

**The role of angiopoietins and their mediators in symptomatic small bowel
angiodysplasia; identifying novel diagnostic and therapeutic targets
in chronic anaemia and obscure gastrointestinal bleeding.**

A thesis submitted for the degree of Doctor in Philosophy (Ph.D), Clinical Medicine
2021

Dr Grainne Holleran

Supervisor:

Professor Deirdre McNamara

Consultant Gastroenterologist/Associate Professor in Clinical Medicine

Trinity Centre for Health Sciences

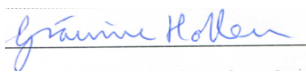
Tallaght Hospital

Dublin 24

Declaration:

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.



Grainne Holleran

Summary

The over-arching aim of this thesis was to investigate the underlying pathophysiology of SBA (small bowel angiodysplasia) formation. Chapter 1 outlines how despite SBA being more commonly encountered, there are still many difficulties including diagnostic delays, the unknown natural history and prognosis, poor management options, and their resultant poor quality of life for patients. These difficulties all stem from the same issue, significant limitations in our knowledge of SBA pathophysiology, and the results from each section of this thesis will hopefully contribute to the knowledge base of SBA, and allow us to improve the clinical challenges faced by SBA patients in the future.

In chapter 2, we attempted to determine which angiogenic pathway might be of most importance in SBA formation, through our putative assessment. We used information from the literature regarding angiogenesis in gastrointestinal angiodysplasia (GIAD) to guide this assessment, and broadened our inclusion of other factors based on initial results. Unexpectedly, we found no association between VEGF and SBA, despite the published findings of both serum and tissue associations with VEGF previously, and the fact that the VEGF pathway is the most commonly understood of the angiogenic pathways (6, 107). Interestingly, we detected a positive association with serum Ang-2 in SBA patients, which was directed by a publication by Ojeda-Fernandez et al in HHT patients (85). However, we found no association with sEnd which was also shown to be lower in HHT patients. Further assessments performed in this chapter on serum and tissue samples allowed us to conclude that the Angiopoietin pathway and specifically Ang-1, Ang-2, and their common receptor Tie2 were likely to be key factors in the pathophysiology of SBA formation.

In chapter 3 we assessed how the use of clinical factors, or our recently discovered serum factors (Ang-1 or Ang-2), might be useful in predicting diagnosis, clinical severity, or prognosis, in affected patients. We collaborated with researchers in Radboud University in the Netherlands, who were carrying out similar clinical research in their SBA and GIAD cohorts. Collectively, we performed a systematic literature review assessing all published studies of GIAD to determine whether clinical factors could be used to aid diagnosis, classify disease severity, or predict prognosis. In essence, the literature review didn't yield any incremental clinical information regarding risk

assessment or predictive factors to our own respective cohort studies, which although a reassuring validation of our own research findings, was not clinically helpful. The second study in this chapter assessed the use of serum measurements of Ang-1 and Ang-2 as a diagnostic aid in a group of patients undergoing CE for the investigation of suspected small bowel bleeding. This assessment allowed us to address the issues around confounding factors affecting serum Ang-1 and Ang-2 levels which were raised by reviewers of our published findings in Chapter 2. We also found that either serum Ang-2 alone, or a combination of the factors in an Ang-1/Ang-2 ratio produced a clinically acceptable negative and positive predictive value for a diagnosis of SBA, and established a cut-off serum Ang-2 level of 2600 pg/ml to be predictive of a diagnosis of SBA in this cohort with an AUC of 0.695.

Chapter 4 used a commercially available multiplex assessment array which measured serum levels of 55 angiogenic factors. The results from the multiplex assessment were reassuring, as they validated our already detected association of SBA with Ang-1 and Ang-2, and further out ruled any significant association with the factors suggested by the literature, including VEGF and sEnd. Two new factors were identified, TIMP1 and Endostatin, and we assessed the significance of both factors in the same way we had examined Ang-1 and Ang-2, by firstly using commercially available ELISA kits to measure serum levels in SBA patients and healthy controls, and secondly by assessing their use as diagnostic aids or discriminative factors by measuring serum levels in patients with suspected small bowel bleeding. Both factors were found to be significantly associated with SBA, with decreased levels of TIMP1 and increased levels of Endostatin, both anti-angiogenic factors, found in patients with SBA compared to controls, however; they were deemed to be less clinically useful as potential biomarkers than Ang-1 or Ang-2, as there was significant overlap in the ranges of each factor across each diagnosis.

The initial chapters of this thesis focused on identifying several angiogenic factors likely to have a role in SBA pathophysiology. However, the process of angiogenesis is highly complex, dynamic and constantly evolving, which meant that to prove a causative rather than a clinical association, we needed to define levels of these factors in SBA tissue rather than just serum. In chapter 5 we initially decided to do this by measuring relative gene expression levels of each factor by qPCR. This showed elevated relative levels of genes encoding Ang-1, Ang-2, and Tie2 in SBA tissue compared to the background

patient control tissue, while levels of TIMP1 and Endostatin were not significantly different. We went on to attempt Immunohistochemistry (IHC) to more accurately quantify tissue levels of Ang-1, Ang-2, and Tie2 but unfortunately, we were unable to get any clinically useful results, despite many protocol adjustments and the expertise of a senior medical scientist and consultant pathologist. This issue will hopefully be quite easily overcome moving forward due to the acquisition of a new enteroscope in the unit, with a channel which permits use of a larger biopsy forceps, meaning that samples will be almost double the size in future. This may also be helpful for future qPCR work, as we had difficulties obtaining enough DNA in matched SBA and control tissue also, likely due to tissue size. Secondly, we may have been unlucky with the antibodies we selected for use in IHC on this occasion, so further IHC are likely to be a worthwhile pursuit.

We have successfully identified the Angiopoietin pathway as a key angiogenic pathway in SBA pathophysiology, primarily through changes in levels of Ang-1, Ang-2 and their receptor Tie2, both in serum and at a tissue level. Furthermore, we have identified TIMP1 and Endostatin as angiogenic factors associated specifically with SBA, and likely to be closely involved in the pathophysiology of SBA, at least at a serum level, perhaps through their interaction with the Angiopoietins, or other angiogenic stimuli, or inhibitors. These studies provide the basis for future work in SBA, where the assessment of specific serum levels of Ang-1, Ang-2, Endostatin and TIMP-1 may be used to highlight at risk patients who warrant closer monitoring. Furthermore, the assessment of serum levels of each of these factors at various intervals, particularly around bleeding episodes, may prove them as useful prognostic factors which could be used as an adjunct to the current clinical and Hb tools used in disease monitoring. Further work at a tissue level will hopefully identify some of these factors as useful therapeutic targets for more specific disease management.

The below charts summarise the patient cohorts used in each of the following chapters.

Group 1
SBA cohort identified via CE or DBE

Group 2
Non-bleeding controls, identified from CRC screening pilot, negative FIT, normal Hb

Group 3
Normal SB controls – undergoing DBE for any non-bleeding indication, normal SB macroscopically and histologically

Group 4
Patients undergoing CE for suspected small bowel bleeding of any cause. Subsequently divided into 3 groups based on diagnosis at CE – 4a SBA, 4b any other cause of SB bleeding, 4c Normal SB

Chapter 2

- Serum assessment Group 1 vs Group 2
- Gene expression assessment Group 1 (SBA tissue) vs Group 1 (normal tissue) vs Group 3



Chapter 3

- Group 4 - subsequently divided into groups 4a, 4b & 4c



Chapter 4

- Multiplex assessment Group 1 vs Group 2
- ELISA validation Group 1 vs Group 2
- OGIB assessment Group 1 vs Group 4b



Chapter 5

- Tissue assessment Group 1 (SBA tissue) vs Group 1 (normal tissue)

Acknowledgements

Firstly, I would like to thank the Health Research Board and the Health Service Executive for their generous funding of my PhD project and clinical training scholarship over the course of the last 6 years. This has allowed me many unique opportunities which have advanced my clinical and academic career in ways which otherwise would not have been possible.

Secondly, I'd like to sincerely thank my supervisor, Professor Deirdre McNamara for all her time, encouragement, enthusiasm, and unwavering support both in the preparation of this thesis, and in my overall career for the last decade.

There have been several key people involved in the scientific preparation of this thesis and I am particularly grateful to Sinead Smith for all her practical help in the troubleshooting and direction changes along the way, and particularly for her patience while overseeing my laboratory work in the earlier days! I would also like to acknowledge Dr Kevin O'Hare and Sarah Delaney from the Pathology Department in Tallaght University Hospital, for all their efforts with the Immunohistochemistry section of the study. If

nothing else, I have gained a much deeper appreciation for the purple and brown spots at histology meetings in recent years.

There have been many colleagues in Tallaght University Hospital and the Trinity Academic Gastroenterology Group who have contributed in some form to this thesis, from endoscopy nurses and clinical colleagues, to administrative and research staff, and I am grateful to each of them. I am also equally grateful to my colleagues in St James's Hospital, particularly Dr Finbar MacCarthy and Dr David Kevans, who made the difficult task of combining my research and clinical training so much easier by welcoming me into the Gastroenterology Department and including me in their training opportunities over the last 5 years.

Finally, I'd like to thank my husband, John, for his constant support and inflexible belief in my abilities. Without his encouragement, during a pandemic and in the later months of pregnancy, I would easily have convinced myself not to finish this thesis, but I will always be glad I did.

Abbreviations used throughout the thesis

ADAMTS-1 – a disintegrin and metalloprotease with thrombospondin motifs
ALK-1 – Activin-like Kinase -1
Ang-1 – Angiopoietin 1
Ang-2 – Angiopoietin 2
APC – Argon Plasma Coagulation
ARMD – Age Related Macular Degeneration
AUC – Area Under the Curve
CCL – Chemokine Ligand
CD26 – Cluster Differentiation Antigen 26
CE – Capsule Endoscopy
CI – Confidence Interval
CKD – Chronic Kidney Disease
CKD – Chronic Kidney Disease
COPD – Chronic Obstructive Pulmonary Disease
CV – Coefficient of Variation
DBE – Double Balloon Enteroscopy
DM – Diabetes Mellitus
DNA – Deoxyribonucleic Acid
DPPIV – Dipeptidyl Peptidase IV
DU – Densitometric Unit
DY – Diagnostic Yield
ECM – Extra Cellular Matrix
ECM – Extracellular Matrix
EG-VEGF – Endocrine Gland-Derived Vascular Endothelial Growth Factor
EGF – Epidermal Growth Factor
ELISA – Enzyme-linked Immunosorbent Assay
FFPE – Formalin-fixed Paraffin-embedded
FGF – Fibroblast Growth Factor
GAPDH – Glyceraldehyde 3-Phosphate Dehydrogenase
GAVE – Gastric Antral Vascular Ectasia
GDNF – Glial Cell Line-Derived Neurotrophic Factor
GDPR – General Data Protection Regulation
GFR – Glomerular Filtration Rate
GIAD – Gastrointestinal Angiodysplasia
GM-CSF – Granulocyte-Macrophage Colony-Stimulating Factor
Hb – Haemoglobin
HB-EGF – Heparin-Binding Epidermal-like Growth Factor
HGF – Hepatocyte Growth Factor
HHT – Hereditary Haemorrhagic Telangiectasia
HIF – Hypoxia Inducible Factor
HR – Hazard Ratio
HTN – Hypertension
IDA – Iron Deficiency Anaemia
IGF-BP - Insulin Growth Factor-Binding Proteins
IHC - Immunohistochemistry
IHD – Ischaemic Heart Disease

IL-1 β - Interleukin-1 beta
IM – Intramuscular
KGF – Keratinocyte Growth Factor
MASPIN – Mammary Serine Protease Inhibitor
MMP – Matrix Metalloproteinases
NRG – Neuregulin
OGIB – Obscure Gastrointestinal Bleeding
OR – Odds ratio
PAI-1 – Plasminogen Activator Inhibitor -1
PCR – Polymerase Chain reaction
PD-ECGF – Platelet Derived Endothelial Cell Growth Factor
PDGF – Platelet Derived Growth Factor
PEDF – Pigment Epithelium Derived Factor
PF4/CXCL4 – Platelet Factor 4
PIGF – Placental Growth Factor
PPI – Proton Pump Inhibitor
PRISMA – Preferred Reporting Items for Systematic Reviews
QoL – Quality of Life
RNA – Ribonucleic Acid
ROC – Receiver Operator Charactersitic
RR – Relative Risk
SBA – Small Bowel Angiodysplasia
SBE – Single Balloon Enteroscopy
SE – Spiral Enteroscopy
sEnd – Soluble Endoglin
TGF- Transforming Growth Factor
Tie2 – Receptor Tyrosine Kinase 2
TIMP – Tissue Inhibitor of Metalloproteinase
TNF – Tumour Necrosis Factor
TP – Thymidine Phosphorylase
TSP - Thrombospondin
TSP – Thrombospondins
uPA – Urokinase Plasminogen Activator
VEGF – Vascular Endothelial Growth Factor
VHD – Valvular Heart Disease
VTE – Venous Thromboembolism
vWF – von Willebrand Factor

Table of contents

Chapter number	Page number
Chapter 1	
A) An introduction to gastrointestinal angiodysplasia with focus on small bowel angiodysplasia – diagnosis, management, prognosis.	6-23
B) The role of angiogenesis in gastrointestinal angiodysplasia	24-28
Chapter 2	
The assessment of putative angiogenic factors in sporadic small bowel angiodysplasia	
A) the assessment of circulating serum levels of various angiogenic factors based on the available literature on gastrointestinal angiodysplasia	29-60
B) the assessment of tissue levels of angiogenic factors found to be abnormal by the serum assessment	35-60
Chapter 3	
Predictive factors assessment; can clinical factors or angiogenic factors be used as diagnostic aids or provide information regarding prognosis in SBA?	61-80
A) systematic review on the use of clinical factors as predictive factors of disease presence, severity, and prognosis in gastrointestinal angiodysplasia	81-103

B) the assessment of serum levels of Ang-2 as a diagnostic tool/predictive marker in the diagnosis of SBA in a cohort of patients with obscure gastrointestinal bleeding	
Chapter 4 A broader assessment of circulating serum angiogenic factors in patients with SBA A) the use of a multiplex angiogenic array detector to assess 55 angiogenic factors B) the use of ELISA measurements of factors detected by the multiplex study C) the use of ELISA measurements of factors detected by the multiplex study in a cohort of patients with other causes of obscure gastrointestinal bleeding	104-131 116-131 132-137
Chapter 5 Assessment of tissue levels of angiogenic factors suggested to be involved in the pathophysiology of SBA formation by the results of chapters 2-4 A) qPCR assessment of relative gene expression levels of angiogenic proteins B) The use of Immunohistochemistry using commercially available antibodies to detect quantitative and qualitative expression and location of Ang-1, Ang-2 and Tie2	137-161 149-161
Chapter 6	

A) Summary of all findings	162-168
B) Future directions	168-175
References	176-219

Chapter 1

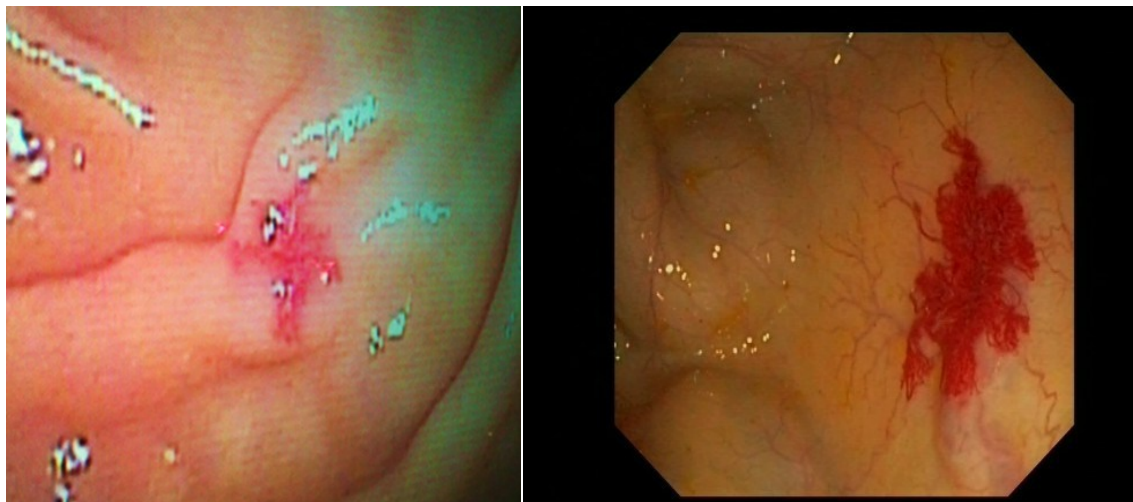
Publication:

1. Holleran G, McNamara D. An overview of angiodysplasia: management and patient prospects. Expert Rev Gastroenterol Hepatol. 2018 Sep;12(9):863-872.

Introduction:

Gastrointestinal angiodysplasias (GIAD) are generally diagnosed at endoscopy by the visualisation of a classical star or fern-shaped, red, mucosal vascular lesion ranging in size from 1mm-5cm at any location of the gastrointestinal tract (figures 1 and 2).

Figures 1 and 2. Endoscopic appearances of GIAD



They are found in the mucosal layer and are composed of abnormal clusters of enlarged, tortuous blood vessels. Histological examination often reveals dilated sub-mucosal veins

and arteries which lack a smooth muscle layer and have overlying mucosal thinning, leaving them weak and prone to easy bleeding (1). However, the pathophysiology behind their formation is currently poorly understood. Improvements in endoscopic techniques over the last two decades, particularly small bowel procedures such as capsule endoscopy (CE) and double balloon enteroscopy (DBE), have led to a rapid increase in the awareness of gastroenterologists that GIAD is a significant disease, with a noteworthy morbidity and mortality. However, so far unfortunately; this increased interest and recognition of the condition has not yet translated to improvements in patient outcomes. Currently the management of the condition continues to be based on empirical and “ad hoc” treatments, due to a lack of established guidelines regarding the condition.

This introduction summarises the currently available literature regarding the medical, endoscopic, radiological, and surgical management of GIADs, and more recent research advances in angiogenesis, which provides the basis for the body of this PhD thesis. Our hope is that by identifying the angiogenic factors involved in the pathophysiology of SBA formation, we may be able to find ways of using these factors as diagnostic and treatment targets which may ultimately improve the prognosis of patients affected by the condition in the future.

Pathophysiology of GIAD formation

Several hypotheses have been put forward regarding the pathophysiology of GIAD formation, which are generally related to hypoxia, and an aging process within the bowel wall. The original hypothesis proposed by Boley et al in 1977, was that GIADs developed due to chronic intermittent low grade obstruction of the sub-mucosal colonic

vessels due to increased bowel wall tensions, resulting in progressive vasculature dilatation and new collateral vessel formation (2). This was based on the law of Laplace, where the colonic wall thickness was thought to reduce as the bowel lumen radius increased, due to higher pressures and muscular contractions. This theory was further supported by Baum et al later the same year, when they proposed that the increased bowel wall tension also led to local ischaemia of the mucosa, causing thinning and ulceration of the mucosa overlying these poorly formed vessels, increasing their propensity towards bleeding (3). Although these hypotheses seem theoretically feasible for the development of lesions in the right colon where bowel wall tensions are at their highest, they do not support the development of small bowel lesions, which were not clinically recognised at that time. More recently in 1998, study by Roskell et al looked at levels of type IV collagen in areas of GIAD from resected colons, and found reduced levels compared to controls (4). They proposed that this deficiency may be secondary to cholesterol emboli and local ischaemia; however, their findings were found to be non-specific as decreased levels of collagen type IV were also present in half of the controls tested. In addition, both patients and controls had undergone colectomies for colorectal cancer, and although the tissue examined appeared to be unaffected, it is possible that the differences in collagen levels may have been due to a field effect associated with their malignancy rather than the angiodysplasia. There has been a long-standing clinical association with GIADs and aortic stenosis, defined as Heyde's syndrome, which was first described in 1958 (5). Although there is much controversy about the scientific evidence behind the association, there does seem to be some basis for the role of von Willebrand factor (vWF) deficiency in angiogenesis in general, and possibly in GIAD formation. The association of colonic angiodysplasias with cardiovascular and other hypoxia-based

clinical conditions, has more recently led to the hypothesis of a hypoxic trigger resulting in the release and imbalance of certain angiogenic factors as the mediators behind angiodysplasia development. In 1999 Junquera et al explored the possible role of vascular endothelial growth factor (VEGF) and other angiogenic factors in the formation of colonic angiodysplasias by assessing their levels in tissue specimens of colonic GIADs (6). The detection of elevated expression of VEGF in colonic angiodysplasia tissue compared to controls was replicated by Tan et al in 2012. However, the identification of potential angiogenic factors in GIAD pathophysiology is a relatively novel area of research at present. If confirmed it offers an exciting area for the future development of potential diagnostic and prognostic markers, and specific treatment targets which could revolutionise our approach to these patients. This hypothesis forms the basis for this PhD thesis, and will be introduced in further detail in a later section of this chapter.

