science 282, 946 (1998).

120. Dupin, N. Absence de detection du virus HHV-8 dans les hemangiomes immatures du nourrisson. Annales de dermatologie et de vénéréologie 125, 98 (1998).

121. Dvorak, H. F. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. Journal of clinical oncology 20, 4368 (2002).

122. Eady, R. A. Mast cell population density, blood vessel density and histamine content in normal human skin. British journal of dermatology 100, 623 (1979).

123. Eccles, S. A. Parallels in invasion and angiogenesis provide pivotal points for therapeutic intervention. The International journal of developmental biology 48, 583 (2004).

124. Edelson, B. T. Mast cell-mediated inflammatory responses require the alpha 2 beta 1 integrin. Blood 103, 2214 (2004).

125. Edgerton, M. T. Steroids and surgery in the management of cutaneous angiomas (Year Book Medical Publishers, Chicago, 1976).

126. Edgerton, M. T. The treatment of hemangiomas: with special reference to the role of steroid therapy. Annals of surgery 183, 517 (1976).
127. Eifert, S. Prevalence of deep venous anomalies in congenital vascular malformations of venous predominance. Journal of vascular surgery 31, 462 (2000).
128. Eimoto, T. Ultrastructure of an infantile hemangiopericytoma. Cancer 40, 2161 (1977).
129. Eivazi, B. Update on hemangiomas and vascular malformations of the head and neck. European archives of oto-rhino-laryngology 266, 187 (2009).
130. Enjolras, O. Classification and management of the various superficial vascular anomalies: hemangiomas and vascular malformations. Journal of Dermatology 24, 701-710 (1997).
131. Enjolras, O. Vascular tumors and vascular malformations: are we at the dawn of a better knowledge? Pediatric dermatology 16, 238 (1999).
132. Enjolras, O. Malformations arterioveineuses: etude de 200 cas. Annales de dermatologie et de vénéréologie 127, 17 (2000).
133. Enjolras, O. Noninvoluting congenital hemangioma: a rare cutaneous vascular anomaly. Plastic and reconstructive surgery 107, 1647 (2001).
134. Enjolras, O., Chapot, R. & Merland, J. J. Vascular anomalies and the growth of limbs: a review. Journal of pediatric orthophaedics 13, 349-57 (2004).
135. Enjolras, O., Deffrennes, D., Borsik, M., Diner, P. & Laurian, C. Vascular 'tumors' and the rules of their surgical management. Annales de chirurgie plastique et esthétique 43, 455-89 (1998).
136. Enzinger, F. M. & Weisse, S. W. Heamangioendotheliona: vascular tumours of intermediate malignancy (ed. F.M., E.) (C.V. Mosby Company, St Louis, 1988).
137. Epstein, A. C. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107, 43 (2001).
138. Erdmann, M. W. Multidisciplinary approach to the management of head and neck arteriovenous malformations. Annals of The Royal College of Surgeons of England 77, 53 (1995).
139. Esterly, N. B. Cutaneous hemangiomas, vascular stains and malformations, and associated syndromes. Current Problems in Pediatrics 26, 3 (1996).
140. Feoktistov, I. Hypoxia modulates adenosine receptors in human endothelial and smooth muscle cells toward an A2B angiogenic phenotype. Hypertension 44, 649 (2004).
141. Ferrara, N. Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocrine reviews 13, 18 (1992).
142. Ferri, C. Circulating endothelin-1 concentrations in patients with chronic hypoxia.

Journal of Clinical Pathology 48, 519 (1995).

143. Finn, M. C. Congenital vascular lesions: clinical application of a new classification. Journal of Pediatric Surgery 18, 894 (1983).
144. Fishman, S. J. Hemangiomas and vascular malformations of infancy and childhood. The Pediatric clinics of North America 40, 1177 (1993).
145. Folkman, J. Tumor angiogenesis: therapeutic implications. New England Journal of Medicine, The 285, 1182 (1971).
146. Folkman, J. The role of heparin in angiogenesis. Ciba Foundation symposium 100, 132 (1983).
147. Folkman, J. Toward a new understanding of vascular proliferative disease in children. Pediatrics 74, 850 (1984).
148. Folkman, J. How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. Cancer research 46, 467 (1986).
149. Folkman, J. Angiogenic factors. Science 235, 442 (1987).
150. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Medicine 1, 27 (1995).
151. Folkman, J. Blood vessel formation: what is its molecular basis?[comment]. Cell 87, 1153 (1996).
152. Folkman, J. Fundamental concepts of the angiogenic process. Current molecular medicine 3, 643 (2003).
153. Folkman, J., Mulliken, J. B. & Ezekowitz, R. A. B. Angiogenesis and haemangiomas (eds. Oldham, K. T., Colombani, R. P. & Foglia, R. P.) (Lippincott-Raven, Philadelphia, 1997).
154. Fong, G. H. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376, 66 (1995).
155. Frangogiannis, N. G. Stem cell factor induction is associated with mast cell accumulation after canine myocardial ischemia and reperfusion. Circulation 98, 687 (1998).
156. Frank, S. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. The Journal of biological chemistry 270, 12607 (1995).
157. Fraser, R. A. Role of mast cells in experimental tumour angiogenesis. Ciba Foundation symposium 100, 120 (1983).
158. Frieden, I. J. Guidelines of care for hemangiomas of infancy. American Academy of Dermatology Guidelines/Outcomes Committee. Journal of the American Academy of

Dermatology 37, 631 (1997).