Clinical presentation

Overall, GIADs are thought to account for 5% of gastrointestinal bleeding, and approximately 50% of cases of obscure gastrointestinal bleeding, defined as persistent overt or occult bleeding following a negative upper and lower endoscopy, or more recently, as suspected small bowel bleeding (7, 8). GIAD is estimated to account for 4%–7% of non-variceal upper GI bleeding, 35%–50% of small bowel bleeding and 3%–40% of lower GI bleeding (9).

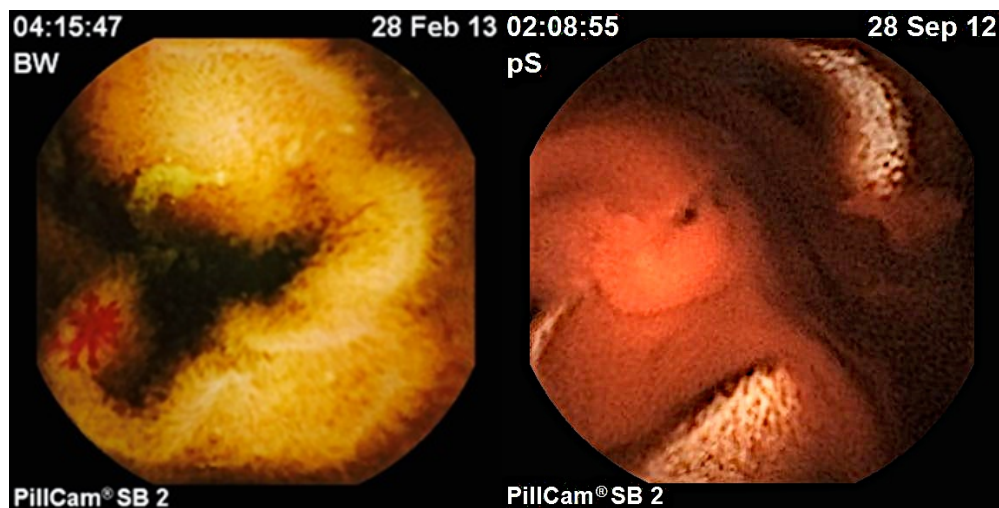
Location and diagnosis of GIADs: Lesions can be commonly seen at conventional endoscopy in the distribution of the colon (80%) and stomach (5%), where they are easily diagnosed and treated (10). However, lesions in the small bowel (accounting for

15%) present much more of a diagnostic and therapeutic challenge. It is difficult to accurately describe the overall distribution of GIADs, as the majority of studies reporting their prevalence do not include small bowel endoscopic assessment. Small bowel endoscopy is a relatively new endoscopic tool, introduced into clinical practice in the last 20 years, meaning that the presence and significance of small bowel lesions, including small bowel angiodysplasias (SBAs), is likely to be grossly underestimated by older literature (figures 3 and 4). A relatively recent study by Bollinger et al in 2012, aimed to assess the anatomical location of GIADs in a large group of patients with GIAD. They examined the entire gastrointestinal tract of 127 patients with bleeding GIADs, by performing conventional upper and lower endoscopy, and CE (11). They determined that GIADs were present in the jejunum in 80%, the duodenum in 51%, the stomach in 22.8%, the right colon in 11.4%, and the ileum in 5.7%, with the majority, 60%, having GIADs in more than one location. We performed a similar assessment of anatomical spread in our own cohort of 101 patients with SBA (shown in Table 1), who had all undergone pan-endoscopic assessment and found contrasting results with ileal lesions in 28%, and a predominance of isolated small bowel disease over co-involvement of upper and lower intestinal lesions. However, it is likely that we encountered some bias in our patient cohort, as all included patients had originally presented with obscure gastrointestinal bleeding, making them more likely to have SBA at the outset (12). Either way, both studies identify the high prevalence of SBA, and at least highlight the importance of full gastrointestinal tract evaluation in patients with confirmed GIAD, in the absence of larger studies at present. Indeed, both studies indicate that in the presence of colonic angiodysplasias which accounted for 11.4% and 10% in both studies respectively, small intestinal lesions were detected in over 80% of patients.

Table 1. The anatomical location of GIADs in our cohort of 101 patients with SBA

	Stomach	Duodenum	Jejunum	Ileum	Colon
% of patients with lesions N=86	7% N=6	28% N=24	83% N=71	28% N=24	10% N=9
% of overall lesions N=274	5% N=15	10% N=27	65% N=178	14% N=38	6% N=16

Figures 3 and 4. SBA at capsule endoscopy and the volume of small bowel bleeding from a different SBA lesion.



Natural history and clinical significance of GIADs: Presently, the clinical course and outcome of patients with GIAD ranges substantially, but can be poor in the most severely affected patients. The clinical presentation of GIADs is varied, and as with all causes of gastrointestinal bleeding, can be influenced by disease location. Gastric or small bowel lesions are more likely to present with melaena, while lesions in the lower gastrointestinal tract more likely present with haematochezia, or a mix of fresh and

altered blood. However, many patients with GIAD do not notice any overt bleeding, which presents a significant clinical challenge in relation to treatment and follow up. Very few studies have been carried out to describe the natural history of GIAD, however the available literature would suggest differences in the clinical significance based on location of disease, with lesions in the small intestine thought to have a poorer prognosis (7, 13, 14).

Although It is accepted that almost all lesions will bleed at some point, it has been reported historically that 90% of these bleeds will stop spontaneously and that most colonic lesions are detected incidentally, with the risk of bleeding from incidentally found lesions thought to be minimal (15). However, most of the available literature is based on single-centre reports of small heterogeneous patient cohorts, with variability in inclusion criteria, definition of bleeding events, and management protocols, making their interpretation difficult. It is known that GIADs are prone to recurrent bleeding, estimated in up to 70%, and although the risk of re-bleeding is thought to be highest within the first two years of diagnosis, there remains a possibility of re-bleeding at any interval from diagnosis. The impact of bleeding from GIADs varies between patients, however data on the clinical characteristics of lesions throughout the gastrointestinal tract has identified that affected patients are likely to be elderly and suffer from multiple comorbidities, many of which can be decompensated by recurrent gastrointestinal bleeding and anaemia, and that overall the quality of life of the more severely affected patients is poor (7, 16-18). In chapter 3 we report the results of a systematic review of the literature which we performed to acquire an up to date assessment of the clinical factors associated with the presence and severity of GIADs, and to determine whether any clinical factors could be used to predict re-bleeding episodes.

Management:

The management of GIADs is focussed upon the control of acute or active bleeding, the initiation of treatment to prevent further, or chronic bleeding, and the management of associated anaemia. Management approaches have varied in more recent decades due to increasing acceptance of GIAD as a significant diagnosis, with a substantial bleeding risk. This shift in acceptance has been due to several reasons. GIAD is a condition known to affect elderly patients, and due to advances in medical care in developed countries, particularly vascular medicine, our population is aging, with many patients dependant on the use of anti-thrombotic medications; known to accentuate gastrointestinal bleeding from any source. Furthermore, the more recent, albeit limited, natural history studies available suggest a higher risk of recurrent bleeding, particularly from lesions in the small intestine which were not recognised in prior decades. However, the management of GIADs still varies substantially, and this can be attributed in part to the lack of any accredited management guidelines for the condition. A recent Dutch study highlighted the size of this problem by performing a national web-based survey assessing the treatment approaches of gastroenterologists in 2015 (19). They noticed significant disparities in the knowledge of the disease, and treatment approaches amongst the participants, highlighting the need for a more unified approach to the condition.

Empirical treatment with oral or parenteral iron remains an option for patients who are asymptomatic with mild anaemia only, however most patients with clinically significant lesions require some form of targeted therapy including endoscopic, medical or surgical treatment, and despite this there are a cohort of patients with treatment-refractory

disease who may remain dependent on frequent blood transfusions to try and treat their anaemia (20).

Endoscopic treatment: The most convincing data regarding treatment outcome comes from the use of endoscopic ablation of GIADs via argon plasma coagulation (APC). APC involves the synchronised delivery of an electric current and the release of ionised argon gas, resulting in the transmission of electric current to an area of GIAD, without direct contact with the mucosa (21) (figures 5 and 6).

Figures 5 and 6 showing an SBA pre-treatment with APC and the appearances following ablation



APC has been shown to be extremely successful at treating GIAD in the colon, an Argentinian group compared the outcome following APC of colonic lesions in both patients with actively bleeding and non-bleeding lesions and found that re-bleeding rates of only 2% and 10% at 1 and 2 years follow up respectively (22, 23). Proximal lesions in the small bowel may be amenable to treatment via push enteroscopy, however; more distal lesions generally require the use of device assisted enteroscopy,

including single balloon (SBE), DBE and spiral enteroscopy (SE). The efficacy of DBE and APC for the treatment of small bowel lesions, has been associated with much higher re-bleeding rates than in colonic GIADs, of up between 20 and 64%, as summarised in table 2 below.

Table 2. The efficacy of DBE at reducing re-bleeding rates and RCC requirements and improving Hb levels at follow-up.

Study	Number of patients	Location of lesions	Mean follow up (months)	Re-bleeding rate %	Change in Hb	RCC requirements
May et al 2011	44	SB	55	44	Pre-7.6g/dL Post -11g/dL	Pre-60% Post-16%
Madisch et al 2008	23	SB	2	20	n/a	n/a
Arakawa et al 2009	19	SB	20	32	n/a	n/a
Landi et al 2002	18	SB	32	56	n/a	n/a
Gerson et al 2009	40	SB	30	45	n/a	n/a
Rahmi et al 2014	183	SB	12	35	n/a	n/a
Samaha et al 2012	98	SB	36	46	n/a	n/a

However, despite relatively high re-bleeding rates, it has been shown to have a definite beneficial effect on haemoglobin levels and transfusion requirements at follow-up, which are other markers of disease activity. The largest study to date, a multi-centre study of 183 patients in 2014, has identified that APC is a relatively short term measure, with re-bleeding rates of 34.5% at one year, and between 42%-56% beyond two years (24-26). In our own cohort of patients who underwent DBE and APC we detected re-bleeding rates of up to 78% after a mean follow-up period of 9.1 months (7).

The success of APC in SBA is thought to be inversely proportional to the number of lesions present, and due to the multiplicity of these lesions in the small intestine, often not all can be successfully treated endoscopically, even via a dual approach using antegrade and retrograde DBE (27). It is also recognised that along with the recurrence of previously treated lesions, new lesions grow sporadically at other sites in predisposed patients. For this reason, it was suggested by Bauditz et al that APC combined with an effective medical therapy would be the optimum treatment for SBA (28, 29). APC has been shown to be relatively safe in the small bowel, with reported complication rates of 0%-1.2% (30). This is comparable to safety reports from colonic APC, where pre-treatment submucosal injection with saline or a colloid solution has been recommended to reduce perforation rates (31, 32).

Other endoscopic haemostatic techniques including Nd:YAG laser therapy and band ligation have been used to control bleeding in the small bowel but there have been no randomised controlled trials, and case reports have suggested similar rates of re-bleeding to APC (33, 34). There are also concerns about the safety of using band ligation in the small bowel with perforation rates of 100% in one study using excised intestines,

though no safety studies have been performed on living specimens to date. The benefit of endoclips for haemostasis in SBA has not been formally evaluated, although there are a few case reports detailing their successful use in isolation or in synergy with APC.

Medical Treatments

Hormonal therapies: Oestrogen with or without progesterone has been used in gastrointestinal bleeding since the early 90s. Its exact mechanism of action in GIAD is unknown but it has been hypothesised that it may be due to a combination of a reduction in bleeding time, inhibition of angiogenesis, increased keratinisation and decreased mesenteric blood flow (35, 36). Earlier studies based on uncontrolled case series suggested a beneficial effect of hormonal therapy at reducing re-bleeding rates by up to 100% after a variable follow up period (37-39). However, this was not confirmed by larger controlled studies which showed no benefit at reducing re-bleeding rates or transfusion requirements despite combination therapy and escalated doses (40). There are also several unwanted side effects of hormonal therapy including thromboembolic events, weight gain, and an increased risk of endometrial cancer, which considerably restrict their use in clinical practice.

Thalidomide: Thalidomide has been shown to be effective at reducing re-bleeding in patients with obscure gastrointestinal bleeding in numerous case reports and case series. Its mechanism of action in GIAD is thought to be due to its inhibition of VEGF, and it has been shown to have a promising role in reducing re-bleeding episodes and transfusion requirements in refractory cases of GIAD (41-46). However, the case studies published all included small patient numbers and had very different measurements of outcome, making their comparison difficult. There has only been one randomised

controlled study to date, where Ge et al treated 52 patients with GIAD with oral thalidomide for a period of four months and found a considerably lower rate of re-bleeding in the treatment group compared to controls (54% vs 100%) after a mean follow up of 39 months. They also found a significant reduction in transfusion requirements and hospitalisations for the treatment group. SBA lesions have been shown to reduce in number, size and colour intensity using capsule endoscopy while taking thalidomide (47). There are many recognised side effects of Thalidomide, with high rates of occurrence in up to 50% of patients, which need to be considered prior to treatment, including; teratogenicity, hepatotoxicity and peripheral neuropathy. Given that most publications are case reports, there is a risk of publication bias and to date there has been only one published case report of treatment failure with thalidomide in angiodysplastic bleeding (48). However, it may represent a useful therapeutic option in a subset of patients who have failed or are unsuitable for endoscopic therapy.

Somatostatin analogues: Octreotide, a somatostatin analogue, has been validated for use in gastrointestinal bleeding due to varices, however; its use in bleeding due to GIAD is still being evaluated. Its effect is thought to be due to a combination of increased platelet aggregation, reduced splanchnic blood flow, increased vascular resistance, and down regulation of VEGF (49, 50). Its beneficial effect in GIAD overall has been reported by numerous case reports and cohort studies, and earlier reports showed that its administration as a subcutaneous injection three to four times daily reduced re-bleeding rates by up to 77% (51, 52). More recent studies reported the use of long acting intramuscular (IM) octreotide given as a monthly injection, and showed a significant reduction in transfusion requirements in patients with resistant GIAD, including those with small bowel disease (53-55). Our group published a pilot proof of concept study on

the use of long acting somatostatin analogues in patients with refractory SBA and demonstrated a decreased bleeding rate, decreased transfusion dependency and increase in mean haemoglobin levels in 70% of patients after a mean treatment duration of 8.8 months (56). To date no randomised controlled trials evaluating the role of long acting somatostatin analogues in GIADs have been published, although a Dutch group published their protocol and began recruitment in 2016 (57). A recently published double blinded randomised non-comparative study examined the use of 60mg of Pasireotide-LAR in 22 patients with refractory GIAD for 6 months, followed by a further observation non-treatment period of 6 months (58). They observed success rates (defined as the decrease in transfusion requirements by at least 30%) in 83% and 50% in the per protocol and intention to treat analyses respectively.

Tranexamic acid: Tranexamic acid works by preventing clot breakdown by inhibiting the action of plasmin which is needed for fibrinolysis. It has been reported in many clinical trials that tranexamic acid can reduce the risk of death in acute upper gastrointestinal haemorrhage (59, 60). However, most trials are of poor quality which has limited its widespread uptake into clinical practice among gastroenterologists. Concerns have been raised regarding the possible association of tranexamic acid with inducing thromboembolic or ischaemic events in patients and these potential adverse events have not been evaluated in gastrointestinal bleeding to date (61). A double blinded randomised controlled trial, the HALT-IT trial, is currently underway in the UK, which should have the power to determine the risk of these side effects in patients with gastrointestinal bleeding. A single case report has been published reporting the successful use of tranexamic acid as a rescue treatment in a patient with uncontrolled GIAD bleeding (62). In the meantime, tranexamic acid is not currently recommended for

treatment of SBA but remains a treatment option in acute life-threatening haemorrhage (63).

Bevacizumab: Bevacizumab, an antibody against VEGF has been used in small series of patients with Hereditary Haemorrhagic Telangiectasia (HHT) with some described benefit. A case report from France describe its use in a 47-year-old patient with SBA refractory to medical and endoscopic therapies who had persistent bleeding following surgical resection of bleeding GIADs (64). They reported significant improvements in haemoglobin levels and decreased blood transfusion requirements, along with a reduction in endoscopic lesions following induction and maintenance with Bevacizumab for a 14-month period.

Radiological embolization: Mesenteric embolization is generally used as a rescue therapy for gastrointestinal bleeding which cannot be controlled by endoscopic treatment or in patients who are unable to undergo endoscopic assessment due to acute bleeding causing haemodynamic compromise. GIADs can be recognised by a characteristic blush sign of slowly filling veins, which persists after other mesenteric veins have emptied, which is seen in >90% of cases (65). Embolization has been reported in several case reports and series of patients with bleeding from GIAD, however its use is limited to massive acute bleeding, due to the requirement of an active bleeding rate of >0.5ml/minute to be detected radiologically, which means that imaging can also be falsely negative (66). Recent advances in angiography using an intravenous methylene blue technique has shown some potential in improving the diagnostic yield and specificity of imaging.

Surgical management: surgical resection of localised areas of GIAD is reserved for patients with uncontrollable and debilitating bleeding which is unresponsive to other

treatment options. Due to the recent awareness of a likely field effect of angiogenesis it is important to fully assess the entire gastrointestinal tract to rule out synchronous areas of bleeding, although the possibility of sporadic growth of new lesions in other locations remains still post-resection of a current lesion. Often in the case of uncontrollable bleeding SBAs at enteroscopy there is the opportunity to inject tattoo ink into the submucosal space which can be seen at laparoscopy and identify a specific area for resection. However, in cases of obscure bleeding a combined approach of laparoscopy with intra-operative enteroscopy should be employed to identify the actively bleeding source with a view to preserving as much small intestinal tissue as possible.

Prognosis and patient prospects

Clinical presentation: The mode of presentation of GIADs is very variable, ranging from those who remain asymptomatic, to those with acute life threatening overt bleeding. Despite advances in diagnosis and treatment of GIADs, the prognosis for those with bleeding refractory to the above treatment options remains poor. The development of more specific treatments against GIADs has been limited by our still relatively poor understanding of the pathophysiology of GIAD formation. Lesions are known to bleed recurrently at unpredictable intervals, making decisions regarding treatment and follow up very difficult. It is also very difficult to counsel patients regarding their prognosis, and to make a risk benefit assessment regarding the continuation of anti-thrombotic medications without any validated prognostic indices. GIADs are known to be associated with high levels of morbidity and have an in-hospital mortality rate of at least 2%, with small intestinal lesions known to have a worse prognosis than lesions elsewhere (67).

Clinical characteristics: Information regarding the clinical characteristics of patients with GIADs has been collected and may facilitate the earlier detection and subsequent treatment of these patients if certain risk factors are identified. Some work on patients with colonic GIADs has shown that they occur more commonly in elderly patients, occurring rarely before 60 years, and have an equal gender incidence (68). Similarly, a Korean study looking at the clinical characteristics associated with gastric GIADs identified a mean age of 68 years with no gender predisposition (69). There have been suggestions of associations with cardiovascular disease, specifically aortic stenosis, in the form of Heyde's syndrome, although this association has been challenged in more recent years, and wasn't detected in our own assessment of SBA (70). In addition, case reports of GIADs overall suggest an increased prevalence of angiodysplasias in patients with chronic kidney disease (CKD) and an association with the use of anticoagulants and specific coagulopathies (71). Our own cohort of SBA is the largest to date and shows an association with increasing age, median of 71.5 years, female gender, and a higher prevalence of all co-morbidities. We found that specific clinical factors associated with SBA include cardiovascular disease, chronic respiratory disorders, CKD and previous VTE (72).