159. Friedlander, M. Definition of two angiogenic pathways by distinct alpha v integrins. Science 270, 1500 (1995).
160. Frischer, J. S. Biomolecular markers and involution of hemangiomas. Journal of Pediatric Surgery 39, 400 (2004).
161. Gaber, T. Hypoxia inducible factor (HIF) in rheumatology: low O2! See what HIF can do! Annals of the Rheumatic Diseases 64, 971 (2005).
162. Gale, N. W. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes & development 13, 1055 (1999).
163. Gampper, T. J. Vascular anomalies: hemangiomas. Plastic and reconstructive surgery 110, 572 (2002).
164. Ganju, R. K. CD10/neutral endopeptidase 24.11 is phosphorylated by casein kinase II and coassociates with other phosphoproteins including the lyn src-related kinase. Blood 88, 4159 (1996).
165. Garzon, M. C. Vascular tumors and vascular malformations: evidence for an association. Journal of the American Academy of Dermatology 42, 275 (2000).
166. Garzon, M. C. Hemangiomas: when to worry. Pediatric annals 29, 58 (2000).
167. Gerber, H. P. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. The Journal of biological chemistry 272, 23659 (1997).
168. Gerety, S. S. Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. Molecular cell 4, 403 (1999).
169. Glowacki, J. Mast cells in hemangiomas and vascular malformations. Pediatrics 70, 48 (1982).
170. Gonzales-Crussi, F., Hull, M. T., Grosfeld, J. L. & Mirkin, L. D. Congenital hemangioendothelioma; immunologic and ultrastructural studies. Laboratoty investigations, 38-387 (1978).
171. Gordon, J. R. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. Nature 346, 274 (1990).
172. Graf, K. Regulation and differential expression of neutral endopeptidase 24.11 in human endothelial cells. Hypertension 26, 230 (1995).
173. Gruber, B. L. Angiogenic factors stimulate mast-cell migration. Blood 86, 2488 (1995).
174. Gupta, R. A. Cyclooxygenase-1 is overexpressed and promotes angiogenic growth

factor production in ovarian cancer. Cancer research 63, 906 (2003).

175. Hand, J. L. Vascular birthmarks of infancy: resolving nosologic confusion. American journal of medical genetics 108, 257 (2002).

176. Happle, R. Lethal genes surviving by mosaicism: a possible explanation for sporadic birth defects involving the skin. Journal of the American Academy of Dermatology 16, 899 (1987).

177. Hasan, Q. Steroid therapy of a proliferating hemangioma: histochemical and molecular changes. Pediatrics 105, 117 (2000).

178. Hattori, K. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nature Medicine 8, 841 (2002).

179. Hatva, E. Tie endothelial cell-specific receptor tyrosine kinase is upregulated in the vasculature of arteriovenous malformations. Journal of Neuropathology and Experimental Neurology 55, 1124 (1996).

180. Hein, K. D. Venous malformations of skeletal muscle. Plastic and reconstructive surgery 110, 1625 (2002).

181. Hermans, C. Malformations veineuses et coagulopathie. Annales de chirurgie plastique et esthétique 51, 388 (2006).

182. Hidano, A. Earliest features of the strawberry mark in the newborn. British journal of dermatology 87, 138 (1972).

183. Hirschi, K. K. Pericytes in the microvasculature. Cardiovascular research 32, 687 (1996).

184. Hirschi, K. K. Cell-cell interactions in vessel assembly: a model for the fundamentals of vascular remodelling. Transplant immunology 5, 177 (1997).

185. Hj, L. Pathomorphology of vascular malformations. Analysis of 310 cases. International Angiology 9, 147 (1990).

186. Hohenleutner, U. Hemangiomas of infancy and childhood. Journal der Deutschen Dermatologischen Gesellschaft 5, 334 (2007).

187. Holmdahl, K. Cutaneous hemangiomas in premature and mature infants. Acta Paediatr Scan 44, 370-9 (1955).

188. Hood, J. D. Differential alphav integrin-mediated Ras-ERK signaling during two pathways of angiogenesis. The journal of cell biology 162, 933 (2003).

189. Houdart, E. A proposed angiographic classification of intracranial arteriovenous fistulae and malformations. Neuroradiology 35, 381 (1993).

190. Houseman, N. D. The angiosomes of the head and neck: anatomic study and clinical

applications. Plastic and reconstructive surgery 105, 2287 (2000).

191. Howell, K. Chronic hypoxia causes angiogenesis in addition to remodelling in the adult rat pulmonary circulation. Journal of physiology 547, 133 (2003).
192. Hu, C. J. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Molecular and cellular biology 23, 9361 (2003).
193. Huang, H., Bhat, A., Woodnutt, G. & Lappe, R. Targeting the ANGPTâ€“TIE2 pathway in malignancy. Nat Rev Cancer 10, 575.
194. Hughes, P. E. Integrin affinity modulation. Trends in cell biology 8, 359 (1998).
195. Huynh-Do, U. Surface densities of ephrin-B1 determine EphB1-coupled activation of cell attachment through alphavbeta3 and alpha5beta1 integrins. EMBO Journal 18, 2165 (1999).
196. Hyodoh, H. et al. Peripheral vascular malformations: imaging, treatment approaches, and therapeutic issues. Radiographics 25, S159-71 (2005).
197. Imtiyaz, H. Z. Hypoxia-inducible factors as essential regulators of inflammation. Current topics in microbiology and immunology 345, 105.
198. Irani, A. A. Two types of human mast cells that have distinct neutral protease compositions. Proceedings of the National Academy of Sciences of the United States of America 83, 4464 (1986).
199. Irani, A. M. Human mast cell heterogeneity. Allergy proceedings 15, 303 (1994).
200. Isik, F. F. Monocyte chemoattractant protein-1 mRNA expression in hemangiomas and vascular malformations. The Journal of surgical research 61, 71 (1996).
201. Isner, J. M. Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. The journal of clinical investigation 103, 1231 (1999).
202. Ivan, M. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292, 464 (2001).
203. Ivanov, S. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. The American journal of pathology 158, 905 (2001).
204. Iyer, N. V. The human hypoxia-inducible factor 1alpha gene: HIF1A structure and evolutionary conservation. Genomics 52, 159 (1998).
205. Ja, F. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Molecular and cellular biology 16, 4604 (1996).
206. Jaccoud, S. Nouveau Dictionnaire de Medecine et de Chirurgie Pratiques (Bailliere, Paris, 1870).
207. Jackson, I. T. Hemangiomas, vascular malformations, and lymphovenous malformations: classification and methods of treatment. Plastic and reconstructive

surgery 91, 1216 (1993).

208. Jackson, I. T. Compartmentalization of massive vascular malformations. Plastic and reconstructive surgery 115, 10 (2005).

209. Jackson, J. E. Treatment of high-flow vascular malformations by venous embolization aided by flow occlusion techniques. Cardiovascular and interventional radiology 19, 323 (1996).

210. Jacobs, A. H. Strawberry hemangioma: natural history of the untreated lesion. Calif Med 83, 8-10 (1957).

211. Jacobs, A. H. The incidence of birthmarks in the neonate. Pediatrics 58, 218 (1976).

212. Jeltsch, M. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 276, 1423 (1997).

213. Jg, W. Systemic hypoxia increases leukocyte emigration and vascular permeability in conscious rats. Journal of applied physiology 89, 1561 (2000).

214. Jiang, B. H. Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. Journal of biological chemistry 272, 19253 (1997).

215. Jin, H. Integrins: roles in cancer development and as treatment targets. British Journal of Cancer 90, 561 (2004).

216. Johnson, W. C. Pathology of cutaneous vascular tumors. International journal of dermatology 15, 239 (1976).

217. Kaban, L. B. Vascular anomalies of the maxillofacial region. Journal of oral and maxillofacial surgery 44, 203 (1986).

218. Kaplan, E. N. Hamartomas (eds. Kernahan, D. A. & Vistnes, L. M.) (Little Brown & Company, Boston, 1977).

219. Kelly, B. D. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circulation research 93, 1074 (2003).

220. Kieran, M. W. Angiogenesis inhibitors and hypoxia. Nature medicine 9, 1104 (2003).

221. Kim, J. Y. Surgical treatment for congenital arteriovenous malformation: 10 years' experience. European journal of vascular and endovascular surgery 32, 101 (2006).

222. Kim, K. H. Sclerotherapy for congenital lesions in the head and neck. Otolaryngology--head and neck surgery 131, 307 (2004).

223. Kim, Y. W. Risk factors for leg length discrepancy in patients with congenital vascular malformation. Journal of vascular surgery 44, 545 (2006).

224. Kimelman, D. Synergistic induction of mesoderm by FGF and TGF-beta and the

identification of an mRNA coding for FGF in the early Xenopus embryo. Cell 51, 869 (1987).

225. Kindblom, L. G. Factor VIII related antigen and mast cells. Acta pathologica, microbiologica et immunologica Scandinavica. Section A, Pathology 90, 437 (1982).
226. Kishnani, P. Hemangioma, supraumbilical midline raphe, and coarctation of the aorta with a right aortic arch: single causal entity? American journal of medical genetics 59, 44 (1995).
227. Kitamura, Y. Heterogeneity of mast cells and phenotypic change between subpopulations. Annual review of immunology 7, 59 (1989).
228. Klagsbrun, M. Regulators of angiogenesis. Annual Review of Physiology 53, 217 (1991).
229. Kleinman, M., Tepper, O. & Capla, J. Increased circulating AC133+CD34+endothelial progenitor cells in children with haemangioma. Lymphatic research and biology, 1:301 (2003).
230. Kohout, M. Arteriovenous Malformations of the Head and Neck: Natural History and Management. Plastic and reconstructive surgery 102, 643 (1998).
231. Korpelainen, E. I. Endothelial receptor tyrosine kinases activate the STAT signaling pathway: mutant Tie-2 causing venous malformations signals a distinct STAT activation response. Oncogene 18, 1 (1999).
232. Kubota, H. Spontaneous disappearance of a renal arteriovenous malformation. International journal of urology 10, 547 (2003).
233. Kurihara, H. [Hypertension and endothelin]. 84, 15 (1995).
234. Laberge-le Couteulx, S. Truncating mutations in CCM1, encoding KRIT1, cause hereditary cavernous angiomas. Nature genetics 23, 189 (1999).
235. Lando, D. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science 295, 858 (2002).
236. Law, R. & Bukwirwa, H. The Physiology of Oxygen Delivery. Physiology, 1 (1999).
237. Leavesley, D. I. Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. The journal of cell biology 121, 163 (1993).
238. Lee, B. B. New experiences with absolute ethanol sclerotherapy in the management of a complex form of congenital venous malformation. Journal of vascular surgery 33, 764 (2001).
239. Lee, B. B. Advanced management of congenital vascular malformations: a multidisciplinary approach. Cardiovascular surgery 10, 523 (2002).

240. Lee, B. B. Management of arteriovenous malformations: a multidisciplinary approach. Journal of vascular surgery 39, 590 (2004).
241. Lee, B. B. Critical issues in management of congenital vascular malformation. Annals of vascular surgery 18, 380 (2004).
242. Lee, B. B. New approaches to the treatment of congenital vascular malformations (CVMs)--a single centre experience. European journal of vascular and endovascular surgery 30, 184 (2005).
243. Lee, B. B. Vascular malformations. Journal of the American College of Surgeons 200, 638 (2005).
244. Lee, B. B. Current concepts in lymphatic malformation. Vascular and endovascular surgery 39, 67 (2005).
245. Lee, B. B. Advanced management of arteriovenous shunting malformation with transarterial lung perfusion scintigraphy for follow-up assessment. International angiology 24, 173 (2005).
246. Lee, B. B. Congenital vascular malformations: general treatment principles. Phlebology 22, 258 (2007).
247. Lee, B. B. Congenital vascular malformations: general diagnostic principles. Phlebology 22, 253 (2007).
248. Lee, B. B. Terminology and classification of congenital vascular malformations. Phlebology 22, 249 (2007).
249. Lee, S. Y. Neovastat (AE-941) inhibits the airway inflammation via VEGF and HIF-2 alpha suppression. Vascular pharmacology 47, 313 (2007).
250. Levi-Schaffer, F. Mast cells and angiogenesis. Clinical and experimental allergy 31, 521 (2001).
251. Levy, A. P. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. The Journal of biological chemistry 270, 13333 (1995).
252. Li, C. H. Risk factors associated with intra-operative major blood loss in patients with hepatocellular carcinoma who underwent hepatic resection. Journal of the Chinese Medical Association 66, 669 (2003).
253. Li, J. Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. The Journal of biological chemistry 270, 308 (1995).
254. Li, L., Reddel, W. & Krills, S. A. The phenotypic similarities and differences between human basophils and mast cells (eds. Marone, G., Lichtenstein, L. M. & Galli, S. J.) (Academic Press, London, 2000).