Clinical outcome studies: The natural history of GIADs is poorly defined in the literature, although it is reported that the risk of bleeding from incidentally found lesions is minimal and that in most cases of bleeding lesions, the bleeding will stop spontaneously (15). The location of GIADs seems to be associated with re-bleeding risk. A Korean study assessing upper GIADs identified a re-bleeding rate of 40%, which is consistent with a previous meta-analysis which showed re-bleeding rates of 36% over a median follow up period of 22 months (73). In contrast, a Japanese study estimated the re-bleeding rates

in colonic GIADs to be less than 1% over a 3 year follow up period, although these patients were also asymptomatic at diagnosis (74). More recently a different Japanese group identified re-bleeding in 31% of patients, most which were located in the right colon (75). SBAs appear to have the highest rates of re-bleeding, likely due to their inaccessibility. Our own group's natural history study is the largest study to date describing re-bleeding rates in predominantly SB lesions, of up to 80% after a mean follow-up period of 31.9 months (12). We found a median interval between diagnosis and the first re-bleeding event of 7 months, and 71% and 87% occurred within 12 and 24 months of diagnosis respectively, which was despite the use of some form of specific treatment in 57% of patients. Although limited in number, these natural history studies highlight the need for improved treatments and predictive indicators to improve patient prospects. Cox survival analysis was performed in our study to identify whether any clinical factors were predictive of an increased risk of re-bleeding, with the presence of multiple lesions and valvular heart disease found to be predictive of re-bleeding episodes on logistic regression analysis. However, due to an overall re-bleeding rate of 80% it was difficult to determine any further significant associations with disease severity or mortality. This is comparable to results obtained from studies of upper and lower GIADs where only multiplicity and size of lesions, along with the use of anti-thrombotics appears to be predictive of re-bleeding events. The mortality rate of GIADs is poorly defined in the literature, and estimated at 2% overall, although we identified a 3.5% mortality rate during follow up as a direct consequence of bleeding in our SBA cohort.

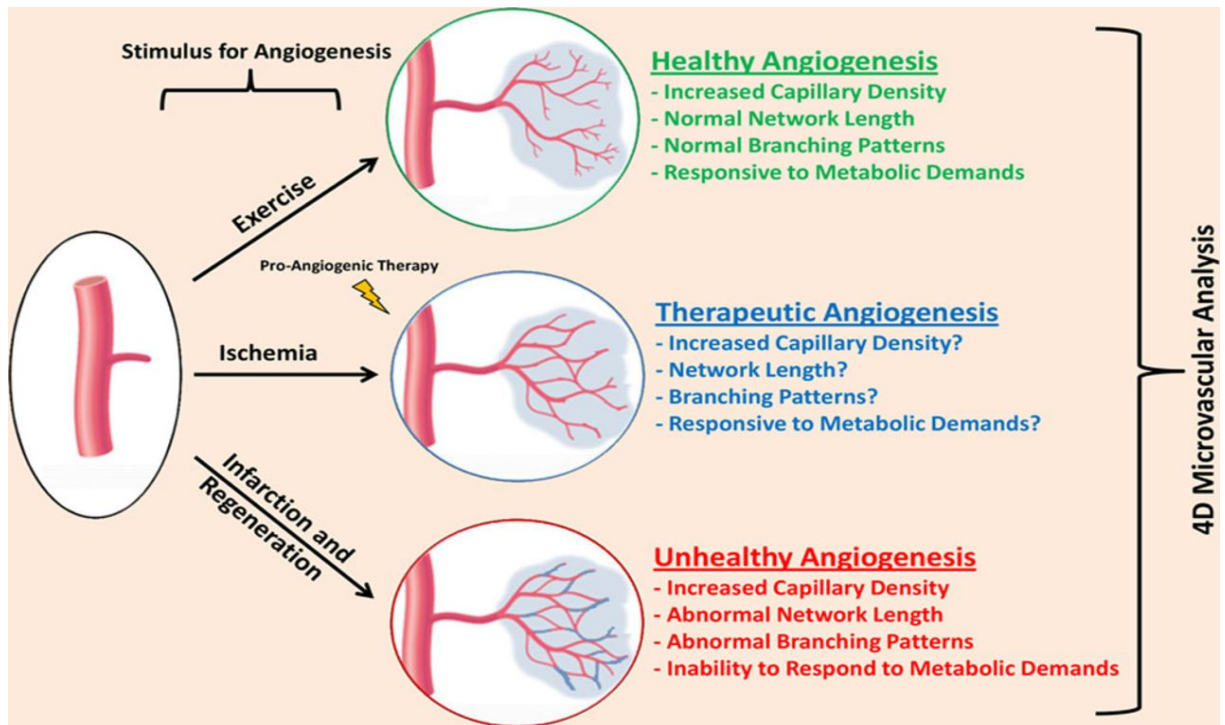
Overall, the quality of life for (QoL) patients with significant GIAD is thought to be poor, due to persistent anaemia which worsens their coexistent comorbidities. A Dutch study

performed a patient-reported outcome study to determine the QoL of 144 patients with GIAD using an age-matched unaffected control group, and to determine any perceived benefits of treatments on QoL (76). They observed a significant decreased QoL in patients with GIAD, which was influenced by their disease severity, defined by the need for treatment, frequent hospital visits or the presence of anaemia, when compared to unaffected controls.

The role of angiogenesis in GIAD:

Angiogenesis in disease: Angiogenesis refers to the process of new blood vessel development, specifically involving the migration, growth, and differentiation of endothelial cells, which line the inside wall of blood vessels. It is an essential factor for the growth of new normal tissue, healing and repair of existing tissue, and the growth and development of malignant tissue. Angiogenesis is known to be controlled by a delicate balance of a multitude of pro-angiogenic and anti-angiogenic factors (77). Although angiogenesis is essential for normal tissue growth and function, a switch in balance of these angiogenic factors can switch the process from a healthy to an unhealthy one as outlined in the below figure (78).

Figure 7. An image borrowed from a publication by Durand MJ, et al. (78) Illustrating the different types of angiogenesis and the triggers for each type.



In healthy tissue development angiogenesis occurs in a balanced and regulated fashion controlled by an intricate relationship between angiogenic stimuli and inhibitors. In unhealthy tissue development, an often-unknown stimulus leads to an imbalance between the pro and anti-angiogenic factors, leading to excessive, and disordered blood vessel growth. A shift from organised to disorganised angiogenesis is associated with many clinical conditions, including age-related macular degeneration (ARMD), Psoriasis, Rheumatoid arthritis, obesity, Diabetic retinopathy, ischaemic cardiomyopathy, and most malignancies (79). Angiogenesis plays a critical role in tumour development, as most solid-organ cancers cannot grow larger than a few millimetres without stimulating new blood vessel development to supply their increasing oxygen demands. Research in cancer angiogenesis has led to the development and approval of several anti-angiogenic therapies which will hopefully improve patient outcomes in cancer care. Furthermore, the valuable information provided by this research in other organ systems has led to a

greater understanding of angiogenesis overall, although this continues to evolve and develop in a rapid fashion. We have observed a substantial increase in the number of publications regarding angiogenesis over the timescale of this thesis preparation, and it is likely that many of the initial theories regarding angiogenic factor interactions and regulations have been modified since their initial discovery, and will continue to do so with increasing knowledge. The most widely studied angiogenic factor in medicine overall, and very importantly in gastrointestinal disorders, is VEGF. VEGF is released in response to hypoxia and has been identified as a central mediator in the early phases of angiogenesis (80). The identification of specific angiogenic factors involved in a specific disease process allows treatments to target a specific and hopefully earlier stage of disease development than other therapies, and the use of angiogenic factors as adjuncts to standard therapies in cancer therapy has shown significant results in survival outcomes so far. The identification of VEGF as a key factor in angiogenesis has led to the development of anti-angiogenic treatments directed against VEGF which have been used successfully in several conditions found to be associated with increased VEGF activity, including; ARMD, colorectal cancer, gliomas, renal cell carcinoma, and to control gastrointestinal bleeding in HHT (81-84). VEGF has a number of different subtypes and receptors which will be discussed in more detail in later chapters.

Angiogenesis in the pathophysiology of GIAD formation: Very little is known to date about the role of specific angiogenic factors in the pathophysiology of GIAD formation. The association of colonic GIAD with cardiovascular and other hypoxia-based clinical conditions led to the more recent hypothesis of a potential hypoxic trigger for the release and imbalance of certain angiogenic factors as the mediators behind GIAD

development. Prior to the initiation of this PhD study, only a small number of studies had been published detailing the potential role of a few angiogenic factors in GIAD or related conditions, which we used as a basis for our initial work. The first group to explore the role of angiogenic factors in GIAD were Junquera et al, who identified markedly elevated levels of VEGF and fibroblast growth factor (FGF) in colonic mucosal samples from patients with GIAD compared to healthy controls. VEGF is known to interact with many of the angiogenic factors which have been implicated in GIAD, including vWF and integrin. Furthermore, down-regulation of VEGF is the presumed mechanism of action of both Thalidomide and Somatostatin analogues, which have both been shown to have some success at reducing re-bleeding in GIAD. Newer targeted therapies against VEGF (e.g. Bevacizumab) are currently used in the hereditary form of GIAD, HHT. Further basis for the likely role of angiogenic factors in GIAD can be surmised from work done in HHT, where an association was found with reduced serum and plasma levels of Angiopoietin-2 (Ang-2) and soluble Endoglin (sEnd) (85). Endoglin is a transmembrane glycoprotein highly expressed by endothelial cells during angiogenesis (86). Endoglin has been used as a diagnostic and prognostic serum biomarker in certain angiogenic malignancies, as a target for a monoclonal antibody to aid diagnosis in radiological imaging and as a direct anti-angiogenic therapy. Ang-2 is member of the Angiopoietin family, and is known to have an important role in angiogenesis (87). Ang-2 is produced by endothelial cells and stored in Weibel Palade bodies within the cells. It is rapidly released in response to various angiogenic stimuli, and an excess of Ang-2 is thought to promote the production of unstable, immature blood vessels (88). It is currently an attractive focus for translational research due to the emerging availability of anti-Ang-2 therapies. vWF is also stored in Weibel Palade bodies of endothelial cells, and

appears to regulate the release of Ang-2 and integrin, through a complex interaction with VEGF receptor-2 (89). Outside of the identification of VEGF as a factor in colonic GIAD, and the identification of Ang-2 and sEnd as factors in the HHT GIAD model, no other angiogenic factors had been examined in GIAD prior to the development of this thesis proposal, and the undertaking of our laboratory experiments. Our over-arching aim was to identify the key angiogenic factors involved in the pathophysiology of SBA formation. By confirming angiogenic targets we hoped that we may be able to identify key targets for the development of diagnostic and prognostic markers, and uncover disease specific therapeutic targets.

Conclusion:

GIADs are an important cause of gastrointestinal bleeding and current treatments are lacking, particularly in the case of SBAs, where endoscopic treatment is limited. Improvements in the diagnosis and the development of targeted treatments could drastically improve outcomes for affected patients. A major limiting factor in progressing these improvements to date has been a very limited amount of published research regarding the role of angiogenesis in GIAD pathophysiology, and the identification of the key factors involved. The identification of key mediators of angiogenesis in other angiogenic-driven disease processes has led to the development of targeted anti-angiogenic medications, with improved patient outcomes. Over the course of the next four chapters we will investigate the role of angiogenesis in SBA pathophysiology and attempt to determine the key mediators of the condition, which we hope may be useful as diagnostic or prognostic markers, or as therapeutic targets in the future.

Chapter 2 – assessment of putative angiogenic factors in sporadic small bowel angiodysplasia

Publication:

Holleran G, Hall B, O'Regan M, Smith S, McNamara D. Expression of Angiogenic Factors in Patients with Sporadic Small Bowel Angiodysplasia. J Clin Gastroenterol. 2015 Nov-Dec;49(10):831-6

Oral Presentations:

1. United European Gastroenterology Week, Berlin, 2013. Elevated angiopoietin 2 levels in small bowel angiodysplasia; future biomarker or therapeutic target **(awarded National Scholar and Best oral presentation prizes and travel grant)**.
2. Irish Society of Gastroenterology Summer meeting 2014. Gene expression levels of angiogenic factors in small bowel angiodysplasia.

Poster presentations:

1. Irish Society of Gastroenterology Winter meeting 2013. Expression of serum angiogenic factors in patients with sporadic small bowel angiodysplasia.
2. Irish Society of Gastroenterology Winter meeting 2013. TNF- α dependent Angiopoietin mediated angiogenesis in sporadic small bowel angiodysplasia; novel pathophysiology and potential clinical marker.
3. United European Gastroenterology Week 2014. Gene expression levels of angiogenic factors in small bowel angiodysplasia **(awarded travel grant)**.
4. British Society of Gastroenterology, Liverpool, 2014. Tnf- α dependent angiopoietin mediated angiogenesis in sporadic small bowel angiodysplasia; novel pathophysiology and potential clinical marker.

Introduction:

Small bowel angiodysplasias (SBAs) account for up to 50% of cases of OGIB, and IDA and have become increasingly recognised in the last decade due to the wider use of small bowel CE (90, 91). GIADs are known to bleed recurrently and although 90% of bleeding resolves spontaneously, it recurs despite treatment in almost 70% within 2 years (92).

Due to the intermittent nature of bleeding, radiological imaging such as CT mesenteric angiography and red cell scans, are often falsely negative, leading to a delay in diagnosis (93-95). CE has been proven to be the most reliable diagnostic tool for SBA, but its availability at present, particularly in Ireland, is still relatively limited¹. Due to a combination of a lack of awareness of the significance of the condition, and the difficulties in diagnosis, Patients generally undergo many unnecessary and unyielding endoscopic and radiological procedures; with an average duration from presentation with OGIB to diagnosis of 18 months (97).

Without an accurate diagnosis, treatment is limited to empirical iron replacement and blood transfusions, which is generally suboptimal for correction of anaemia. Chronic IDA is associated with a high mortality and morbidity, and accounts for a significantly impaired quality of life, causing a huge burden on healthcare resources (98). Patients with angiodysplasias are known to be more elderly and have numerous co-morbidities, particularly cardiovascular diseases, which are exacerbated by their recurrent anaemia. There is known to be a worse prognosis for patients with small intestinal lesions, anti-coagulant use, and those dependent on red cell transfusions, and the condition overall carries an in-hospital mortality rate of 2% (99, 100). The development of more specific and effective medical therapies for SBA is restricted by a lack of understanding of the pathophysiology behind the condition.

Angiodysplasias are thought to occur due to a dysregulation in the angiogenic pathway. Angiogenesis is controlled by a delicate balance between the initiation and maturation phase of new vessel development; mediated by a number of angiogenic factors, which can be either pro-angiogenic or anti-angiogenic. It has been hypothesised that in angiodysplasias, a stimulus; either hypoxia or inflammation, leads to a shift in the angiogenic cascade, resulting in the overproduction of poorly formed blood vessels which lack a smooth muscle layer and are prone to easy-bleeding (101). However, to date, the specific angiogenic factors responsible for sporadic SBA formation have not been identified, although other forms of GIAD have been examined. Junquera et al identified VEGF as a key factor in sporadic colonic angiodysplasia formation, and Ojez-Fernandez et al identified abnormal serum levels of both sEnd and Ang-2 in patients with HHT, and Platelet Derived Growth Factor (PDGF-BB) has also been implicated as an important factor in angiogenesis in HHT (102, 103). Angiogenic factors are emerging as useful molecular targets and have been used successfully as diagnostic and prognostic biomarkers in a range of conditions, and for the development of specific anti-angiogenic therapies in several malignancies (104, 105).

Aim:

1. To identify any putative serum angiogenic factors associated with sporadic SBA, using the available literature on angiogenic factors in sporadic colonic angiodysplasia and HHT.
2. To determine whether the serum abnormalities in angiogenic factors found to be associated with SBA correlated with abnormalities in tissue levels of these angiogenic factors.

Methods:

1. Serum angiogenic factor measurement

Patient selection: A review of our small bowel CE database was undertaken and patients with a diagnosis of SBA were identified. At the time of reporting of capsule endoscopy a P value is assigned to any areas of possible angiodysplasia with P1 being equivocal and P2 being definitive for a diagnosis of angiodysplasia. Only patients with a definite (P2) diagnosis were included in the study. Any patients with a known diagnosis of HHT or those unable to provide informed consent were excluded. Patient information including: demographics, previous endoscopy reports, extent and severity of disease, past medical and medication history, were collected.

Control selection: Controls were identified from our Institution's colorectal cancer screening pilot database. We selected participants who had submitted two negative faecal immunochemical tests, meaning that they were not bleeding from their gastrointestinal tract, making an underlying diagnosis of asymptomatic SBA very unlikely. Information including: demographics, medical history and medication usage, was collected. A full blood count was checked at initial screening and any control who was anaemic, according to our laboratory cut off limits (Haemoglobin (Hb) <11.5g/dL for females and <13g/dL for males) was excluded from the study.

Patients and controls were contacted by telephone and invited prospectively to participate in the study. An information leaflet was then posted to any interested participants prior to meeting with them to collect blood samples. Ethical approval was obtained from our institution's ethics committee prior to commencing the study and written informed consent was obtained from all participants prior to inclusion.

Sample collection: Phlebotomy was performed in a standard fashion on all patients and controls and two serum samples (5mls each) were taken. Serum tubes were left to clot for at least 30 minutes prior to undergoing centrifugation for 15 minutes at 1000rpms. The resulting supernatant was then extracted, anonymised, and stored in aliquots at -80°C for batch analysis. An additional 10mls of plasma was taken at initial phlebotomy and sent to our institution's laboratory where a full blood count and renal profile were measured for comparison between the groups.

Selection of angiogenic factors: Based on the previous literature available on angiogenic factors in gastrointestinal angiodysplasias, we initially chose to measure serum levels of VEGF, sEnd, Ang-2, and PDGF-BB. Guided by the results of this initial analysis we proceeded to further evaluate the Angiopoietin pathway by measuring levels of Ang-1 which is known to be a competitive antagonist of Ang-2 for a shared receptor, and Tumour necrosis factor- α (TNF α) and vWF, which are both known to interact closely with this pathway. To ensure that the initial finding of significant differences in serum Ang-2 levels between SBA patients and controls was a reproducible result we repeated the measurement of Ang-2 in the second analysis.

Measurement of angiogenic factors: All measurements were performed according to the manufacturer's guidelines, using commercially available solid phase sandwich enzyme linked immunosorbent assay (ELISA) kits for VEGF (DVE00), sEnd, Ang-1, Ang-2, TNF- α , PDGF-BB, and vWF (R and D systems, Minneapolis, MN).

Assay procedure: The assay procedure for each of the ELISA kits was similar, with minor differences, depending on the antibody used, in serum sample dilutions, volume of sample or diluent used, the number of washes with buffer required and the incubation

with antibody durations. The general assay procedure according to the manufacturer's instructions was as follows:

1. Preparation of all reagents and samples– dilution of serum samples, wash buffer, calibrator and substrates. Reconstitution of the antibody and preparation of the antibody standard.
2. Specified volume of assay diluent was added to each well of the 96 well microplate.
3. Specified volume of standard or sample was added to each well, with each standard or sample being prepared in duplicate. The microplate was then covered with an adhesive cover and incubated for 2hrs at room temperature on a horizontal orbital microplate shaker set at 500 +/- 50rpm.
4. Following this, each well was aspirated and washed with the buffer solution a total of 4 times and then the microplate was dried.
5. Specified volume of the antibody was then added to each well, and the microplate was covered with an adhesive cover again and incubated for 2 hours at room temperature on the shaker.
6. Each well was then washed with the buffer solution 4 times again.
7. 200µL of substrate solution was then added to each well the microplate was then covered in tinfoil to protect it from light and incubated at room temperature on the benchtop for 30 minutes.
8. 50µL of stop solution was then added to each well, resulting in a colour change from blue to yellow.
9. The optical density of each well was determined within 30 minutes using a microplate reader set to 450nm absorbance.

10. The duplicate readings for each standard and sample was averaged and the average zero standard optical density was subtracted. A standard curve was then created using computer software supplied by the manufacturer and the concentration levels for each factor were generated based on this and multiplied by the dilution factor to give a final level.

The intra-assay coefficient of variation (CV) was calculated as an average of all the individual CVs for the sample concentration duplicates analysed by ELISA and were compared to the manufacturer's specifications in the table below.

Table 3. The Intra-assay coefficient of variation for each of the measured angiogenic factors for our results and as per the manufacturer's specifications.