255. Li, Z. Expression of endothelin-1 in macrophages and mast cells in hyperplastic human tonsils. FEBS letters 457, 381 (1999).
256. Lim, M. Characterization of the integrin alpha v beta3 in arteriovenous malformations and cavernous malformations. Cerebrovascular diseases 20, 23 (2005).
257. Limaye, N. Somatic mutations in angiopoietin receptor gene TEK cause solitary and multiple sporadic venous malformations. Nature genetics 41, 118 (2009).
258. Lin, T. J. SDF-1 induces IL-8 production and transendothelial migration of human cord blood-derived mast cells. International Archives of Allergy and Immunology 124, 142 (2001).
259. Liu, J. et al. Identification and characterization of small-molecule inhibitors of Tie2 kinase. FEBS Letters 582, 785 (2008).
260. Loncaster, J. A. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer research 61, 6394 (2001).
261. Loose, D. A. The surgical treatment of predominantly venous defects. International angiology 9, 189 (1990).
262. Loose, D. A. Surgical treatment in predominantly arterial defects. International angiology 9, 183 (1990).
263. Loose, D. A. Congenital vascular defects. International angiology 9, 133 (1990).
264. Loose, D. A. Surgical treatment of predominantly venous defects. Seminars in vascular surgery 6, 252 (1993).
265. Loose, D. A. Combined treatment of congenital vascular defects: indications and tactics. Seminars in vascular surgery 6, 260 (1993).
266. Loose, D. A. Operative Korrekturen vaskularer Malformationen an der unteren Extremitat. Kongressband/Deutsche Gesellschaft fur Chirurgie 118, 507 (2001).
267. Loose, D. A. Surgical management of venous malformations. Phlebology 22, 276 (2007).
268. Lorentz, A. Regulatory effects of stem cell factor and interleukin-4 on adhesion of human mast cells to extracellular matrix proteins. Blood 99, 966 (2002).
269. Lyttle, D. J. Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. Journal of virology 68, 84 (1994).
270. Maeda, K. Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. Cancer 77, 858 (1996).
271. Maftei, N. The surgical management of 73 vascular malformations and preoperative predictive factors of major haemorrhage--a single centre experience. European journal

of vascular and endovascular surgery 38, 488 (2009).

272. Magazine, H. I. Vascular contractile potency of endothelin-1 is increased in the presence of monocytes or macrophages. American journal of physiology 266, H1620 (1994).

273. Mahnke, P. F. Haufigkeit und mogliche Bedeutung von Gewebsmastzellen in kindlichen Hamangiomen. Gegenbaurs morphologisches Jahrbuch 111, 153 (1967).

274. Maisonpierre, P. C. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277, 55 (1997).

275. Malan, E. Congenital arteriovenous malformations of the face and scalp. Journal of cardiovascular surgery 9, 109 (1968).

276. Malan, E. Vascular malformations (Angiodysplasias) (Carlo Erba Foundation, Milan, 1974).

277. Maltepe, E. Oxygen, genes, and development: an analysis of the role of hypoxic gene regulation during murine vascular development. Journal of molecular medicine 76, 391 (1998).

278. Manalo, D. J. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood 105, 659 (2005).

279. Mandriota, S. J. HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron. Cancer cell 1, 459 (2002).

280. Marchiano, A. V. Embolizzazione transcatetere in 39 casi di malformazione artero-venosa iperattiva. Radiologia medica 91, 440 (1996).

281. Margilath, A. M. & Museles, M. Current concepts in diagnosis and management of congenital cutaneous hemangiomas. Pediatrics 36, 410-6 (1965).

282. Marietta, M. Pathophysiology of bleeding in surgery. Transplantation proceedings 38, 812 (2006).

283. Marler, J. J. Current management of hemangiomas and vascular malformations. Clinics in plastic surgery 32, 99 (2005).

284. Marler, J. J. Increased expression of urinary matrix metalloproteinases parallels the extent and activity of vascular anomalies. Pediatrics 116, 38 (2005).

285. Marschel, P. Control of fluid shear response in circulating leukocytes by integrins. Annals of biomedical engineering 30, 333 (2002).

286. Masaki, T. Historical review: Endothelin. Trends in Pharmacological Sciences 25, 219 (2004).

287. Masson-Gadais, B. Integrin alphavbeta3, requirement for VEGFR2-mediated activation of SAPK2/p38 and for Hsp90-dependent phosphorylation of focal adhesion kinase in

endothelial cells activated by VEGF. Cell stress & chaperones 8, 37 (2003).

288. Mattle, H. P. Dilemmas in the management of patients with arteriovenous malformations. Journal of neurology 247, 917 (2000).

289. Mazereeuw-Hautier, J. Extensive venous/lymphatic malformations causing life-threatening haematological complications. British journal of dermatology 157, 558 (2007).

290. Meininger, C. J. Mast cells and angiogenesis. Seminars in cancer biology 3, 73 (1992).

291. Meininger, C. J. Increased stem cell factor release by hemangioma-derived endothelial cells. Laboratory Investigation 72, 166 (1995).

292. Mencarelli, M. Endothelin receptor A expression in human inflammatory cells. Regulatory peptides 158, 1 (2009).

293. Mertens, A. C. Congenital abnormalities in children with acute leukemia: a report from the Children's Cancer Group. The journal of pediatrics 133, 617 (1998).

294. Metcalfe, D. D. Mast cells. Physiological reviews 77, 1033 (1997).

295. Meyer, J. S. Biological classification of soft-tissue vascular anomalies: MR correlation. AJR, American journal of roentgenology 157, 559 (1991).

296. Michaloudi, H. Parallel development of blood vessels and mast cells in the lateral geniculate nuclei. Developmental brain research 140, 269 (2003).

297. Minchenko, A. Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. Laboratory Investigation 71, 374 (1994).

298. Miyamoto, S. Fibronectin and integrins in cell adhesion, signaling, and morphogenesis. Annals of the New York Academy of Sciences 857, 119 (1998).

299. Moazzam, F. The leukocyte response to fluid stress. Proceedings of the National Academy of Sciences of the United States of America 94, 5338 (1997).

300. Moller, A. Human mast cells produce IL-8. The journal of immunology 151, 3261 (1993).

301. Morelli, J. G. Treatment of ulcerated hemangiomas infancy. Archives of pediatrics & adolescent medicine 148, 1104 (1994).

302. Moroz, B. Long term follow-up of hemangiomas in children (ed. Williams, H. B.) (CV Mosby, St Louis, 1983).

303. Mueller, B. U. The infant with a vascular tumor. Seminars in perinatology 23, 332 (1999).

304. Mulliken, J. B. Management of a vascular malformation of the face using total circulatory arrest. Surgery, gynecology & obstetrics 146, 168 (1978).

305. Mulliken, J. B. Classification of VASCULAR BIRTHMARKS (eds. Grainger, R. G. &

Allison, D. J.) (WB Saunders, Philadelphia, 1988).

306. Mulliken, J. B. Cutaneous vascular anomalies. Seminars in vascular surgery 6, 204 (1993).
307. Mulliken, J. B. Vascular anomalies. Current Problems in Surgery 37, 517 (2000).
308. Mulliken, J. B. et al. Pharmacologic therapy foe endangering hemangiomas. Current Opinion in Dermatology, 109-13 (1995).
309. Mulliken, J. B., Fishman, S. J. & Burrows, P. E. Vascular anomalies (ed. Wells, S. A.) (Mosby, 2000).
310. Mulliken, J. B. & Glowacki, J. Hemangiomas and vascular malformations in infants and children: a classification based on endothelial characteristics. Plastic and reconstructive surgery 69, 412 (1982).
311. Mulliken, J. B. & Young, A. E. Vascular birthmarks (W.B. Saunders, Philadelphia, 1988).
312. Muramatsu, M. Chymase mediates mast cell-induced angiogenesis in hamster sponge granulomas. European Journal of Pharmacology 402, 181 (2000).
313. Murdoch, C. Hypoxia regulates macrophage functions in inflammation. The journal of immunology 175, 6257 (2005).
314. Nakanishi, K. Expression of hypoxia-inducible factor-1alpha protein predicts survival in patients with transitional cell carcinoma of the upper urinary tract. Clinical cancer research 11, 2583 (2005).
315. Nakatsu, M. N. VEGF(121) and VEGF(165) regulate blood vessel diameter through vascular endothelial growth factor receptor 2 in an in vitro angiogenesis model. Laboratory Investigation 83, 1873 (2003).
316. Nauck, M. Corticosteroids inhibit the expression of the vascular endothelial growth factor gene in human vascular smooth muscle cells. European Journal of Pharmacology 341, 309 (1998).
317. Nguyen, V. A. Infantile hemangioma is a proliferation of beta 4-negative endothelial cells adjacent to HLA-DR-positive cells with dendritic cell morphology. Human Pathology 35, 739 (2004).
318. Nikkari, S. T. Macrophages contain 92-kd gelatinase (MMP-9) at the site of degenerated internal elastic lamina in temporal arteritis. The American journal of pathology 149, 1427 (1996).
319. Nisato, R. E. alphav beta 3 and alphav beta 5 integrin antagonists inhibit angiogenesis in vitro. Angiogenesis 6, 105 (2003).
320. Norrby, K. Mast cells and de novo angiogenesis: angiogenic capability of individual

mast-cell mediators such as histamine, TNF, IL-8 and bFGF. Inflammation research 46, S7 (1997).

321. North, P. E. GLUT1: a newly discovered immunohistochemical marker for juvenile hemangiomas. Human Pathology 31, 11 (2000).
322. Olofsson, B. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proceedings of the National Academy of Sciences of the United States of America 93, 2576 (1996).
323. Opitz, J. M. & Gilbert, E. F. CNS anomalies and the midline as a 'developmental field'. Am J Med Genet, 443-55 (1982).
324. Paltiel, H. J. Soft-tissue vascular anomalies: utility of US for diagnosis. Radiology 214, 747 (2000).
325. Pascarella, L. Venous angiomata: treatment with sclerosant foam. Annals of vascular surgery 19, 457 (2005).
326. Pascual-Castroviejo, I. Hemangiomas of the head, neck, and chest with associated vascular and brain anomalies: a complex neurocutaneous syndrome. AJNR, American journal of neuroradiology 17, 461 (1996).
327. Pasyk, K. Quantitative Evaluation of Mast Cells in Cellularly Dynamic and Adynamic Vascular Malformations. Plastic and reconstructive surgery 73, 69 (1984).
328. Pasyk, K. A. Cellular haemangioma. Light and electron microscopic studies of two cases. Virchows Archiv. A, Pathological anatomy and histology 396, 103 (1982).
329. Pasyk, K. A. Ultrastructure of mast cells in growing and involuting stages of hemangiomas. Human Pathology 14, 174 (1983).
330. Pasyk, K. A. Familial vascular malformations. Report of 25 members of one family. Clinical genetics 26, 221 (1984).
331. Pasyk, K. A. Quantitative evaluation of mast cells in cellularly dynamic and adynamic vascular malformations. Plastic and reconstructive surgery 73, 69 (1984).
332. Pasyk, K. A. Classification and clinical and histopathological featuresof haemangiomas and other vascular malformations (eds. Ryan, T. J. & Cherry, G. W.) (Oxford University Press, New York, 1987).
333. Pepper, M. S. Biphasic effect of transforming growth factor-beta 1 on in vitro angiogenesis. Experimental cell research 204, 356 (1993).
334. Pepper, M. S. Angiogenesis-regulating cytokines: activities and interactions. Current topics in microbiology and immunology 213, 31 (1996).
335. Pepper, M. S. Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. Enzyme & protein 49, 138 (1996).