	VEGF	sEnd	Ang-1	Ang-2	TNF- α	PDGF-BB
Our results						
Intra-assay CV	4.33 pg/ml	6.8 ng/ml	13.9 pg/ml	8.7 pg/ml	23.3 pg/ml	4.6 pg/ml
Manufacturer's specifications						
Intra-assay CV	5.75 pg/ml	4.85 ng/ml	7.9 pg/ml	7.8 pg/ml	4.4 pg/ml	9.8 pg/ml

2. Gene expression of angiogenic factor measurement

Patient selection: As per the serum assessment, patients with a definitive (P2) diagnosis of SBA were identified from our SBCE database. Patients awaiting treatment with APC via DBE were identified and invited to participate in the second part of the study. Agreeable patients were enrolled upon providing written informed consent on the day of their endoscopy procedure.

Control selection: Patients undergoing DBE for any indication were considered for participation in the study as non-bleeding controls. Any patient aged between 18-80 years of age, who had undergone a recent CE which was negative for SBA, was invited to participate in the study and agreeable patients were enrolled upon providing written informed consent on the day of their endoscopy procedure. Only patients who had macroscopically normal small bowel mucosa, confirmed by histologically normal biopsies were used as controls in the analysis.

Sample collection: Small bowel mucosal biopsies were taken from patients and controls during DBE. From patients with SBA, one standard biopsy (2mm in size) was taken from a single angiodysplasia lesion, and a further biopsy was taken from macroscopically normal adjacent mucosa. In controls, two small bowel mucosal biopsies were taken at random from an area of macroscopically normal mucosa. One of these was sent to the laboratory for histological examination and any controls found to have histological abnormalities were excluded from the study. All other biopsy samples were immediately placed in individual aliquots of RNAlater solution, and stored in a fridge overnight before being stored at -80°C for later batch analysis.

Selection of angiogenic factors for gene expression analysis: Based on the results of the putative serum angiogenic factor analysis we proceeded to measure gene levels of Ang-1, Ang-2 and their receptor Tie2. As we found significant differences in serum levels of TNF- α between patients and controls we included TNF- α in the gene expression assessment. Although we found no significant differences in serum levels of VEGF between patients and controls we felt it may still be valuable to measure tissue gene expression levels as VEGF is one of the most widely recognised angiogenic factors, and it

had been strongly implicated in the pathophysiology of colonic angiodysplasia in previous literature.

Measurement of gene expression:

1. Each biopsy sample was disrupted by the addition of a lysis buffer for 30 minutes at room temperature and then homogenized using the TissueRuptor (Qiagen, UK) at full speed for 30 seconds. The lysate was then centrifuged at full speed for 3 minutes and the resultant supernatant was used for further steps.
2. Total RNA was isolated using the RNeasy Midi Kit (Qiagen) according to the manufacturer's instructions. 70% ethanol was added to the lysate in an equal volume to the original volume of lysate supernatant obtained in step 1 and mixed by pipetting and the mix was transferred to an RNeasy spin column within a 2ml collection tube. This was then centrifuged for 15 seconds at >10,000 rpm and the flow-through in the collection tube was discarded. The RNeasy spin column was then washed three times using various volumes of two different buffers and dried by centrifugation at >10,000 rpm for 2 minutes. 30µL of RNase-free water was then added to the spin column membrane and centrifuged at 10,000 rpm for 1 minute.
3. Following this, a reverse transcription reaction was performed on each RNA sample using the Fermentas first strand cDNA synthesis kit (Thermo Scientific, MA). The RNA was added to primers, a buffer, an RNase inhibitor, a dNTP mix and reverse transcriptase. This was then mixed gently and incubated for 60 minutes at 37°C. The reaction was then terminated by heating to 70°C for 5 minutes, resulting in cDNA.
4. The resulting cDNA was then used in quantitative Polymerase Chain Reactions (qPCR) to determine the relative expression of Ang-1, Ang-2, Tie2, VEGF, and TNF- α using Taqman Gene Expression Assays, and the TaqMan Universal PCR Mastermix for qPCR

(Life Technologies, CA). technique. A PCR reaction mixed was made by combining the gene expression assay, the PCR mastermix, the cDNA, and RNase-free water. 20µL of this mix was then transferred into a well-reaction plate and each sample was prepared in quadruplicate. The well-reaction plate was then loaded into the PCR system (Applied Biosystems 7900HT Real-time PCR System, Thermo Scientific, MA), and the plate was run. An amplification curve was then generated for each sample.

5. Relative gene expression in patient samples compared to controls was calculated using the comparative cycle threshold (CT) method (automatically generated by the PCR system software according to the amplification curve), and were then normalised to the control gene GAPDH. Fold differences of each gene were expressed as a mean and compared between groups.

Statistical analysis: Results of all assays and patient demographics were expressed as a mean and/or median and compared between patients and controls using a student's t-test, Mann-Whitney, or Chi-squared test as appropriate, with a difference of <0.05 considered significant. All analyses were performed using SPSS version 20 (SPSS Inc, Chicago,IL).

Results:

Demographics: A total of 80 serum samples were measured for each factor, in 40 SBA patients and 40 non-bleeding controls. The mean age of the patient and control groups were 71 years (range 36 to 91 years) and 66 years (range 46 to 75 years), and 53.2% (n=22) and 51.1% (n=23) were female respectively. These differences were not statistically significant. The average Hb level for patients and controls overall was 10.8g/dL (11.5g/dl for males and 10.3 g/dL for females) and 12.6g/dL (14.1g/dL for males and 12.7g/dL for females) respectively.

Of the SBA patients, 90% (n=36) had isolated small bowel lesions with previous negative gastroscopies and colonoscopies. With regard to co-morbidities, there was no significant difference in the presence of a significant co-morbidity between the two groups, with 75% (n=30) in the patient group versus 58% (n=23) of controls (p=0.10) (table 4).

Table 4. The medical co-morbidities (and frequencies) present in SBA patients compared to controls.

	SBA Patient N=40	Control N=40	P value
Any Co-morbidity	30 (75%)	23 (58%)	0.10
Hypertension	26 (65%)	21 (53%)	0.26
Ischaemic heart disease	22 (55%)	5 (12.5%)	0.0001
Valvular heart disease	8 (20%)	1 (2.5%)	0.013
Chronic obstructive pulmonary disease	16 (40%)	5 (12.5%)	0.005
Chronic kidney disease	15 (37.5%)	3 (7.5%)	0.001
Anticoagulant use	4 (10%)	1 (2.5%)	0.04
Antiplatelet use	14 (35%)	11 (27.5%)	0.89

The most common co-morbidity in both groups was hypertension, present in 65% (n=26) and 53% (n=21) of patients and controls respectively, with no differences between groups (p=0.26). There were however, significantly higher rates of ischaemic heart disease (IHD) (p<0.001), valvular heart disease (VHD) (p<0.013), chronic obstructive pulmonary disease (COPD) (p<0.005), and chronic kidney disease (CKD) (p<0.001) in the patient group versus controls.

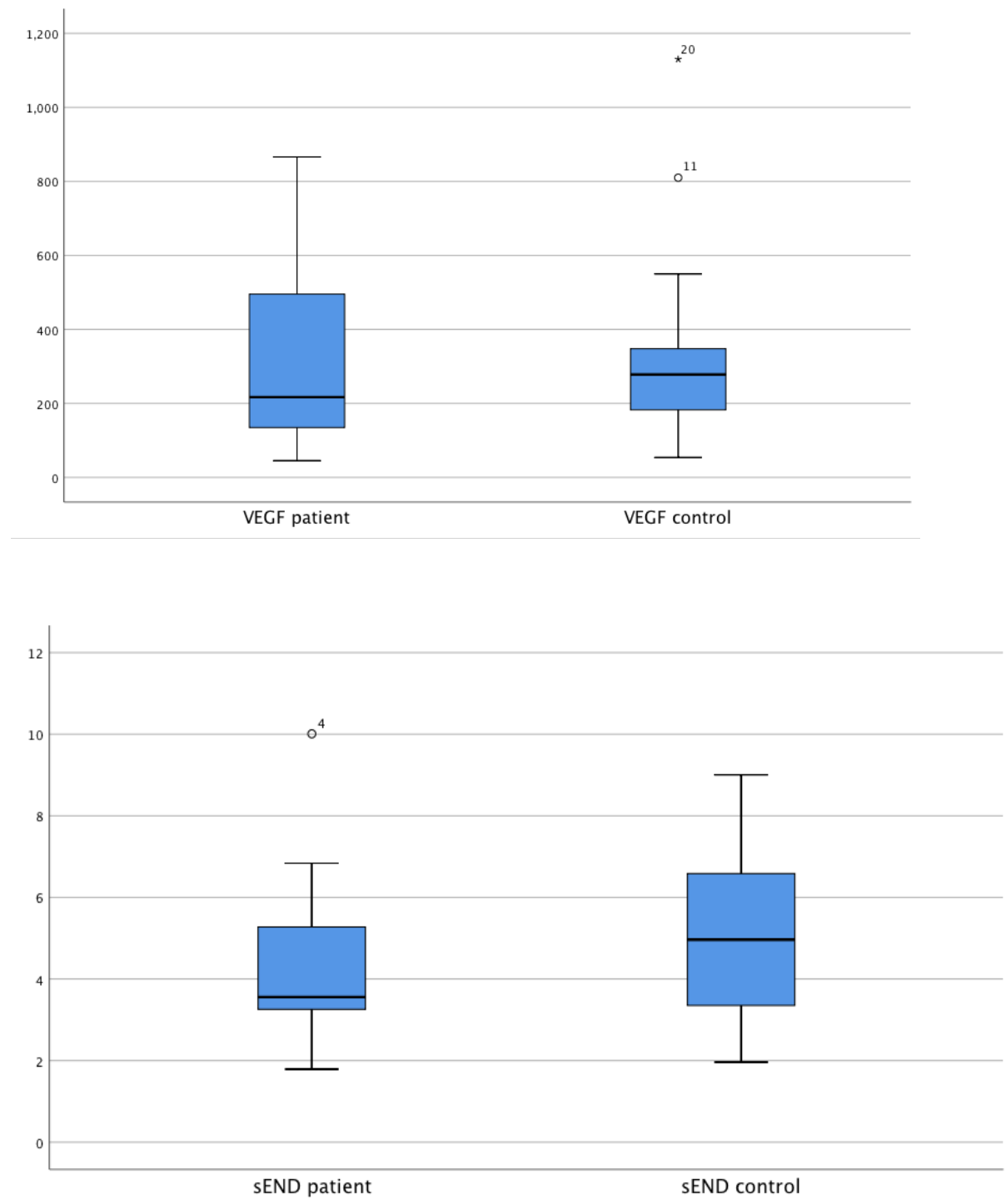
Angiogenic factors:

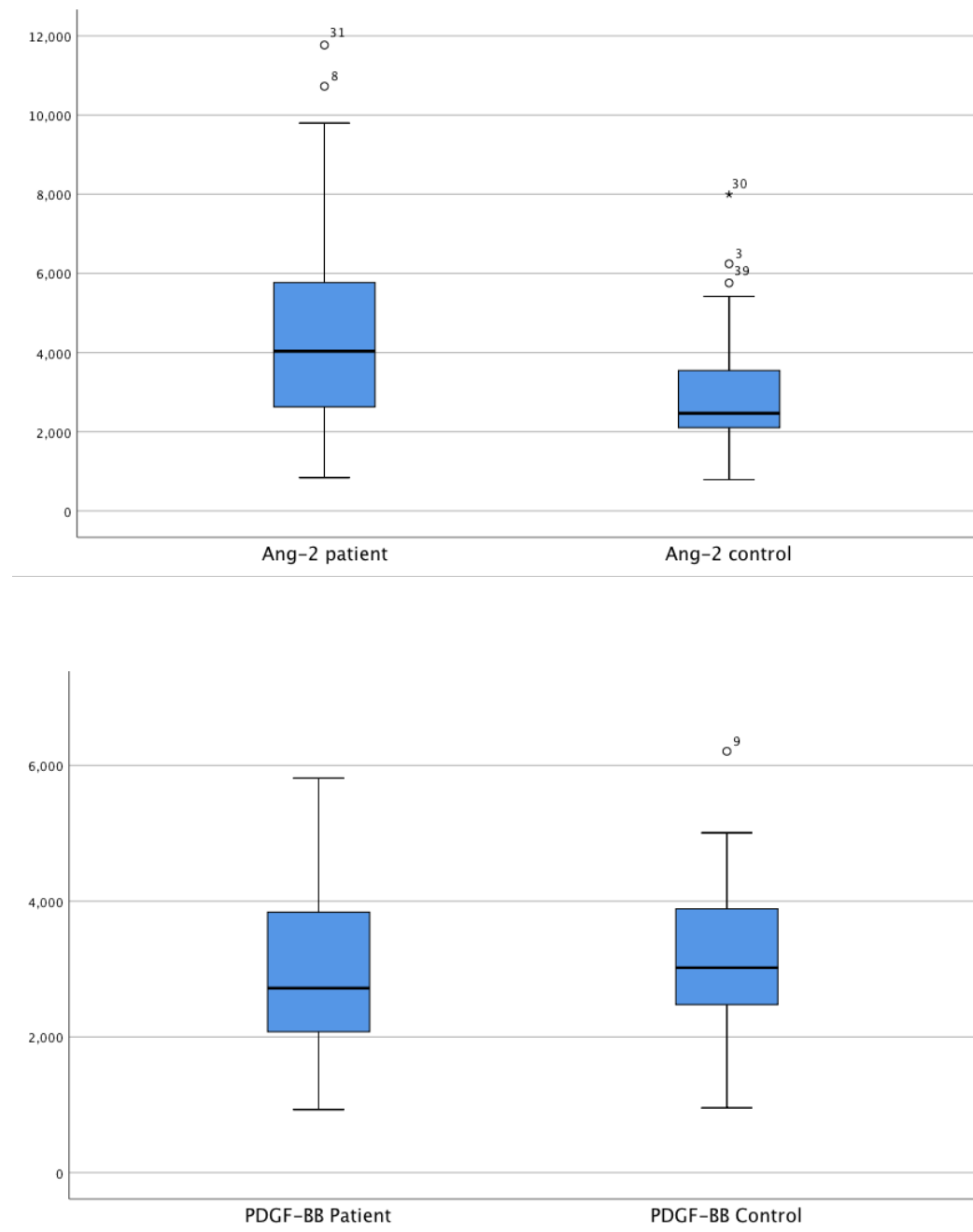
The mean and median serum levels and ranges of the first serum factor analysis of VEGF, sEnd, Ang-2, and PDGF-BB in SBA patients and controls is shown in table 5. Both mean and median levels of Ang-2 were found to be significantly higher in patients than in controls (4521.33 pg/ml and 4035.47 pg/ml vs 2973.5 pg/ml and 2506.78 respectively). Serum levels of VEGF, sEnd and PDGF-BB appeared to be lower in patients vs. controls with median levels of (254 vs. 272.25), (3.49 vs. 3.49) and (2727.03 vs. 2971.01) respectively, however, these differences were not found to be significant.

Table 5. Results of the serum ELISA measurements and statistical comparisons for VEGF, sEnd, Ang-2 and PDGF-BB in SBA patients and controls

SBA Patient	VEGF (pg/mL)	sEnd (ng/mL)	Ang-2 (pg/mL)	PDGF-BB (pg/mL)
Mean	355.88	3.71	4521.33	4229.25
Median	254.14	3.43	4035.47	2960.47
Range	45 – 929	1.79 – 10.01	842.40-11767.70	929.20 - 39978.42
Standard deviation	254.11	1.67	2568.57	6856.21
Control	VEGF (pg/mL)	sEnd (ng/mL)	Ang-2 (pg/mL)	PDGF-BB (pg/mL)
Mean	319.3	4.95	2973.5	3113.14
Median	272.35	3.49	2506.78	2971.04
Range	54 – 1130	0.06 – 9.0	792.05 – 7995.10	955.15 – 6208.40
Standard deviation	254.3	2.15	1481.31	1315.56
	VEGF	sEnd	Ang-2	PDGF-BB
T test				
p value	0.222	0.811	0.003	0.257
95% CI	(181.92) - 108.75	(0.154) - 2.32	(2437.20) - (658.46)	(4169.97) - 1937.75
One way Anova				
f ratio	0.256	6.108	11.98	0.539
p value	0.615	0.161	0.001	0.466

Figure 8. Boxplots showing mean serum levels of VEGF, sEND, Ang-2 and PDGF-BB in SBA patients and non-bleeding controls.





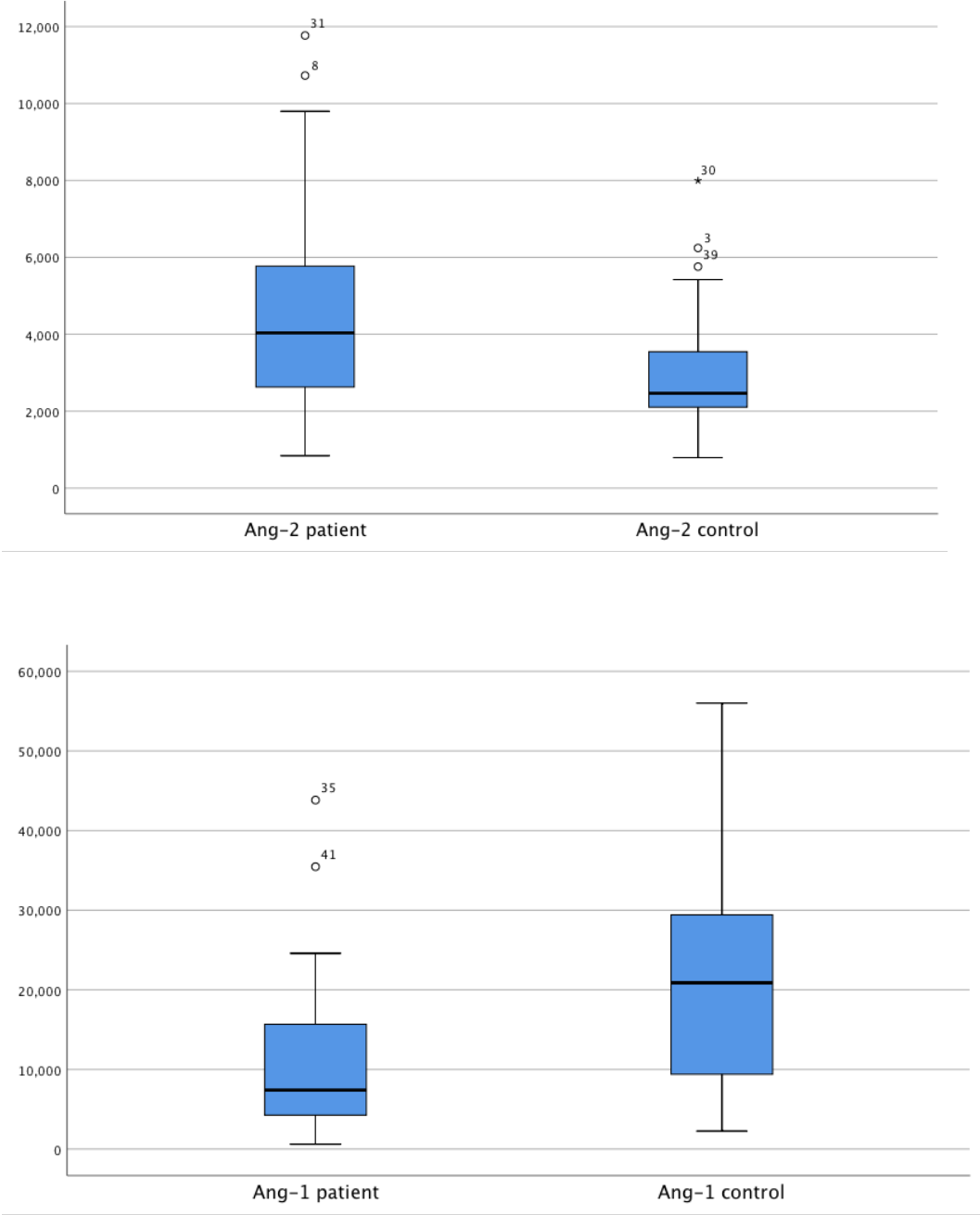
Based on this initial assessment we went on to measure serum Ang-1, TNF- α , and vWF levels in both groups. Mean levels of Ang-2 were again significantly higher in SBA patients compared to controls (4600.75 ng/ml vs 2973.49 ng/ml), while Ang-1 and TNF- α levels were significantly lower in SBA patients compared to controls (12665.72 ng/ml and 6.73 pg/ml vs 21147.97 ng/ml and 12.44 pg/ml respectively). There was no