336. Peters, K. G. Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. Proceedings of the National Academy of Sciences of the United States of America 90, 8915 (1993).
337. Phillips, J. H., Tang, C. L., Armstrong, D., Chalain, T. D. & al., e. Congenital arteriovenous malformations: A follow-up of treatment. Canadian Journal of Plastic Surgery 13, 23 (2005).
338. Piali, L. CD31/PECAM-1 is a ligand for alpha v beta 3 integrin involved in adhesion of leukocytes to endothelium. The journal of cell biology 130, 451 (1995).
339. Polverini, P. J. Activated macrophages induce vascular proliferation. Nature 269, 804 (1977).
340. Powell, J. Update on hemangiomas and vascular malformations. Current opinion in pediatrics 11, 457 (1999).
341. Powell, T. G. Epidemiology of strawberry haemangioma in low birthweight infants. British journal of dermatology 116, 635 (1987).
342. Pratt, A. G. Birthmarks in infants. A.M.A. archives of dermatology and syphilology 67, 302 (1953).
343. Preusser, M. Vascularization and expression of hypoxia-related tissue factors in intracranial ependymoma and their impact on patient survival. Acta neuropathologica 109, 211 (2005).
344. Puri, M. C. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. EMBO Journal 14, 5884 (1995).
345. Ra, C. Fibronectin receptor integrins are involved in mast cell activation. Journal of allergy and clinical immunology 94, 625 (1994).
346. Rak, K. M. MR imaging of symptomatic peripheral vascular malformations. AJR, American journal of roentgenology 159, 107 (1992).
347. Rakusan, K. Mast cells in the rat heart during normal growth and in cardiac hypertrophy. Circulation research 66, 511 (1990).
348. Ramsauer, M. Getting Tie(2)d up in angiogenesis. The journal of clinical investigation 110, 1615 (2002).
349. Rao, V. K. Angiomatosis of soft tissue. An analysis of the histologic features and clinical outcome in 51 cases. The American journal of surgical pathology 16, 764 (1992).
350. Rapisarda, A. Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer research 62, 4316 (2002).

351. Rastinejad, F. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell 56, 345 (1989).

352. Re, N. alphav beta 3 and alphav beta 5 integrin antagonists inhibit angiogenesis in vitro. Angiogenesis 6, 105 (2003).

353. Reid, M. R. Abnormal arteriovenous communications acquired and congenital. IV: The treatment of abnormal arteriovenous communications. Prognosis. Archives of Surgery 11, 237 (1925).

354. Remuzzi, G. New therapeutics that antagonize endothelin: promises and frustrations. Nature reviews. Drug discovery 1, 986 (2002).

355. Requena, L. Cutaneous vascular anomalies. Part I. Hamartomas, malformations, and dilation of preexisting vessels. Journal of the American Academy of Dermatology 37, 523 (1997).

356. Restrepo, M. Birth defects among children born to a population occupationally exposed to pesticides in Colombia. Scandinavian Journal of Work, Health & Environment 16, 239 (1990).

357. Rhoten, R. L. Specific repression of the preproendothelin-1 gene in intracranial arteriovenous malformations. Journal of neurosurgery 86, 101 (1997).

358. Ribatti, D. Angiogenesis and mast cell density with tryptase activity increase simultaneously with pathological progression in B-cell non-Hodgkin's lymphomas. International journal of cancer 85, 171 (2000).

359. Riles, T. S. & Jacobowitz, G. R. Surgical management of vascular malformations (ed. Rutherford, R. B.) (WB Saunders, Philadelphia, 2006).

360. Risau, W. & Rubanyi, G. M. Morphogenesis of endothelium (ed. Rubanyi, G. M.) (Harwood Academic Publishers, California, 2000).

361. Ritter, M., Dorrell, M. & Edomonds, J. Insulin-like growth factor 2 and potential regulators of haemangioma growth and involution adentified by large-scale expression analysis. Proc Natl Acad Sci USA, 99-7455 (2002).

362. Ritter, M., Moreno, S. & Dorrell, M. Identifying potential regulators of infantile hemangioma progression through large-scale expression analysis: a possible role for the immune system and indoleamine 2,3 dioxygenase (IDO) during involution. Lymphatic research and biology, 1:291 (2003).

363. Roberts, W. G. Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. Journal of cell science 108, 2369 (1995).

364. Roda, J. M. Hypoxia-Inducible Factor-2{alpha} Regulates GM-CSF-Derived Soluble Vascular Endothelial Growth Factor Receptor 1 Production from Macrophages and

Inhibits Tumor Growth and Angiogenesis. The journal of immunology 187, 1970.

365. Roganovic, J. Increased prevalence of minor anomalies in children with hematologic malignancies. Medical and pediatric oncology 38, 128 (2002).
366. Rosai & Ackerman. Rosai and Ackerman's Surgical Pathology (Mosby, 2004).
367. Rosen, J. R. & Riles, T. S. in Vascular Surgery (ed. B, R. R.) 1452-64 (WB Saunders, Philadelphia, 2000).
368. Ruegg, C. Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. Cellular and molecular life sciences 60, 1135 (2003).
369. Ruger, B. M. Mast cells and type VIII collagen in human diabetic nephropathy. Diabetologia 39, 1215 (1996).
370. Ruoslahti, E. Integrin signaling and matrix assembly. Tumor biology 17, 117 (1996).
371. Ryan, H. E. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO journal 17, 3005 (1998).
372. Salani, D. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. The American journal of pathology 157, 1703 (2000).
373. Salani, D. Role of endothelin-1 in neovascularization of ovarian carcinoma. The American journal of pathology 157, 1537 (2000).
374. Sarihan, H. A new treatment with bleomycin for complicated cutaneous hemangioma in children. European journal of pediatric surgery 7, 158 (1997).
375. Sato, T. N. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 376, 70 (1995).
376. Schwartz, L. B. Mast cells: function and contents. Current opinion in immunology 6, 91 (1994).
377. Sebai, N. E. et al. Malformations arterioveineuses: etude de 54 patients. La Tunisie Medicale 85, 871 (2007).
378. Semenza, G. L. Targeting HIF-1 for cancer therapy. Nature Reviews. Cancer 3, 721 (2003).
379. Semenza, G. L. Regulation of gene expression by HIF-1. Novartis Foundation symposium 272, 2 (2006).
380. Serafin, W. E. Mediators of immediate hypersensitivity reactions. New England Journal of Medicine, The 317, 30 (1987).
381. Shalaby, F. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376, 62 (1995).
382. Shattil, S. J. Function and regulation of the beta 3 integrins in hemostasis and vascular

biology. Thrombosis and haemostasis 74, 149 (1995).