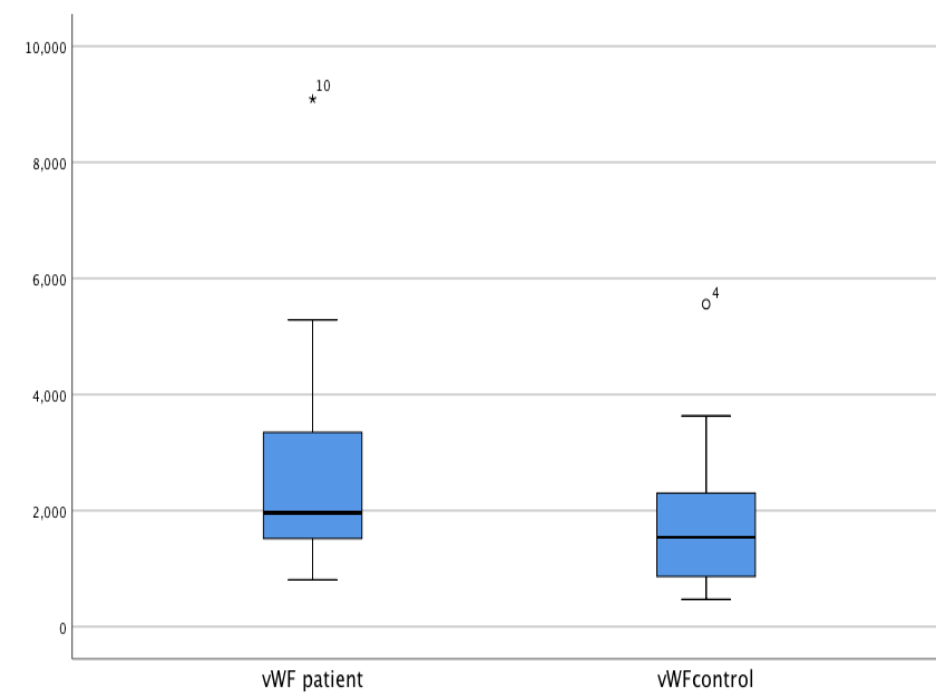
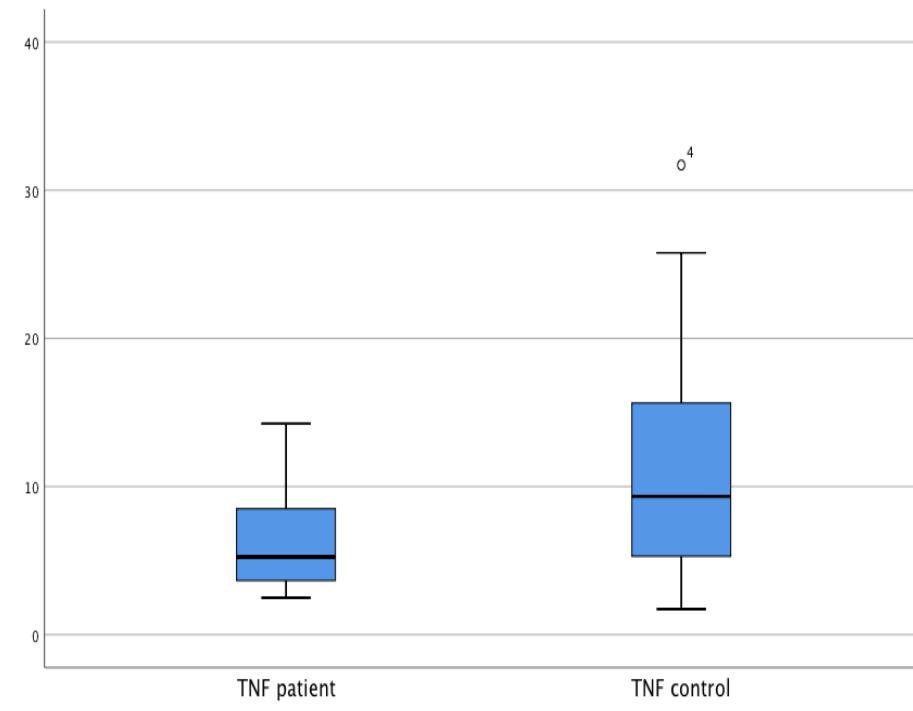
difference in the mean level of vWF between the groups. Results are shown in table 6 below.

Table 6. Results of the serum ELISA measurements and statistical comparisons for Ang-2, Ang-1, TNF- α , and vWF in SBA patients and controls

SBA Patient	Ang-2	Ang-1	TNF- α	vWF
Mean	4600.75	12665.72	6.73	2679.37
Median	4035.47	7850.75	5.46	1962.75
Standard deviation	2762.09	11276.95	3.83	1911.80
Range	842.40 – 11767.70	615.33 – 43833.51	1.97 – 16.54	805.77 – 53587.37
Control	Ang-2	Ang-1	TNF- α	vWF
Mean	2973.49	21147.97	12.44	1854.63
Median	2506.78	20968.69	9.49	1539.07
Standard deviation	1481.31	13008.16	9.11	1266.34
Range	792.05 – 7995.10	2253 – 56010.52	1.73 – 42	469.90 – 37092.55
	Ang-2	Ang-1	TNF- α	vWF
T test				
p value	0.0008	0.004	0.001	0.116
95% CI	693.78 – 2560.74	2721.74 - 14242.76	2.19 - 9.22	(213.30) – 1862.78
Mann Whitney U test				
U value	625	394	317.5	141
Z score	(2.86)	(2.84)	(2.89)	(1.58)
P value	0.004	0.005	0.004	0.183

Figure 9. Boxplots showing mean serum levels of Ang-2, Ang-1, TNF- α , and vWF in SBA patients and controls.



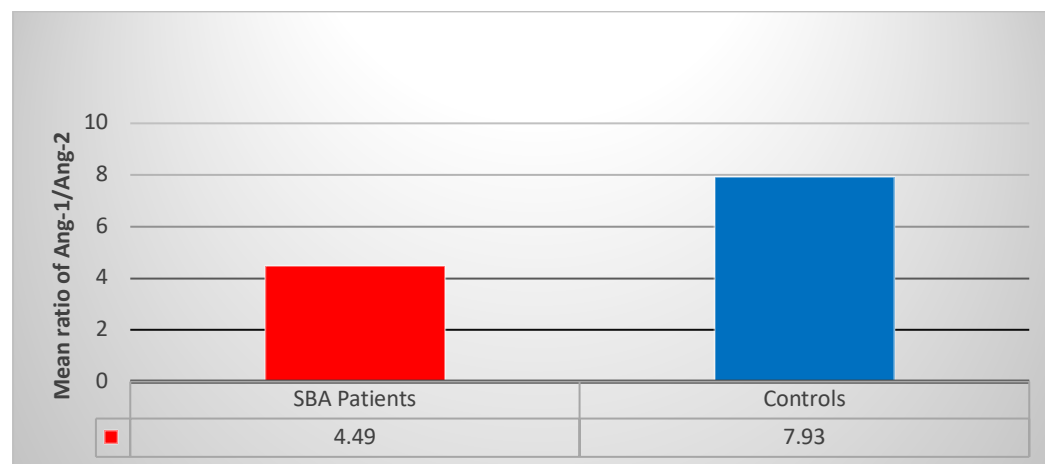


Overall, significant differences were found in median levels of Ang-2, Ang-1 and TNF α . Ang-2 levels were found to be significantly elevated in the patient group compared to controls, and Ang-1 and TNF- α levels were found to be significantly lower in patients

compared to controls. No significant difference was determined in levels of vWF between SBA patients and controls. In addition, serum levels of vWF appeared to be higher in SBA patients compared to controls, however; none of these trends reached statistical significance.

The ratio of mean levels of Ang1/Ang-2 was significantly lower in patients at 4.49 vs controls at 7.93 [$p=0.0272$, 95% CI (6.494) – (0.401)] , Mann-Whitney U value 311, z-score -3.53 (figure 10).

Figure 10. Ratio of mean serum levels of Ang-1/Ang-2 in SBA patients and controls



Angiopoietin levels according to anaemia and co-morbidities: At the time of Phlebotomy, 75% (n=30) of the patient group were anaemic, compared to none of the controls. When Ang-1 and Ang-2 levels were controlled for anaemia, there appeared to be a trend towards lower mean levels of Ang-1 and higher mean levels of Ang-2 in patients with anaemia (11431pg/ml and 4941.85pg/ml) compared to those without anaemia (16123pg/ml and 3374.56pg/ml) but these differences did not reach statistical significance, with p values of 0.264 and 0.83 respectively.

When Ang levels were controlled for co-morbidities, although there were a higher number of medical conditions in the patient group compared to controls, there was no significant difference in levels of Ang-1, Ang-2 or the Ang-1/Ang-2 ratio when controlled for the number of medical conditions per patient, using Fisher's scoring algorithm. Controlling of angiogenic factors for comorbidities was difficult as 50% of patients had at least 2 significant conditions, compared with only 11% of controls. However, when patients with CKD were removed, Ang-2 levels were still significantly higher in patients than controls with mean levels of 4186.96 pg/mL in patients compared with 2784.12 pg/mL in controls ($P=0.002$; 95% CI, 531.72 - 2273.95). Levels of Ang-2 seemed to be slightly higher in SBA patients with CKD, but this difference was not statistically significant, with mean levels of 5476.47 pg/mL ($SD=2843.68$) in patients with CKD, compared with 4186.96 pg/mL ($SD=2389.52$) in patients without CKD ($P=0.161$).

Angiogenic Factor Gene Expression Levels in Tissue:

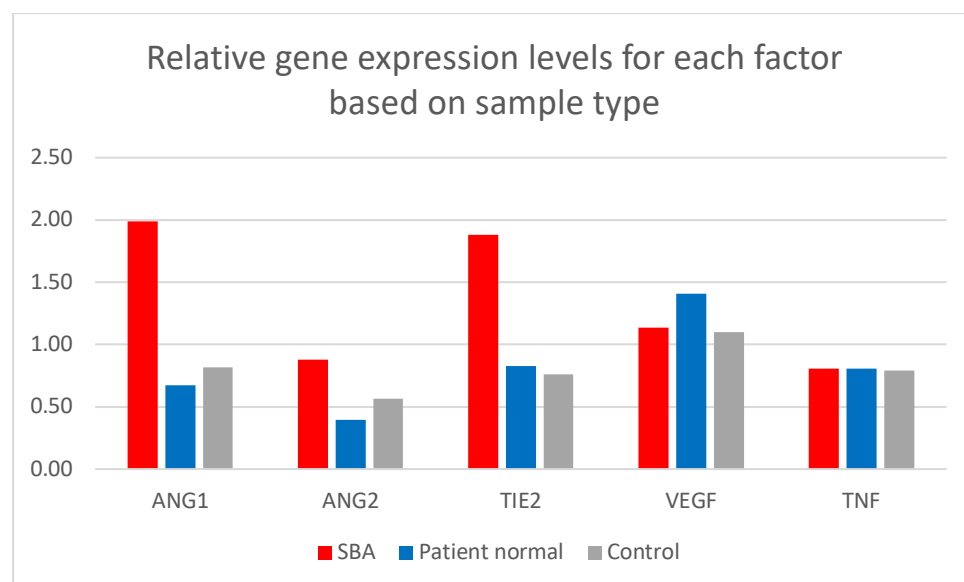
In total, 20 biopsy samples were collected. This included 9 patients – 7 with both angiodysplasia tissue and tissue from adjacent normal mucosa, and a further 2 with solely angiodysplasia tissue. Biopsies were taken from normal mucosa in 4 controls. Detectable levels of genes encoding Ang-1, Ang-2, Tie2, TNF- α and VEGF were found in all biopsy samples. Significantly higher levels of Ang-1, Ang-2 and their receptor Tie2 were found in SBA samples compared to both the macroscopically normal patient tissue and to non-bleeding controls (table 7). There were no differences in gene expression levels of VEGF or TNF- α between all 3 groups when tested using a student's T test and one way anova testing. There was no difference in gene expression levels for any of the

five factors when compared between macroscopically normal patient tissue and non-bleeding controls.

Sample	Ang1	Ang2	TIE2	VEGF	TNF
SBA 1	1.12	0.94	1.18	1.28	0.73
SBA 2	1.70	0.67	1.80	0.84	0.75
SBA 3	0.94	0.61	0.98	1.21	0.73
SBA 4	4.79	1.79	4.03	2.33	0.53
SBA 5	1.22	0.94	2.42	1.11	1.42
SBA 6	1.21	0.89	1.86	0.94	0.74
SBA 7	1.49	0.82	1.17	0.77	0.52
SBA 8	3.92	0.46	2.14	1.17	0.61
SBA 9	1.51	0.78	1.36	0.58	1.25
Mean	1.99	0.88	1.88	1.14	0.81
	ANG1	ANG2	TIE2	VEGF	TNF
Normal 1	0.66	0.40	0.72	0.67	0.81
Normal 2	0.59	0.38	0.61	0.99	0.63
Normal 3	0.59	0.04	0.46	4.50	0.66
Normal 4	0.99	0.42	1.27	0.86	1.34
Normal 5	0.69	0.24	1.03	0.85	0.53
Normal 6	0.98	0.84	1.19	0.79	0.74
Normal 7	0.19	0.45	0.52	1.18	0.93
Mean	0.67	0.40	0.83	1.41	0.81
	ANG1	ANG2	TIE2	VEGF	TNF
Control 1	1.00	1.00	1.00	1.00	1.00
Control 2	0.89	0.76	1.14	1.30	1.02
Control 3	0.90	0.23	0.50	1.10	0.54

Control 4	0.48	0.26	0.40	1.00	0.60
Mean	0.82	0.56	0.76	1.10	0.79
T test SBA vs Normal					
	ANG1	ANG2	TIE2	VEGF	TNF
p value	0.0266	0.011	0.0138	0.593	0.983
95% CI	(2.461) - (0.177)	(0.835) - (0.1289)	(1.856) - (0.251)		
One way Anova test					
	ANG1	ANG2	TIE2	VEGF	TNF
f ratio	4.679	4.509	6.647	0.269	0.006
p value	0.023	0.026	0.007	0.077	0.1

Figure 11. Relative gene expression level for each angiogenic factor in SBA patients, patient normal tissue and in healthy control tissue.



Discussion:

These findings constitute the first published study, to our knowledge, to explore the role of serum or tissue angiogenic factors in sporadic SBA. The very limited previous work around GIAD pathophysiology has mainly been based on a presumption of VEGF involvement, and carried out in very small patient numbers. The focus on VEGF was mainly driven by observed changes in circulating VEGF levels following treatment with empiric treatments, rather than being directly attributable to a role in the pathophysiology of SBA formation, although most of these treatments are known to target VEGF for their therapeutic effect, making it difficult to determine a causal association with GIAD (106, 107). Through our identification of decreased serum Ang-1 and elevated Ang-2 levels, we have determined an association between the Angiopoietin pathway and sporadic SBA. The detection of a significant difference in mean ratios of serum levels of Ang-1/Ang-2 in SBA patients compared to controls, supports our hypothesis that these factors may be directly related to the pathophysiology of SBA formation. The further elucidation of elevated gene expression of Ang-1, Ang-2 and their receptor Tie-2 in angiodysplasia tissue further supports this role of the angiopoietin pathway in SBA pathophysiology, as a potential regulator of angiodysplasia formation. This novel identification is a necessary rudimentary step towards learning more about the pathophysiology behind SBA formation.

The Angiopoietin pathway, identified in the mid-1990s, is currently an attractive area of focus for translational research, particularly in Oncology, as several anti-angiogenic therapies directed at the Angiopoietins have recently become available. Of the four members of the Angiopoietin pathway, Ang-1 and Ang-2 are the two most well characterised, and are both known to have an important role in angiogenesis (108, 109).

Ang-1 is expressed by smooth muscle and other perivascular cells, and is thought to promote stabilisation and maturation of new blood vessels by binding with the receptor Tyrosine Kinase 2 (Tie2)(110, 111). Ang-2 is almost exclusively produced by endothelial cells and stored in Weibel Palade bodies within the cells, and is rapidly released in response to various angiogenic stimuli (112). Ang-2 is a competitive antagonist of Ang-1 for binding of the Tie2 receptor within endothelial cells, while Ang-1 is an agonist for the receptor (113). In an over-simplified model of explanation, Ang-2 is thought to have its vessel destabilising role predominantly by preventing binding of Ang-1 to Tie2, and thereby preventing the vessel maturing action of Ang-1 (114). The Tie2 receptor is expressed by endothelial cells, haematopoietic cells and tumour cells (115). Tie2 expression is known to be increased in larger vessels over smaller ones, however its upregulation has specifically been associated with the pathological angiogenesis of small vessels of tumours (116, 117).

Much of the insight into the specific roles of Ang-1, Ang-2 and Tie2 has been generated using mouse studies, as each factor has been shown to have threshold quantities associated with fatality at various levels of embryonic development, making their specific study in humans unachievable (118). Using knock-out mouse studies, various groups have demonstrated that Ang-1 deficiency results in fatality at a very early point of embryonic development. A similar outcome has been demonstrated in Tie2 deficiency, although at a much later point of development. Fatalities in both instances have been shown to be due to disturbances in vascular development within the myocardium. Ang-1 overexpression has also been associated with fatality in mice, again due to poorly developed cardiovascular tissue, although at a later stage of development than Ang-1 deficiency (119). Ang-1 overexpression in humans conversely has not been

associated with either cardiovascular developmental disorders or death. Similar vascular abnormalities have been observed in Ang-2 transgenic mice, with Ang-2 overexpression demonstrating fatalities comparable to that of Ang-1 and Tie2 deficiency (120). This hypothesis of an imbalance of the agonist/antagonist relationship between Ang-1 and Ang-2 for Tie2 binding in favour of Ang-2 overexpression leading to dysregulated vascular development has been proven in mouse-endothelial cells in various anatomical locations (121). Our results, showing elevated serum levels of Ang-2 and decreased levels of Ang-1 in patients with SBA are in keeping with the defined roles of each factor. Our observed reduction in the ratio of circulating Ang-1/Ang-2 in SBA patients also supports the hypothesis that a dysregulation in the Angiopoietin pathway is a key factor in the pathogenesis of SBA formation. Confirmation of elevated relative expression levels of genes encoding Ang-1, Ang-2 and Tie2, but not of VEGF or TNF- α , adds further weight to our hypothesis.

However, these findings are a rudimentary step towards defining the pathophysiology of SBA formation and a few limitations need to be acknowledged. Firstly, elevated serum Ang-2 levels are not specific to SBA, and have also been reported in patients with sepsis and CKD, which suggests that it may be representative of a systemic inflammatory response rather than a causal association with angiodysplasia formation (122, 123).

Patients with SBA are known to have numerous co-morbidities, including CKD, and other ischaemic conditions. An analysis of our SBA patient group, after controlling for hypertension which affected both groups relatively equally, showed that 76% of patients had at least one further significant condition, with 15% having four or more conditions, compared to 29% and 0% of controls with one and four or more conditions respectively. However, when Ang-1 and Ang-2 levels and their resultant ratio were controlled for the

presence of, or number of, significant medical conditions, there was no statistically significant association. This suggests that despite the association of Ang-2 with other hypoxic conditions, it may still be clinically useful as a diagnostic and prognostic biomarker in SBA, although further analysis with larger patient numbers will be required to assess this fully.

Another limitation of this study which came to light in the analysis of our results was the identification of anaemia as a possible confounding factor. We compared a group of SBA patients, of whom the majority (75%) were anaemic, with a control group who were not. The role of the Angiopoietins in angiogenesis, particularly what triggers their production and release; is still being defined and it could be argued that anaemia alone, irrespective of the cause, may precipitate imbalances in Ang-1 and Ang-2 release. Our sub-group analysis of mean levels of Ang-2 in SBA patients who were anaemic (n=30) versus those who were not (n=10), suggested a trend towards higher levels in the anaemia group, although this did not reach statistical significance. Further work is required to compare levels of Ang-1 and Ang-2 in patients with other causes of anaemia and gastrointestinal bleeding, to determine whether the association is specific for SBA. However, as the first study to identify a link between SBA and a specific angiogenic factor, it does suggest a legitimate pathway for angiodysplasia formation and provides direction and targets for future research on the condition.