383. Sheth, S. Benign vascular tumors and tumor-like proliferations. Seminars in diagnostic pathology 25, 1 (2008).

384. Shih, S. C. Hypoxia-mediated regulation of gene expression in mammalian cells. International journal of experimental pathology 79, 347 (1998).

385. Shima, D. T. Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. Molecular medicine 1, 182 (1995).

386. Shing, Y. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science 223, 1296 (1984).

387. Shubich, M. G. K gistokhimicheskoi kharakteristike tuchnykh kletok v gemangiomakh. Arkhiv patologiĭ 38, 32 (1976).

388. Shweiki, D. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359, 843 (1992).

389. Sidhu, M. K. Ultrasound-guided endovenous diode laser in the treatment of congenital venous malformations: preliminary experience. Journal of vascular and interventional radiology 16, 879 (2005).

390. Sie, K. C. Subglottic hemangioma: ten years' experience with the carbon dioxide laser. The Annals of otology, rhinology & laryngology 103, 167 (1994).

391. Siemeister, G. Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel-Lindau tumor suppressor protein. Cancer research 56, 2299 (1996).

392. Simonson, M. S. Cell signaling by endothelin peptides (ed. Warner, T. D.) (Springer).

393. Simpson, J. R. Natural history of cavernous haemangiomata. Lancet, The 2, 1057 (1959).

394. Singh, B. Vascular expression of the alpha(v)beta(3)-integrin in lung and other organs. American journal of physiology. Lung cellular and molecular physiology 278, L217 (2000).

395. Sluimer, J. C. Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. Journal of the American College of Cardiology 51, 1258 (2008).

396. Soffer, D. Multifocal vascular tumors in fowl induced by a newly isolated retrovirus. Cancer research 50, 4787 (1990).

397. Sonstein, W. J. Expression of vascular endothelial growth factor in pediatric and adult cerebral arteriovenous malformations: an immunocytochemical study. Journal of

neurosurgery 85, 838 (1996).

398. Stead, R. H. Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. Proceedings of the National Academy of Sciences of the United States of America 84, 2975 (1987).

399. Stout, A. P. Hemangioendothelioma: a tumour of blood vessels featuring vascular endothelial cells. Annals of Surgery, 445-64 (1943).

400. Stout, A. P. & Lattes, R. S. Tumors of the Soft Tissue (Armed Forces Institute of Pathology, Washington, 1967).

401. Stout, A. P. & Murray, M. R. Hemangiopericytoma: a vascular tumour featuring Zimmerman's pericytes. Annals of Surgery, 26-33 (1942).

402. Stupack, D. G. Integrins and angiogenesis. Current Topics in Developmental Biology 64, 207 (2004).

403. Suh, J. S. Venous malformations: sclerotherapy with a mixture of ethanol and lipiodol. Cardiovascular and interventional radiology 20, 268 (1997).

404. Sumitomo, M. Synergy in tumor suppression by direct interaction of neutral endopeptidase with PTEN. Cancer cell 5, 67 (2004).

405. Sun, Z. J. Mast cells in hemangioma: a double-edged sword. Medical hypotheses 68, 805 (2007).

406. Sunderkotter, C. Macrophages and angiogenesis. Journal of leukocyte biology 55, 410 (1994).

407. Suri, C. A Magyar Honvedseg Kozponti Honvedkorhaz Szajsebeszeti Osztalya arc- es allcsontserult betegeinek ellatasa (10 ev beteganyaganak elemzese). Fogorvosi szemle 88, 233 (1995).

408. Suri, C. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 87, 1171 (1997).

409. Suri, C. Increased vascularization in mice overexpressing angiopoietin-1. Science 282, 468 (1998).

410. Svendsen, P. A. Direct puncture of large arteriovenous malformations in head and neck for embolisation and subsequent reconstructive surgery. Scandinavian journal of plastic and reconstructive surgery and hand surgery 28, 131 (1994).

411. Szilagyi, D. E. Congenital arteriovenous anomalies of the limbs. Archives of surgery 111, 423 (1976).

412. Takahashi, K. Cellular markers that distinguish the phases of hemangioma during infancy and childhood. The journal of clinical investigation 93, 2357 (1994).

413. Talks, K. L. The expression and distribution of the hypoxia-inducible factors HIF-

1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. The American journal of pathology 157, 411 (2000).

414. Tan, S. T. Cellular and extracellular markers of hemangioma. Plastic and reconstructive surgery 106, 529 (2000).
415. Tan, S. T. A novel in vitro human model of hemangioma. Modern pathology 13, 92 (2000).
416. Tan, S. T. Mast cells and hemangioma. Plastic and reconstructive surgery 113, 999 (2004).
417. Tasnadi, G. Epidemiology and etiology of congenital vascular malformations. Seminars in vascular surgery 6, 200 (1993).
418. Taxy, J. B. Cellular angiomas of infancy: an ultrastructural study of two cases. Cancer 43, 2322 (1979).
419. Taylor, A. M. Stem-cell factor, the kit ligand, induces direct degranulation of rat peritoneal mast cells in vitro and in vivo: dependence of the in vitro effect on period of culture and comparisons of stem-cell factor with other mast cell-activating agents. Immunology 86, 427 (1995).
420. Taylor, G. I. The vascular territories (angiosomes) of the body: experimental study and clinical applications. British Journal of Plastic Surgery 40, 113 (1987).
421. Taylor, S. Protamine is an inhibitor of angiogenesis. Nature 297, 307 (1982).
422. Thornton, S. C. Human endothelial cells: use of heparin in cloning and long-term serial cultivation. Science 222, 623 (1983).
423. Tille, J. C. Hereditary vascular anomalies: new insights into their pathogenesis. Arteriosclerosis, thrombosis, and vascular biology 24, 1578 (2004).
424. Tipoe, G. L. Expression and functions of vasoactive substances regulated by hypoxia-inducible factor-1 in chronic hypoxemia. Cardiovascular & hematological agents in medicinal chemistry 4, 199 (2006).
425. Treiber, F. A. Endothelin-1 gene Lys198Asn polymorphism and blood pressure reactivity. Hypertension 42, 494 (2003).
426. Tucker, G. C. Alpha v integrin inhibitors and cancer therapy. Current opinion in investigational drugs 4, 722 (2003).
427. Tuder, R. M. Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia. Modulation of gene expression by nitric oxide. Journal of Clinical Investigation 95, 1798 (1995).
428. Turner, A. J. Exploring the structure and function of zinc metallopeptidases: old enzymes and new discoveries. Biochemical Society transactions 31, 723 (2003).