An interesting finding in our study was that in contrast to the work done by Junquera et al on sporadic colonic angiodysplasias, we found no association between VEGF and sporadic SBA. VEGF has been one of the most widely studied angiogenic factors to date with a longer recognised history than that of the Angiopoietins. It has been become accepted that the development of healthy and pathological vasculature is dependent as

some level on the presence and action of VEGF (124). The tyrosine kinase VEGF receptors are located exclusively on endothelial cells, and VEGF has a reportedly high affinity for endothelial directed action as a result (125). The recognition of VEGF as an important mediator of angiogenesis has been established due to the detection of overexpression of VEGF and its receptors in a variety of angiogenic malignancies, and in hypoxia driven systemic conditions including myocardial ischaemia, pulmonary hypertension and proliferative retinopathy (126-130). Junquera et al performed immunohistochemistry to determine a relative quantity of VEGF expression in surgically resected specimens of colonic angiodysplasia, colorectal cancer, and macroscopically normal colon. They determined that colonic angiodysplasia had comparable levels of overexpression of VEGF to colorectal tumours, and that normal colonic tissue did not have readily detectable levels of VEGF on immunohistochemical examination. Following this, immunohistochemistry using antibodies to VEGF was also performed during a study by Tan et al, who similarly reported overexpression of VEGF in mucosal biopsy specimens from angiodysplasias detected at endoscopy and compared to macroscopically normal tissue from the same patient, where undetectable levels of VEGF were observed (131). The same group had previously observed decreased circulating levels of VEGF in patients with angiodysplasia following treatment with Thalidomide (46). However, as a recognised mode of action of Thalidomide is the down-regulation of VEGF, the observed decreased circulating levels of VEGF cannot be associated with a causal effect in angiodysplasia development. Furthermore, elevated circulating levels of VEGF have been reported in a cohort of patients with HHT affected by gastrointestinal bleeding (132). However, due to the presence of vascular malformations in many different organs in HHT patients, and a recognised alteration in

genes encoding certain angiogenic factors, this observation cannot be directly translated as a factor in sporadic angiodysplasia development. We observed no differences in serum levels of VEGF between a larger number of SBA patients compared to controls than previously reported. As this finding was out of keeping with the above earlier publications, and due to the obvious important role of VEGF in vascular remodelling of any type we wanted to be certain that this was not a chance finding and for this reason we chose to include VEGF in the tissue measurement of gene expression levels. The confirmation of similar expression levels of the gene encoding VEGF in SBA tissue compared to both macroscopically normal tissue from SBA patients and non-bleeding controls validated our serum findings and allowed us to disregard the previously reported role of VEGF as a key mediator of angiogenesis in SBA and move forward with our hypothesis of an Angiopoietin-driven pathway. While still acknowledging VEGF as a hugely important regulator of angiogenesis we felt that our contrasting data could partially be explained by the fact that that 90% of our patients had isolated SBA, and no studies to date have looked at angiogenic factors in this location alone. It has been suggested that small bowel lesions may behave differently in clinical terms, to lesions located elsewhere in the gastrointestinal tract, and perhaps the differences in the angiogenic factors may also represent a slightly different underlying pathophysiology (133).

We also interestingly found no association with SBA and alterations in serum levels of sEnd, PDGF-BB, or vWF. Endoglin (End) is a transmembrane glycoprotein expressed on activated vascular endothelial cells and is an accessory protein of the transforming growth factor (TGF) receptor (134). Mutations in the gene encoding End, its downstream signalling mediators, or endoglin-associated protein activin-like kinase (ALK-1) has been

associated with HHT (135-137). Immunohistochemistry studies have shown that End is undetectable in normal tissues and identified the presence of End as a poor prognostic factor for patient survival and response to chemotherapy in various malignancies (138-141). sEnd, the soluble form of End, is thought to be generated by proteolytic cleavage of the membrane anchored endoglin, a TGF- β auxiliary receptor involved in angiogenesis and vascular remodelling and development, and is detectable in serum (142). Elevated serum levels of sEnd have been associated with angiogenic driven conditions such as pre-eclampsia, myocardial ischaemia and metastatic malignancies (143, 144). However, decreased serum levels of sEnd have been shown to be associated with HHT in a study by Ojeda-Fernandez et al, presumably due to the genetic mutations associated with the condition (145). Due to the similarities in the endoscopic appearances of vascular lesions in patients with sporadic SBA and HHT, we decided to measure serum levels of sEnd in our SBA cohort to see if it may be linked to the pathophysiology of SBA formation. However, we found no significant differences in serum levels of sEnd between patients and controls, with our mean levels for both groups being directly comparable to the healthy control group used in the above publication. Ojeda-Fernandez et al also found differences in circulating levels of Ang-2 in HHT patients, which prompted us to also include Ang-2 measurement in our initial putative assessment which did yield significant findings in our SBA cohort compared to controls. However, in contrast to the lower circulating levels of Ang-2 in HHT patients compared to controls, we have consistently demonstrated higher levels of Ang-2 in our patient group, with a reciprocal decrease in Ang-1 levels. This finding suggests that the gastrointestinal angiogenesis in HHT is likely to have a different underlying pathophysiology to that of sporadic SBA, and allows us to discontinue further experimental comparisons between the two conditions.

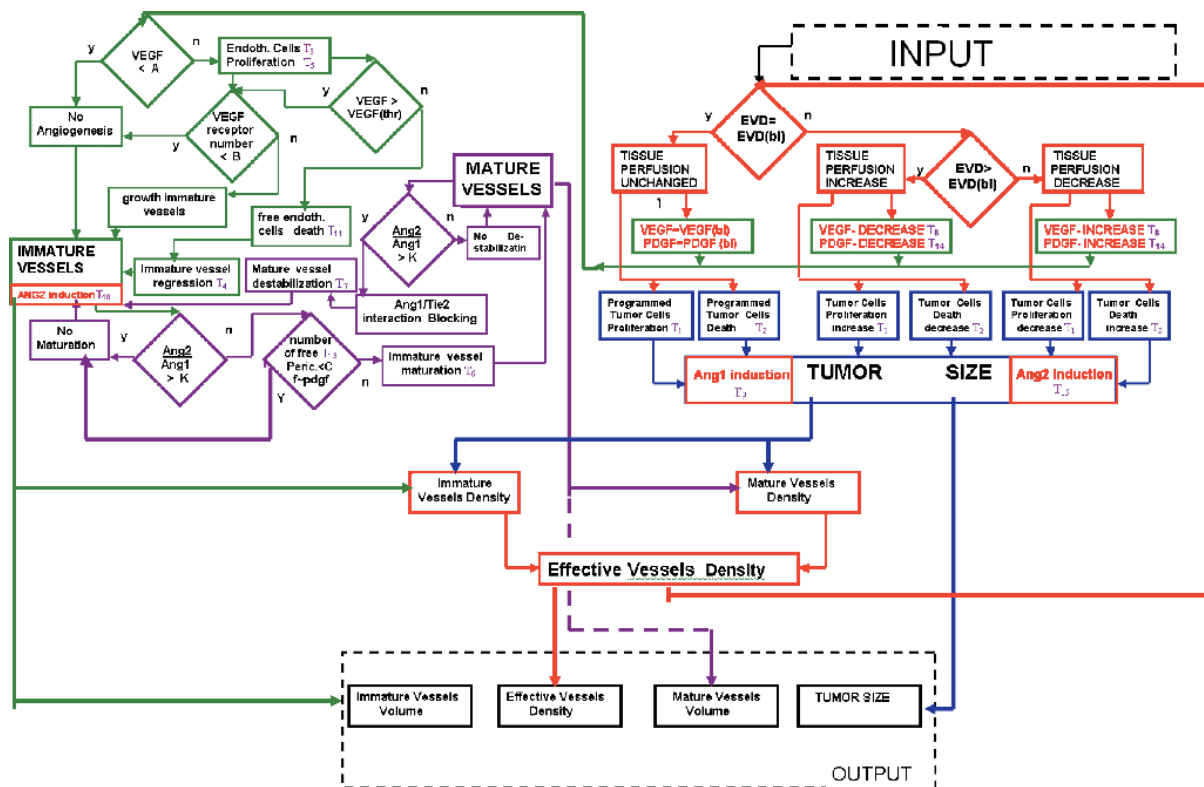
Another clinical condition often associated with the development of multiple gastrointestinal angiodysplasias is von Willebrand disease, or von Willebrand Syndrome, a similar condition caused by an acquired deficiency of vWF. vWF is a multimeric glycoprotein synthesised by endothelial cells that mediates the adhesion of platelets to the sub endothelium of damaged blood vessels under conditions of high shear (146). Large vWF multimers are stored in Weibel-Palade bodies and a deficiency of these large multimers occurs in von Willebrand Syndrome type 2a, which is associated with bleeding from gastrointestinal angiodysplasias (147, 148). Historically, gastrointestinal angiodysplasias have been associated with aortic valve stenosis in a condition referred to as Heyde's syndrome (149). Patients with aortic-valve stenosis, were found to have loss of the large vWF multimers and abnormalities in platelet adhesion and aggregation in vitro (150). These abnormalities were observed to resolve considerably after aortic-valve replacement. The cause of deficiency of the large vWF multimers in Heyde's syndrome is believed to be due to the shear effect of a stenosed aortic valve on the vWF multimers which causes them to elongate and increases their binding to ADAMTS13, a Metalloproteinase which causes the multimers to become smaller and less efficient at haemostasis (151). In recent years, the relevance of Heyde's Syndrome has been challenged and it has been largely disregarded as a clinical entity. In our previously published cohort study which determined the clinical characteristics of patients with SBA, we found no association between SBA and aortic valve disease, although significant associations were found with cardiovascular disease in general. Despite this, our initial putative assessment showed significant differences in serum Ang-2 levels in SBA patients and as Ang-2 is also stored in Weibel-Palade bodies we felt it was worth assessing levels of vWF in our sporadic SBA cohort to determine whether changes in

Ang-2 levels may lead to an alteration in the co-located vWF. We found no significant differences in serum vWF levels between SBA patients and healthy controls, in fact SBA patients had a trend towards higher levels of vWF. This negative association was probably not unexpected due to our previously determined absent clinical association with Heyde's Syndrome and on reviewing the literature it appears that previous studies suggesting an association between abnormalities in circulating levels of vWF and gastrointestinal angiodysplasia have been performed in very small patient cohorts of 21 and 4 patients respectively (152, 153).

In addition to the initial hypothesis of a single mode of action of Ang-2 via the antagonistic prevention of Ang-1/Tie2 binding, more recent evidence examining angiogenesis in corneal cells suggests a dual mode of action of Ang-2, depending on environmental stimuli and the presence of other angiogenic factors (154, 155). It has been proposed that Ang-2 may have both a vessel stabilising and destabilising role depending on different threshold levels of TNF- α , and requiring the presence of VEGF. For this reason, we included TNF- α measurements in our second phase of serum assessments and found significant differences in TNF- α levels in patients compared to controls, which supports the dual mode of action theory. These findings also identify a highly complex interaction between each of the factors and suggest that despite not being elevated in serum in SBA, VEGF is still likely to have a critical role in SBA formation. A very useful study in helping to decipher the relevance of our positive and negative findings in the putative assessment has been a study on roles of various angiogenic factors in relation to the Angiopoietin pathway in influencing the dynamic process of angiogenesis in vessel maturity, which supports the concept of a dual mode of action of angiogenic factors in vessel maturation (156). Although this study was mainly focussed

on identifying the interactions of a variety of factors with the central factors of the Angiopoietin pathway in tumour vessel angiogenesis, it had many similarities to our findings in SBA. The study set out to use computer technology to develop an interactive algorithm to explain the shift from vessel immaturity to maturity, and vice versa, in tumour vessels. This model as shown in the borrowed image (figure 12), incorporates a complex and “oscillating” feedback interaction between the key players, the Angiopoietins and several other angiogenic factors, including VEGF and PDGF. Although it appears to be a relatively complicated algorithm, the four key simplified findings from the paper are 1) a threshold level of VEGF exists above which endothelial cell development can occur, 2) a threshold level of VEGF exists below which endothelial cells undergo apoptosis, 3) a threshold level of basement membrane supportive pericytes exists, above which immature vessels can undergo maturation, and 4) an Ang-1/Ang-2 ratio exists above which immature vessels mature and below which mature vessels are destabilised.

Figure 12. A borrowed image from Arekelyan et al (156) showing an algorithm for the interaction of VEGF, Ang-1, Ang-2 and PDGF in angiogenesis showing the feedback mechanisms and threshold levels affecting various stages of vessel development.



A crucial next step in determining the significance of the serum abnormalities of Ang-1 and Ang-2 is to assess whether these factors, their receptors and potential stimuli are actually expressed in SBA tissue. This was the aim of the second part of this study; the quantitative PCR assessment of gene expression of the factors in both angiodysplasia tissue and unaffected tissue in SBA patients, and in controls. Although our initial numbers are small, we have demonstrated significant differences in gene expression levels of Ang-1, Ang-2 and the Tie2 receptor in affected angiodysplasia areas compared to controls. This further strengthens the likely role of these factors in the pathogenesis of SBA formation, and reduces the chance of the serum abnormalities being explained by other medical conditions or anaemia. Further work on localisation of these factors by immunohistochemical analysis with antibody detection will be useful to delineate their exact location and expression in gastrointestinal endothelial cells.

Conclusion:

This study is the first to explore the role of angiogenic factors, receptors and stimuli in sporadic SBA, and has identified a novel association between the Angiopoietins and SBA. We have identified decreased serum levels of Ang-1, elevated serum levels of Ang-2 and a resultant decreased ratio of Ang-1/Ang-2 in patients with SBA compared to controls. We have also demonstrated elevated gene expression of these factors along with their receptor, Tie2 in angiodysplasia tissue. Our initial findings provide an exciting and innovative basis for further work in this area with a view to the potential use of Ang-1 and Ang-2 as clinical biomarkers and molecular targets for specific treatments, which will hopefully improve the prognosis and quality of life for patients with SBA.

Chapter 3 – Predictive factors assessment; can clinical factors or angiogenic factors be used as aids in diagnosis of SBA or as prognostic markers?

Publications:

1. Grooteman KV, Holleran G, Matheeuwsen M, van Geenen EJM, McNamara D, Drenth JPH. A Risk Assessment of Factors for the Presence of Angiodysplasias During Endoscopy and Factors Contributing to Symptomatic Bleeding and Rebleeds. Dig Dis Sci. 2019 Jun 12.
2. Holleran G, Hussey M, Smith S, McNamara D. Assessment of serum angiogenic factors as a diagnostic aid for small bowel angiodysplasia in patients with obscure gastrointestinal bleeding and anaemia. World J Gastrointest Pathophysiol. 2017 Aug 15;8(3):127-132.

Oral Presentations:

1. Irish Society of Gastroenterology Winter meeting 2016. Serum Angiopoietin-2 is an accurate capsule endoscopy screening tool for the detection of small bowel angiodysplasia.
2. Irish Society of Gastroenterology Winter meeting 2015. Identification of predictive factors for positive capsule endoscopy findings in obscure gastrointestinal bleeding and anaemia.

Poster Presentations:

1. United European Gastroenterology Week, Barcelona, 2015. Assessment of serum angiopoietin levels in small bowel disorders; are they predictive of Angiodysplasia? **(awarded travel grant).**

2. United European Gastroenterology Week, Vienna, 2016. Serum Angiopoietin-2 is an accurate capsule endoscopy screening tool for the detection of small bowel angiodysplasia.

Introduction:

Small bowel angiodysplasia (SBA) accounts for over 50% of cases of obscure gastrointestinal bleeding (OGIB) and iron deficiency anaemia (IDA) (157). It is more common in elderly patients and in those with multiple comorbidities including chronic kidney disease (CKD), cardiovascular disease and chronic obstructive pulmonary disease (COPD) (158). The clinical presentation of SBA varies from those who remain asymptomatic or with mild IDA only, to those with acute life threatening overt haemorrhages. SBAs are thought to have a worse prognosis than angiodysplasias found in the stomach or colon, mainly due to their relatively inaccessible location, providing challenges in both diagnosis and treatment (159). Some lesions are incidentally detected and never bleed, however; once a lesion does bleed, the bleeding tends to recur despite treatment, and at unpredictable intervals (160, 161). A proportion of patients, generally elderly patients with multiple comorbidities and on anti-platelet or anticoagulant medications, develop a more chronic and refractory form of angiodysplasia, suffering from recurrent bleeding episodes.

Although there are no standardised guidelines for the treatment of SBA, the general consensus is that ablation via argon plasma coagulation (APC) with the use of device-assisted endoscopy is the most effective treatment. However, patients with refractory SBA usually have multiple lesions which can be scattered throughout their small bowel, making treatment of all lesions difficult. In addition, despite the use of APC, studies have shown that up to 54% will have recurrent bleeding within two years (162-164). Small bowel enteroscopy is a procedure with a limited availability worldwide, due to a combination of a limited number of experienced endoscopists, availability of the endoscopic equipment, and the lengthy procedure duration. It can also often be poorly

tolerated by patients, and this combination of factors means that repeated enteroscopy for APC of recurrent or re-bleeding SBAs is generally not a feasible long-term management plan. Medical treatments have shown some success in SBA, including thalidomide and more recently somatostatin analogues (165-167). However, although these medications have shown promising reductions in re-bleeding rates, generally when used in combination with endoscopic treatment, rates of persistent anaemia and significant re-bleeding events are still quite high. As a result a number of patients with refractory SBA become dependent on regular blood and iron transfusions which are generally inadequate at maintaining their haemoglobin levels within normal ranges and are associated with high levels of morbidity and reduced quality of life.

The difficulties in managing patients with SBA is that not all patients are symptomatic, and the timing of re-bleeding events is unpredictable. Almost half of patients with significant re-bleeding events do not notice any overt gastrointestinal bleeding, with their condition deteriorating silently until they become symptomatic of their anaemia unless they are monitored by very frequent haemoglobin (Hb) testing. For elderly patients with multiple co-morbidities this makes them extremely vulnerable and can lead to cumulative episodes of cardiac and respiratory decompensation, requiring regular hospital admissions and a resultant poor quality of life. An early and timely diagnosis, and some form of prognostic indicator allowing the identification of high-risk patients, would provide clinicians the opportunity to arrange more vigilant follow up, and to prevent progression to severe anaemia and subsequent pressure on other organs.

The more widespread availability of small bowel CE, which is the most sensitive diagnostic tool for SBA, and increasing awareness of the condition, means that more people are receiving a diagnosis of SBA. SBCE is a very useful diagnostic tool for a number of indications in gastroenterology and is the first second-line investigation recommended for unexplained IDA and OGIB (168). However, there are a number of other indications for which SBCE is also useful, including suspected Crohn's disease, malabsorptive conditions, polyposis syndromes, and many more. This means that the demand for the procedure far outweighs the available resources to perform it, particularly in countries such as Ireland, where availability of CE is limited to only a few centres nationally. Currently there are no published guidelines on how to prioritise patients for CE and due to the common absence of overt bleeding in patients with SBA they are often not prioritised for the procedure. Recent publications have suggested a predisposition for small bowel malignancies in younger patients with IDA or OGIB, however; it is still unknown what factors should direct prioritisation in elderly patients, who account for the majority of referrals (169, 170). Although a number of publications have focussed on identifying clinical factors predictive of re-bleeding events after a negative CE in OGIB, there have been very few studies looking at the clinical characteristics and potential predictive factors for a specific diagnosis in those presenting with OGIB (171-176). Furthermore, there is very limited data on whether or not certain clinical factors may be predictive of prognosis in angiodysplasia, in terms of initial bleeding risk, or risk of re-bleeding. The first part of this chapter outlines a systematic review we undertook to identify patient risk factors for gastrointestinal angiodysplasias, and determine whether any of these risk factors could be useful as prognostic indicators in predicting the likelihood of bleeding and re-bleeding events.