429. Vargesson, N. Smad7 misexpression during embryonic angiogenesis causes vascular dilation and malformations independently of vascular smooth muscle cell function. Developmental biology 240, 499 (2001).
430. Vihanto, M. M. Hypoxia up-regulates expression of Eph receptors and ephrins in mouse skin. The FASEB journal 19, 1689 (2005).
431. Vijayaraghavan, J. The hydrolysis of endothelins by neutral endopeptidase 24.11 (enkephalinase). Journal of biological chemistry 265, 14150 (1990).
432. Vikkula, M. Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. Cell 87, 1181 (1996).
433. Vikkula, M. Molecular basis of vascular anomalies. Trends in cardiovascular medicine 8, 281 (1998).
434. Vink, A. HIF-1 alpha expression is associated with an atheromatous inflammatory plaque phenotype and upregulated in activated macrophages. Atherosclerosis 195, e69 (2007).
435. Virchow, R. Die krankhaften Geschwulste (August Hirschwald, Berlin, 1863).
436. Vittori, F. L'angiome superficiel extensif ulcero-mutilant du nouveau-ne. Etude d'un cas et revue de la litterature. Pédiatrie 32, 691 (1977).
437. Walter, J., North, P. E. & Waner, M. Somatic mutation of vascular endothelial growth factor receptors in juvenile hemangioma. Genes Chromosomes Cancer, 33-295 (2002).
438. Walter, J. W. Genetic mapping of a novel familial form of infantile hemangioma. American journal of medical genetics 82, 77 (1999).
439. Wananukul, S. Clinical manifestation and management of hemangiomas of infancy. Medical journal of the Medical Association of Thailand 85, S280 (2002).
440. Wang, G. L. Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. Journal of biological chemistry 268, 21513 (1993).
441. Wang, H. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. Circulation research 83, 832 (1998).
442. Wang, H. U. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93, 741 (1998).
443. Wang, Q. K. Update on the molecular genetics of vascular anomalies. Lymphatic research and biology 3, 226 (2005).
444. Wang, Y. M., Cheng, L. F. & Li, N. Histological study of vascular changes after intra-arterial and intra-venous injection of N- butyl-2-cyanoacrylate. Chinese Journal of Digestive Diseases 7, 175-9 (2006).

445. Wang, Z. et al. Hypoxia-Induced Down-Regulation of Neprilysin by Histone Modification in Mouse Primary Cortical and Hippocampal Neurons. PLoS ONE 6, e19229.
446. Wautier, M. P. Cultured endothelial cells from human arteriovenous malformations have defective growth regulation. Blood 94, 2020 (1999).
447. Wedemeyer, J. Mast cells and basophils in acquired immunity. British Medical Bulletin 56, 936 (2000).
448. Weisse, S. W. & Goldbloom, J. R. Enzinger and Weiss's Soft Tissue Tumours (2001).
449. West.J.B. Respiratory physiology (ed. West, J. B.) (Williams and Wilkens, Baltimore, 2004).
450. White, M. V. Histamine and asthma. The American review of respiratory disease 135, 1165 (1987).
451. White, M. V. Mast cells secretagogues (eds. Kaliner, M. A. & Metcalfe, D. D.) (Dekker, New York, 1993).
452. White, R. I., Jr. Long-term outcome of embolotherapy and surgery for high-flow extremity arteriovenous malformations. Journal of vascular and interventional radiology 11, 1285 (2000).
453. Widlus, D. M. Congenital arteriovenous malformations: tailored embolotherapy. Radiology 169, 511 (1988).
454. Wiegand, S. Surgery in patients with vascular malformations of the head and neck: value of coagulation disorders. Phlebology 24, 38 (2009).
455. Willenberg, T. Vascular birthmarks. VASA 37, 5 (2008).
456. Wilson, I. H. & Baskett, P. J. F. in World Anaesthesia Online 1-2 (World Anaesthesia and The World Federation of Societies of Anaesthesiologists, 1992).
457. Woodrow, S. L. Enlarging congenital haemangioma in an adult--a new entity? Clinical and experimental dermatology 22, 283 (1997).
458. Woolard, H. H. The development of the principal arterial stems in the forelimb of the pig. Contrib Embryol, 139-154 (1922).
459. Wykoff, C. C. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer research 60, 7075 (2000).
460. Yancopoulos, G. D. Vasculogenesis, angiogenesis, and growth factors: ephrins enter the fray at the border. Cell 93, 661 (1998).
461. Yeo, E. J. YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. Journal of the National Cancer Institute 95, 516 (2003).
462. Yoshimura, H. Prognostic impact of hypoxia-inducible factors 1alpha and 2alpha in

colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. Clinical cancer research 10, 8554 (2004).

463. Yu, F. HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proceedings of the National Academy of Sciences of the United States of America 98, 9630 (2001).

464. Yu, Y., Flint, A. F. & Mulliken, J. B. Endothelial progenitor cells in infantile hemangioma. Blood, 103:1373 (2004).

465. Zhou, B. W., Lin, P. H., Eraso, A. & Lumsden, A. B. in Endovascular Today 85-91 (2005).