Based on the results of this systematic review, in the second part of this chapter we undertook an assessment of whether clinical factors in our SBA patient cohort may be predictive of bleeding risk or as predictors of disease severity. We did this by comparing clinical factors between SBA patients and other causes of OGIB and IDA. Our results in chapter 2 of this thesis outlines the identification of differences in serum levels of Ang-1 and Ang2, and TNF- α in patients with SBA, compared to non-bleeding controls (177). However, the Angiopoietin pathway is known to be a hugely important factor in controlling both normal and pathological angiogenesis in many systems, and abnormalities in levels of Ang-1 and Ang-2 and TNF- α are known to be associated with several hypoxia-driven and inflammatory conditions (178). Due to the important involvement of the Angiopoietins in normal blood vessel development it is possible that our observed association of serum abnormalities with SBA, may be driven by the patient's anaemia or gastrointestinal bleeding rather than being specifically related to the underlying pathophysiology of SBA. Using the same patient cohort we used to assess clinical predictive factors in this chapter, we also measured serum Ang-1 and Ang-2 levels to determine whether the detected serum abnormalities we observed in chapter 2 were present in all patients with OGIB and IDA or whether they are specific for SBA. Finally, we assessed the use of serum levels of Ang-1 and Ang-2 as a diagnostic screening tool to identify patients with underlying SBA, which may be used to prioritise CE and enteroscopy services in patients with anaemia and gastrointestinal bleeding.

Study 1:

A systematic review to identify clinical risk factors for the presence of gastrointestinal angiodysplasias detected during endoscopy and factors predictive of symptomatic bleeding and re-bleeding episodes.

Aim:

To provide an objective, hierarchical assessment of risk factors associated with the presence of angiodysplasias, the occurrence of bleeding episodes or symptomatic disease, and factors which influence re-bleeding and prognosis in patients with angiodysplasias.

Methods:

This study was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (179).

Literature search

A systematic search was undertaken to retrieve relevant publications from MEDLINE, EMBASE, and the Cochrane Library from any date until the 2nd of August 2016, using the following search terms 'gastrointestinal angiodysplasia' (angiodysplasia OR telangiectasia OR ectasia OR angioectasia) and (stomach OR small bowel OR gastrointestinal OR colon OR GI), with synonyms for the outcome 'risk factor' (risk factor OR risk factor OR etiology OR cause OR epidemiology OR severity OR prognosis OR prognoses OR mortality OR predict OR prediction). ns and removed duplicates. Titles, abstracts and subsequent full-text articles were independently screened by two authors based on predefined selection criteria and duplicates were removed. Disagreement on

eligibility was resolved through consensus. The search was completed by hand-searching the references of the selected articles, related reviews, meta-analyses, and guidelines.

Inclusion and exclusion criteria

We selected published articles assessing risk factors for the presence of angiodysplasias or factors that contributing to symptomatic disease or disease severity. The following article types were excluded to reduce the heterogeneity of results: case-reports, case-series < 40 patients, reviews, editorials, opinion papers, animal or children studies, and non-clinical studies. Non-English language articles were excluded. We excluded studies reporting vascular malformations other than angiodysplasias, such as portal hypertensive gastropathy, radiation telangiectasia, hereditary haemorrhagic telangiectasia and gastric antral vascular ectasia.

Data extraction

The following study characteristics were extracted from each study in a standardised manner: year of publication, country of origin, study design and population, sample size, type of control group when applicable, the outcome measures and their 95% confidence intervals (95%-CI), and the adjustments for confounding variables. The preferred outcomes were odd's ratios (OR), relative risks (RR) or hazard ratios (HR). If this data was not given or could not be calculated, the outcome measure used in the article was reported.

Quality assessment

Eligible articles were critically appraised for relevance and internal validity. The quality of each study was assessed with the Newcastle-Ottawa Quality Assessment Scale for nonrandomized studies (180). The following domains of bias were addressed:

representativeness of the cases, adequate selection, comparability, outcome assessment and follow-up. Each of the 8 items is assigned a score (a single X) determined by a binary determination of adequacy, except for the domain comparability, which is assigned two Xs. The overall rating of the risk of bias is determined by the addition of the assigned X score for each study and is classified as low (7-9), moderate (4-6) or high (0-3). In relation to representativeness of each study, one X was assigned for age and sex matched controls, two Xs were assigned for the use of multivariate analysis of risk factors. Adequate mean follow-up was assigned a binary cut-off of ≥ 2 years with acceptable "lost to follow-up" percentage of $<20\%$.

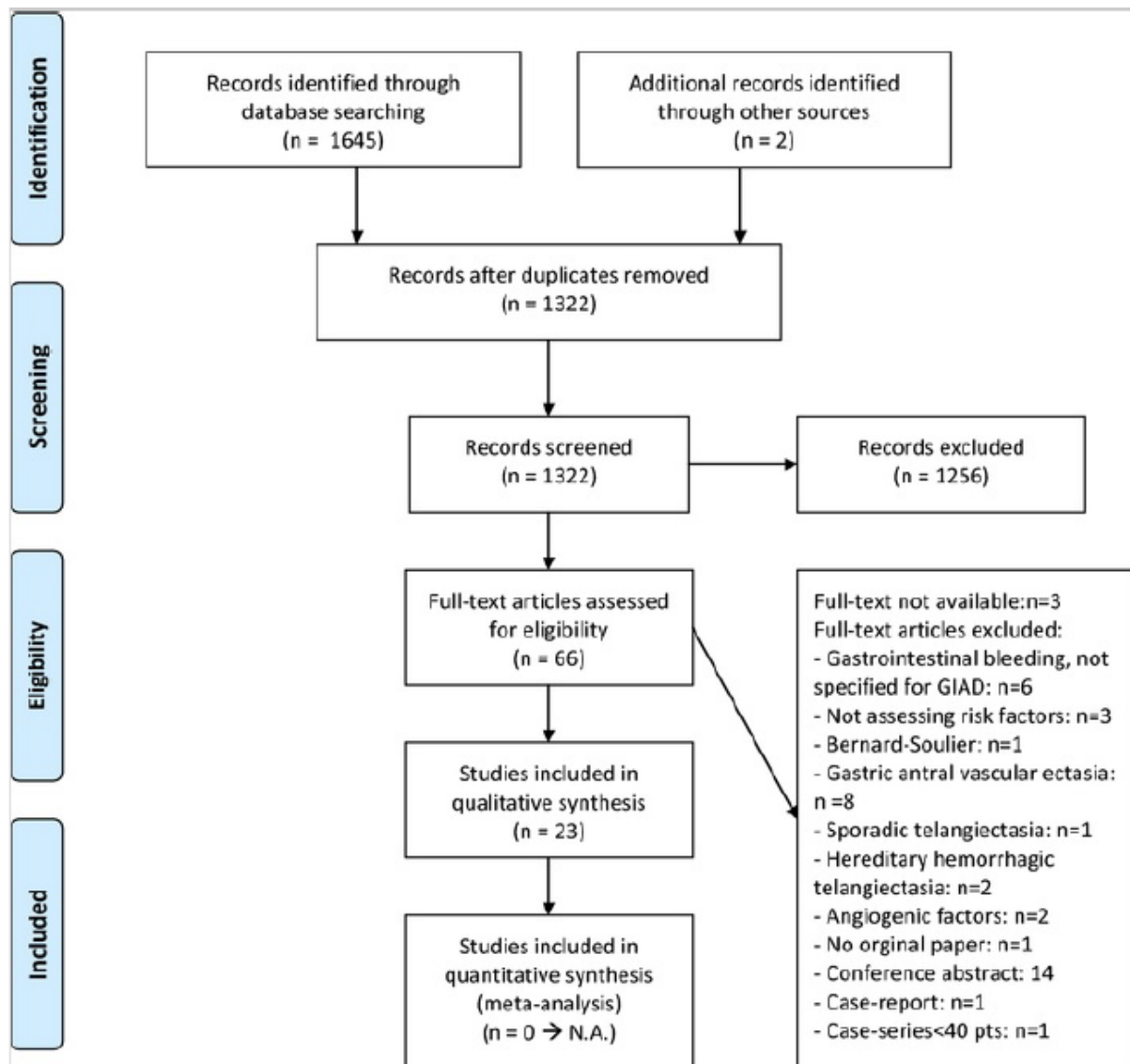
Statistical analysis and hierarchical risk assessment

In the case of the preferred outcomes not being reported, the values were calculated using the raw numbers given in the individual studies where possible. Due to variations in study population, methodologies and measurements between studies, a formal meta-analysis was deemed inappropriate here. A 95% confidence interval (95% CI) not including 0 or a two-sided tested p-value ≤ 0.05 was considered statistically significant. The analyses were performed using SPSS Statistics software version 22.0 (IBM, Armonk, NY, USA). The hierarchical risk assessment identified important risk factors based on two criteria: strength of evidence and effect size as reported previously (181). The strength of evidence was judged valid, when risk factors were identified by at least two independent studies with the same study aim. The effect size was thought to be moderate in cases with a reported OR of 3.5-6.6, and large where the OR ≥ 6 . To be an important risk factor in this study, either strength of evidence and/or effect size had to reach the criteria.

Results:

The original search yielded 1254 citations, with 23 studies passing the stringent inclusion criteria. Most studies were excluded because they did not match our study aim (e.g. only focussing on diagnostic modalities or treatment efficacy etc.) or because they were case-reports or conference abstracts. Two additional studies fulfilling inclusion criteria were discovered by checking references of the included studies. Of 23 reviewed studies included in our systematic review, 13 (n = 3360 patients) reported risk factors associated with the presence of angiodysplasia, 2 reported the risk of symptomatic angiodysplasias (n = 4252 patient) and 6 reported risk factors for re-bleeding episodes and mortality (n = 86366 patients) (Figure 13).

Figure 13. A flow diagram showing the methods involved in determining publications to be included in the literature review



Study assessment

Tables 8 and 9 depict the quality assessment of individual studies indicating the risk of bias. The assessment separates case-control and cohort studies because of the small differences in the scoring systems used.

Table 8. Quality assessment of the case-control studies using the Newcastle-Ottawa Scale.

Study	Case definition	Representativeness of cases	Selection of controls	Definition of controls	Comparability of cases and controls	Ascertainment of exposure	Comparability of exposure	Equality of follow up losses	Total score
Igawa et al	X	-	X	X	XX	-	X	-	6
Holleran et al	X	-	X	-	XX	-	X	-	5
Lanas et al	-	X	-	-	X	-	X	-	3
MacDonald et al	X	-	-	-	XX	-	X	-	4
Chak et al	X	-	X	X	-	-	X	-	4
Clouse et al	X	-	X	X	-	-	X	-	4
Nishimura et al	X	-	X	X	XX	-	X	-	6
Kim et al	X	-	X	X	XX	-	X	-	6
Diggs et al	-	-	X	X	XX	-	X	-	5

Table 9. Quality assessment of the cohort studies using the Newcastle-Ottawa Scale.

Study	Representativeness of exposed cohort	Selection of non- exposed cohort	Ascertainment of exposure	Certain outcome not present at baseline	Comparability of cohorts	Assessment of outcome	Duration of follow up	Adequacy of follow up	Total score
Cochrane et al	-	-	X	X	XX	X	-	X	6
Blackshear et al	X	-	X	-	-	-	-	-	2
French et al	X	-	X	X	-	X	-	-	4
Sotoudehmanesh et al	X	-	X	-	-	X	-	-	3
Duchini et al	X	-	X	-	-	X	-	-	3
Bhutani et al	X	X	X	-	X	X	-	-	5
Marcuard et al	X	-	X	-	-	X	-	-	3
Mai et al	-	X	-	-	XX	X	X	X	6
Kaufman et al	-	X	-	-	XX	X	-	-	4
Jeon et al	-	X	-	-	XX	X	-	X	5
Holleran et al	-	X	-	-	XX	X	X	-	5
Makris et al	X	-	X	-	-	X	-	-	3
Sakai et al	-	X	-	-	XX	X	X	X	6
Saperas et al	-	X	-	-	XX	X	X	X	6
Serrao et al	X	X	-	X	XX	X	-	X	7

Risk of bias was introduced at different levels and were mostly dependent on the design of the study. Overall, the risk of bias was high in 6 studies, moderate in 17 studies and low in 1 study. No statistical tool could be used to assess bias due to the large inter-study heterogeneity. The quality of evidence was too low to perform a meta-analysis.

Risk factors for the presence of angiodysplasias

Thirteen cohort studies assessed risk factors for the presence of angiodysplasias (Table 10) (182-194).

Table 10. Studies assessing risk factors for the presence of gastrointestinal angiodysplasias

Study	Study type	No of controls	No of patients	Significant risk factors	Effect OR (95% CI)/ t-test
Igawa et al	CC	97	64	Liver cirrhosis Cardiovascular disease	4.8 (1.8-14.5) 2.9 (1.4-6.2)
Holleran et al	CC	95	66	Hypertension Ischaemic heart disease Arrhythmia Valvular heart disease Congestive cardiac failure Chronic kidney disease Venous thromboembolism Anticoagulant use Warfarin Proton pump inhibitor	2.8 (1.5-5.4) 4.3 (1.9-9.8) 4.4 (1.7-11.2) 18.8 (2.4-149.6) 4.5 (1.2-17.9) 4.5 (1.9-10.5) 6.4 (1.3-31.3) 2.7 (1.4-5.1) 5.5 (1.1-27.5) 5.4 (2.7-10.7)
Lanas et al	CC	1958	66	Anticoagulants Proton pump inhibitor	p<0.001 p<0.01
MacDonald et al	CC	91	46	Increasing age	1.09 (1.04-1.1)
Chak et al	CC	97	32	Age>65 years Chronic kidney disease	p<0.001 p<0.05
Clouse et al	CC	90	30	Chronic kidney disease	p<0.001
Cochrane et al	RC	56	14	Age	1.3 (1.1-1.6)

				Chronic kidney disease	21.0 (2.5-181)
				Length of hospital stay	5.1 (1.1-23.7)
				Male gender	0.1 (0.01-0.6)
				Diabetes mellitus	0.9 (0.01-0.6)
				Hypertension	0.2 (0.1-0.97)
Blackshear et al	RC	46	7	Mitral valve regurgitation	N/A
French et al	RC	31	-	Left ventricular assist device	N/A
Sotoudehmanesh et al	RC	197	9	End stage kidney disease	N/A
Duchini et al	RC	135	9	CREST syndrome	N/A
Bhutani et al	RC	37	40	Aortic stenosis	N/A
Marcuard et al	-	32	15	Chronic kidney disease	N/A

Six case control studies assessed multiple risk factors for the presence of gastrointestinal angiodysplasias, however as the presence was relatively rare the confidence intervals in most cases were wide. Independent risk factors that were confirmed in two or more studies are increasing age (OR 1.09 per year, 95% CI 1.04-1.1), chronic kidney disease (OR 4.5, 95% CI 1.9-10.5), anticoagulant use (OR 2.7, 95% CI 1.4-5.1) and cardiovascular disease (2.9, 95% CI 1.4-6.2). Independent factors that possessed a moderate to large effect size were valvular heart disease (OR 18.8, 95% CI 2.4-149.6), previous venous thromboembolism (OR 6.4, 95% CI 1.3-31.3), Warfarin use (OR 5.5, 95% CI 1.1-27.5), proton pump inhibitor use (OR 5.4, 95% CI 2.7-10.7), liver cirrhosis (OR 4.8, 95% CI 1.8-14.5), congestive heart failure (OR 4.5, 95% CI 1.2-17.9), arrhythmias (OR 4.4, 95% CI 1.7-11.2) and ischaemic heart disease (OR 4.3, 95% CI 1.9-9.8). Seven cohort studies focussed on the presence of gastrointestinal angiodysplasias in patients with a specific

condition. The findings from the cohort studies supported the determination of CKD as a risk factor having angiodysplasia, and also identified left ventricular assist devices, aortic stenosis, mitral valve regurgitation, and CREST syndrome as risk factors. Hypertension was identified by one study as a risk factor for SBA (OR 2.8 95% CI 0.1-0.97).

Triggers for symptomatic disease causing gastrointestinal bleeding

Only three retrospective cohort studies assessed factors that trigger progression from asymptomatic to symptomatic disease defined as IDA or occult or overt GI bleeding (Table 4).

Table 11. Studies assessing risk factors for progression from asymptomatic to symptomatic disease in gastrointestinal angiodysplasias.

Study	Study type	Number of symptomatic patients	Number of asymptomatic patients	Significant risk factors	Effect OR (95% CI)
Nishimura et al	RC	29	406	Age >80 years	5.15 (1.61-16.5)
				Heart disease	6.88 (1.04-45.5)
				Anticoagulant use	4.22 (1.21-14.7)
				Multiple lesions	6.67 (1.77-25.2)
				Lesion size >5mm	17.7 (4.9-64.0)
Kim et al	RC	35	58	Lesion size >1cm	5.3 (1.04-15.9)
				Location: gastric	6.1 (1.2-12.5)
Diggs et al	RC	2320	1839	Inpatient status	8.74 (5.4-14.1)
				Age >80 years	1.32 (1.1-1.6)
				ASA class >III	1.97 (1.6-2.4)
				Black race	1.95 (1.5-26.6)
				Hispanic ethnicity	1.71 (1.3-2.2)
				2-10 lesions	1.5 (1.3-1.8)
				>10 lesions	2.18 (1.7-2.8)

Independent risk factors identified by two studies for symptomatic disease were age above 80 years (OR 5.2 and 1.3) and multiple lesions (OR 6.7 and 2.2). The study by Diggs et al is a population-based study with a large sample size (n = 4159) investigating only colonic angiodysplasias. Inpatient status had the largest effect size in this study with an OR of 8.7 (95% CI 5.4-14.1). The other two studies had a much smaller sample size (n=435 and n=93 respectively) but investigated bleeding from angiodysplasias in both the stomach and colon. Nishiruma et al. found heart disease (OR 6.9, 95% CI 1.0–45.5)

and lesion size ≤ 5 mm (OR 17.7, 95% CI 4.9–64.0) to be risk factors with a large effect size. In contrast, Kim et al found that lesion size of >1 cm was found to be predictive of symptomatic disease.

Predictors for severe disease in patients with symptomatic angiodysplasias

Six retrospective cohort studies investigated factors associated with rebleeds in a population of symptomatic patients and two studies assessed risk factors for mortality (Table 12).

Table 12. Risk factors for re-bleeding and mortality in patients with symptomatic GIADs.

Study	Study type	Number of patients	Number with rebleed	Follow up % and number of years	Outcome	Risk factor for outcome	Effect OR/HR (95% CI)
Mai et al	RC	87	14	100% >1	Rebleed	Multiple locations Chronic kidney disease Congestive cardiac failure	4.2 (1.1-16.2) 4.5 (1.0-19.6) 4.5 (1.0-19.9)
Kaufman et al	RC	156	46	-	Rebleed 90-day mortality	Longer disease duration Active bleeding at diagnosis Distal small bowel location Inpatient status	1.05 (1.01-1.09) 2.69 (1.15-6.30) 4.29 (1.46-12.56) 17.7 (1.7-185.1)
Jeon et al	RC	66	15	90% Mean 1.8	Rebleed	Liver cirrhosis	4.01 (1.1-15.0)
Holleran et al	RC	56	45	65% Mean 2.7	Rebleed	Multiple lesions Valvular heart disease	- -
Sakai et al	RC	68	23	92% Median 2.5	Rebleed	>3 lesions	3.82 (1.3-11.3)
Saperas et al	RC	57	17	92% Mean 2.8	Rebleed	Over-anticoagulation Multiple lesions	4.15 (1.1-15.4) 8.63 (1.4-52.6)
Makris et al	RC	48	-	-	Rebleed	N/A	N/A
Serrao et al	RC	85971	-	100% N/A	In-hospital mortality	>3 co-morbidities	2.29 (1.2-4.3)

An independent risk factor for re-bleeding episodes identified by at least two studies was the presence of multiple lesions (OR 4.2, 95% CI 1.1–16.2 and 3.8, 95% CI 1.3–11.3 and 8.6, 95% CI 1.4–52.6). In addition, liver cirrhosis (OR 4.0), location in the third small bowel quartile (OR 4.3), chronic kidney disease (4.5), congestive cardiac failure (4.5) and supra-therapeutic anticoagulation (OR 4.2) were detected to be risk factors with a moderate size effect. Multiple (≥ 3) comorbidities led to an increased risk (OR 2.3, 95% CI 1.2–4.3) of in-hospital mortality, and inpatient status led to an increased 90-day mortality (OR 17.7, 95% CI 1.7–185.1) in patients with angiodysplasias.

Discussion:

This systematic review gives an objective assessment of independent risk factors associated with the presence of angiodysplasias, triggers for the progression from asymptomatic to symptomatic disease, and factors that contribute to disease severity as measured by re-bleeding episodes and mortality. Age, chronic kidney disease, anticoagulant use and cardiovascular disease appear to be the most important factors associated with the presence of angiodysplasias. The pathophysiological hypothesis underlying angiodysplasia formation makes it plausible that age, chronic kidney and cardiovascular disease could either be factors contributing to the development of angiodysplasias, or the conditions may share a common aetiological trigger, likely ischaemia. Although anticoagulant use was found to be associated with both the presence of, and risk of bleeding from angiodysplasias, it is most likely that the medication's effects on coagulation precipitates bleeding from already present lesions, rather than contributing to the underlying disease pathophysiology, as proposed by Saperas et al. Furthermore, cardiovascular disease is the most common indication for

anticoagulation, making it more likely that anticoagulation is associated with the presence of an underlying comorbidity rather than having any causative role in angiodysplasia development. This is also the likely case in the detection of an increased use of PPI use in angiodysplasia patients. PPIs are empirically prescribed in patients with gastrointestinal bleeding, and often in patients on antiplatelet agents, which both account for a large proportion of patients with angiodysplasia, making it likely also to be a confounding rather than causative association. Inpatient status was found to be strongly associated with symptomatic disease, however again, it is difficult to extrapolate whether bleeding episodes were the reason for inpatient admission in the first place.

The presence of multiple lesions in contrast, appears to be a more reliable or robust predictive factor for an increased risk of re-bleeding. Surprisingly however, only the presence of multiple comorbidities was found to be associated with mortality. This may be explained due to deficiencies both in recognising or predicting re-bleeding episodes, and to the limited and often ineffective treatment options available. Often re-bleeding episodes require the withdrawal of anticoagulant or antiplatelet agents which albeit a temporary measure, increases the risk of exacerbation of the underlying condition.

Alongside this, anaemia itself is associated with and increased mortality and aggravates underlying cardiovascular, respiratory and neurological conditions.

The over-arching aim of this systematic review was to define risk factors for the presence of gastrointestinal angiodysplasias and/or affect the progression to symptomatic disease. The development of much-needed management guidelines for angiodysplasia requires a thorough assessment of the contribution of each individual risk factor to the overall clinical picture. Patients who develop bleeding angiodysplasias

and possess multiple risk factors which increase the risk of re-bleeding will warrant close monitoring. However, the detection of these risk factors for angiodysplasia, should also prompt close monitoring and potentially modification of the risk factor detected. For example, supra-therapeutic anticoagulation in a patient with known angiodysplasia could be avoided by switching from warfarin to a direct oral anticoagulant which does not require dose adjustment, or the correction of significant valvular heart disease may obviate the need for anticoagulation, thereby reducing the risk of bleeding even in the presence of angiodysplasias. Efforts to lower the clinical risk profile by treating underlying medical conditions may be a useful strategy, complimentary to aggressive endoscopic treatment, and it would be interesting to assess the efficacy of endoscopic and medical treatments in cohorts of clinically defined low and high risk patient groups. At present, one of the major issues in decisions regarding the management of incidentally found or non-bleeding angiodysplasias is that evidence on treatment outcome is based on heterogeneous, small, and often non-controlled studies. Our systematic review provides an objective assessment of the available literature in identifying clinical factors which may be useful in classifying disease severity and offer some prognostic indicators which would be a crucial requirement to facilitate the development of clinical practice guidelines for management of this disease. Our systematic review comes with inherent strengths and limitations. The strength of this study is that it includes a broad search strategy with a thoroughly performed quality assessment. Moreover, we have performed a hierarchical risk assessment based on confirmation of risk factors in a second study and those with a moderate effect size (OR >3.5). To gain further clarity from the heterogeneity of the inclusion criteria of individual studies, we subdivided the risk factors for different stages of disease, i.e. presence,

symptomatic disease/bleeding events, and a more severe course with re-bleeding episodes or mortality. In addition, the risk factors identified for each disease stage includes a mix of data from Western and Asian studies, thereby increasing the external validity of this review. A limitation of the study is that a meta-analysis was not possible due to the methodological heterogeneity between the studies and the moderate risk of bias found in the included studies. Moreover, we excluded case-reports and small case-series which may inadvertently have led to the exclusion of diseases which, although rare, do increase the risk of presence of angiodysplasias, e.g. bone marrow transplantation. Furthermore, we excluded studies reporting on other forms of mucosal vascular abnormalities, which may have introduced bias due to differing opinions regarding the differentiation and diagnosis of vascular lesions endoscopically. Additionally, there were only three studies evaluating risk factors for symptomatic disease, which needs to be considered when drawing conclusions from these studies. In conclusion, this systematic review identified important risk factors for angiodysplasias at different disease stages. The hierarchical risk assessment identified age, chronic kidney disease, anticoagulant and cardiovascular disease as the most important risk factors for the presence of angiodysplasias, and inpatient status and multiple lesions contributing to disease severity. An assessment for the presence of these risk factors may be useful in phenotyping disease at diagnosis and determining prognosis, initial treatment strategies and follow-up. Furthermore, modifying these risk factors where possible, offers an alternative area for treatment target, and may improve the overall outcome.

Study 2

Aims:

1. To identify whether any clinical factors could be used to predict a specific diagnosis of SBA in a group of patients with other causes of IDA and OGIB
2. To determine whether serum abnormalities in Ang1, Ang2, or TNF- α are specific for SBA or are associated with OGIB and IDA in general
3. To assess the use of serum levels of any of the above angiogenic factors as a diagnostic screening tool to identify patients at a higher risk of a diagnosis of SBA.

Methods:

Aim 1: Clinical predictive factor assessment:

After obtaining ethical approval from our Institution's Research Ethics committee a retrospective review of the prospectively collected CE database at Tallaght Hospital was performed to identify patients who underwent CE for IDA or OGIB; either occult or overt between the years of 2009-2015. Data was then collected for each patient from outpatient clinic and initial referral letters, inpatient discharge summaries, and haematological and biochemical laboratory results. Clinical diagnoses were confirmed by a clinical specialist, imaging characteristics diagnostic of certain conditions, or by haematological or biochemical laboratory results. Iron deficiency anaemia was defined as a Hb level of <11.5g/dL for Females or <13g/dL for Males on more than one occasion, along with a decreased serum ferritin or iron studies. CKD was recorded as a clinical condition in patients with a persistent glomerular filtration rate (GFR) of <60ml/min for a minimum of 6 months. Information including: patient demographics, specific

indication for CE (presence of occult or overt bleeding), findings at SBCE, lowest Hb level, interval from referral to undergoing CE, comorbidities and medication usage, was recorded. Patients were then divided into groups based on CE diagnosis, and the presence of clinical factors were compared between groups to establish any factors predictive of SBA compared to other causes of IDA and OGIB and controls i.e. negative CE.

Aim 2: Serum factor assessment:

Any patient over the age of 18 years who was undergoing a CE for either IDA or OGIB was invited to participate in this part of the study prospectively from July 2014-September 2015. Information was collected on patient demographics and clinical history, and approximately 10mls of blood was drawn via standard phlebotomy technique. Plasma samples were sent to our laboratory for a routine Hb level and GFR assessment. The remaining serum samples were left to clot for at least 30 minutes before undergoing centrifugation for 15 minutes at 1000 rpms. The resulting supernatant was then extracted and stored in aliquots at -80C for batch analysis. A database of patients was formed based on subsequent CE findings and patients were divided into three groups; 1) SBA, 2) Abnormal findings (other than SBA) and 3) Normal/negative. Recruitment continued until serum samples had been stored on a minimum of 40 patients in each group.

Serum levels of Ang1, Ang2, and TNF- α in serum were measured using commercially available solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kits (R and D systems, Minneapolis, MN). Samples were prepared in duplicate and results were read at 450 nm absorbance. The intra-assay coefficients of variation (CV) was calculated as an

average of all of the individual CVs for the sample concentration duplicates analysed by ELISA. The specific laboratory technique is described in more detail in chapter 2.

Statistical analysis:

Results of all assays and patient demographics were expressed as a mean and/or median and compared between groups using the Student t test, Mann-Whitney U test, univariate/multivariate logistic regression analysis, Pearson's correlation coefficient, or a relative risk (RR) ratio as appropriate, with a difference of <0.05 considered significant. All analyses were performed using SPSS version 22 (SPSS Inc., Chicago, IL). The potential for the use of serum Ang1, Ang2 or a ratio of Ang1/Ang2 as a diagnostic marker or SBCE screening tool for SBA was explored using Receiver Operator Characteristic (ROC) curve analysis.

Results:

Overall findings:

Over a 66-month period 1379 CEs were performed on the same number of patients in our institution, with 455 (33%) of these for the investigation of IDA or OGIB. The distribution of specific indications within this were: IDA alone: 62% (n=281), occult bleeding: 26% (n=117) and overt bleeding: 12% (n=57). Of the group overall, 51% (n=232) were female and the mean age was 64 years (13-99). The overall diagnostic yield (DY), defined as the detection of a small bowel finding which could explain the degree of bleeding, was 57% (n=258). In cases where more than one diagnosis was reported, the finding thought to be more likely to be associated with bleeding was recorded as the primary diagnosis.

Mode of bleeding:

The DY differed considerably based on the mode of bleeding with a significantly lower DY of 49% (n=138) (p=0.01) in IDA, compared to both occult 64% (n=75) [p=0.0006, 95% CI (0.24) – (0.06)] and overt 79% (n=45) [p=0.0001, 95% CI (0.44 – (0.16))] bleeding.

Specific diagnoses according to mode of bleeding are outlined in table 13. The presence of overt bleeding increased the DY from 53% to 77%, with a RR of 1.46 (p<0.0001). The mode of bleeding however, although predictive of a positive finding overall was not predictive of any specific diagnosis.

Table 13: Positive findings and diagnoses according to mode of bleeding presentation

	OGIB overall n=455	IDA n=281	Occult GIB n=117	Overt GIB n=57
Normal	43% n=197	51% n=144	36% n=43	21% n=12
Positive findings	57% n=258	49% n=138	64% n=75	79% n=45
Inflammation	45% n=115	57% n=78	32% n=24	29% n=13
Angiodysplasia	34% n=88	31% n=43	38% n=28	38% n=17
Active bleeding – source unidentified	12% n=31	5% n=7	16% n=12	27% n=12
Masses/polyps	8% n=21	7% n=10	13% n=10	2% n=1
Coeliac disease	1% n=3	0% n=0	1% n=1	4% n=2

Patient demographics:

Gender was not predictive of a positive CE overall, with n=93 (37%) females and n=104 (63%) males having a negative study vs n=134 (52%) females and n=124 (48%) males having a positive study (p=0.54).

Overall, patients with a negative CE were significantly younger than those with positive findings at 61 vs 67 years [$p < 0.001$, 95% CI (8.91)- (2.93)]. When age was divided into three subgroups of <50 years, 50-69 years and >70 years, the likelihood of having positive findings increased in a linear fashion with increasing age. When analysing predictive factors patients were grouped into binary age groups of <50 and >50 years. The RR of having a positive CE overall if >50 years of age was found to be 2.7 (95% CI 1.0333 to 6.8580), $p = 0.04$. In addition to overall positive findings, there were differences in the specific diagnosis according to age, with inflammation more likely ($p = 0.002$) in younger patients and angiodysplasia more common ($P = 0.0004$) in older patients. The RR for a diagnosis of angiodysplasia was 4.9 in patients >50 years compared to those <50 years (95% CI 1.83-12.9, $p < 0.002$). The use of age as a RR for any other diagnosis was not found to be significant, although there was a trend towards higher levels of inflammation in younger patients than other age groups. Figures 14 & 15.

Figure 14. Ratio of positive to negative findings according to age group

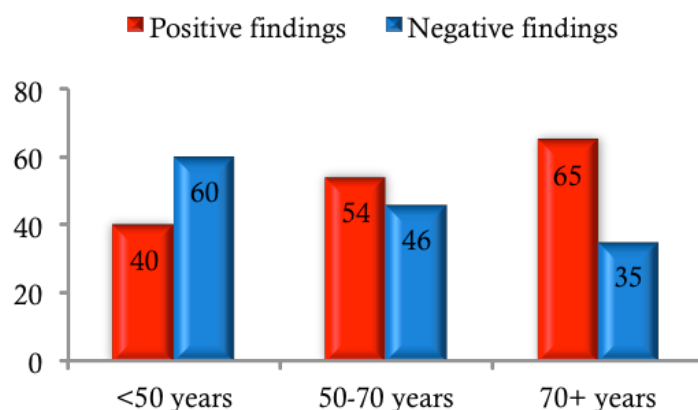
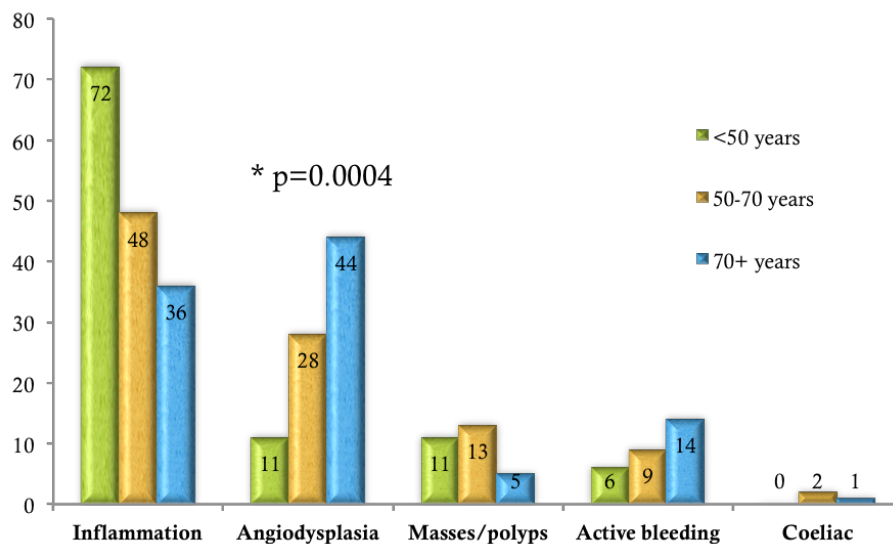


Figure 15. Positive findings according to different age groups



Co-morbidities:

Full clinical information was available in 178 patients, of whom 79 (44.38%) had negative findings at CE and 99 (55.62%) had positive findings. A significant co-morbidity was found in 89.9% of patients overall, with ischaemic heart disease (IHD), COPD, CKD, Diabetes Mellitus (DM) and cirrhosis being the most commonly reported conditions (table 14).

Table 14. Clinical factors as predictive factors for positive or negative findings at CE.

	Significant comorbidity	Cirrhosis	IHD	COPD	DM	CKD	Warfarin	Antiplatelet	Inpatient status
Negative n=79	89.87%	2.53%	29.11%	10.13%	16.46%	10.13%	12.66%	31.65%	22.79%
Positive n= 99	89.90%	8.08%	30.30%	27.27%	4.04%	19.19%	24.24%	46.46%	32.32%
p value	0.8879	0.1114	0.8641	0.00041	0.0049	0.0949	0.0512	0.0451	0.026
95% Confidence Interval	N/S	N/S	N/S	(0.29 - 0.06)	0.04 - 0.21	N/S	N/S	(0.29 - 0.00)	(0.29 - 0.02)

The presence of a single co-morbidity was not found to be predictive of a positive or negative CE with at least one co-morbidity found in 89.9% of patients with both negative and positive studies. The only specific condition found to be predictive of a positive study was COPD with a RR of 1.32 (95% CI 1.1-1.7, $p<0.02$). In contrast, DM was found to be protective with a RR of 0.36 (95% CI 0.15-0.87 $p<0.03$). There were higher rates of antiplatelet and anticoagulant use in patients with positive studies, with only antiplatelet use found to be predictive of a positive study. Significantly more patients with positive findings underwent CE as an inpatient than those with negative findings. We performed a sub-group analysis to determine whether any of the clinical factors identified to be predictive of a positive CE were specific for SBA (n=37) or were predictive only of OGIB in general (n=62) [table 15].

Table 15. Clinical factors as predictive factors for a specific diagnosis of SBA in patients with positive findings in IDA and OGIB overall.

	Significant comorbidity	Cirrhosis	IHD	COPD	DM	CKD	Warfarin	Antiplatelet	Inpatient status
SBA n=37	35%	4%	13%	10%	4%	14%	13%	16%	17%
Other bleeding n=62	53%	4%	17%	17%	0	5%	11%	30%	21%
p value	0.1662	0.4665	0.4242	0.9666	0.0079	0.0002	0.0514	0.6238	0.2363
95% Confidence Interval		N/S	N/S	N/S	0.03 - 0.19	0.15 - 0.45	N/S	N/S	N/S

Although COPD, antiplatelet use and inpatient status were found to be predictive factors of a positive finding on CE, none of these factors were found to be predictive for a specific diagnosis of SBA over other causes of OGIB. In contrast to the findings in OGIB overall, the presence of DM was found to be predictive for a diagnosis of SBA, along with CKD.

Haemoglobin levels and CE timing:

There was a significant difference in the mean Hb levels between patients with positive and negative findings at 8.9g/dL vs 9.7g/dL in males ($p<0.02$, 95% CI 0.39 to 1.32) and 8.9g/dL vs 9.9g/dL in females ($p<0.03$, 95% CI 0.13 to 1.936) respectively. When Hb levels were assessed as a predictive factor for a positive or negative study, a cut-off of $<10\text{g/dL}$ was associated with a positive CE, with a RR of 1.43($p<0.03$ 95% CI 0.27-1.18). The application of this cut-off level as a screening tool in the overall cohort would have led to an increase in the DY from 44% (with a $\text{Hb}>10\text{g/dL}$) to 61%. A further evaluation of Hb levels was undertaken based on gender, with a cut-off of $<10\text{g/dL}$ in males, and $<8\text{g/dL}$ in females. These gender specific cut-off levels gave an overall diagnostic yield of 57.14%, with a RR of 1.54 ($p<0.02$, 95% CI 1.09-2.18). Overall Hb levels could not be used to predict a specific diagnosis, however patients with angiodysplasia were found to have a significantly lower mean Hb level (8.8g/dL) than any other condition (inflammation 9.7g/dL, masses/polyps 10.3g/dL, active bleeding 9.0g/dL).

In the 56 patients who underwent CE within 4 days of presentation as an inpatient, the DY was significantly higher than beyond 4 days with DYs of 68% vs 49% ($p=0.02$, 95% CI 0.03-0.34) respectively. The mode of bleeding was not found to be a confounding variable in this cohort.

Serum biomarker assessment:

Recruitment of participants for the serum collection continued until a minimum of 120 viable samples had been collected, with 40 participants in each group based on CE result; Angiodysplasia, Abnormal findings other than angiodysplasia, and Normal or negative findings. All 120 patients had been referred for the investigation of IDA or

OGIB, with no significant differences between groups in the mode of bleeding at referral.

Patient factors:

Of the group overall, 58% (n=70) were Male, with a mean age of 63 years (range 18-93). Patients in the Normal group were significantly younger than both the Abnormal and Angiodysplasia group with a mean age of 55 years (range 18-81) vs 60 years (range 19-92) and 73 years (range 53-93) respectively. There was no difference in gender between the groups with 42% (n=17), 67% (n=27), and 65% (n=26) Males in each group respectively. There were significant differences in Hb levels with patients with normal findings having a higher mean Hb level (12.0g/dL in Females and 14.0g/dL in Males) compared to both the Abnormal (11.3g/dL in Females and 12.9g/dL in Males) and Angiodysplasia (11.0g/dL in Females and 11.3g/dL in Males) groups. There was no difference between Hb levels in the abnormal and Angiodysplasia groups, p=0.832.

Table 16. Comparison of descriptive factors Age, Gender and mean Hb level in the three groups.

	SBA n=40	Abnormal n=40	Normal n=40
Mean age (years)	72.92	60.16	54.93
Range	53 – 93	19 – 92	18 – 81
p value (Vs SBA)		0.0001	0.0001
95% confidence interval		6.5 – 19.25	(24.63 – 11.36)
Male gender	65%	67%	42%
p value		0.7031	0.0942
95% confidence interval		N/S	N/S
Mean Hb (g/dL)			
Females	11.02	11.29	12.02
p value		0.7230	0.0469
95% confidence interval		N/S	0.014 – 1.984
Males	11.33	12.92	14.02
p value		0.0122	0.0001
95% confidence interval		(2.819 – 0.363)	(3.759 – 1.618)

Serum angiogenic factors:

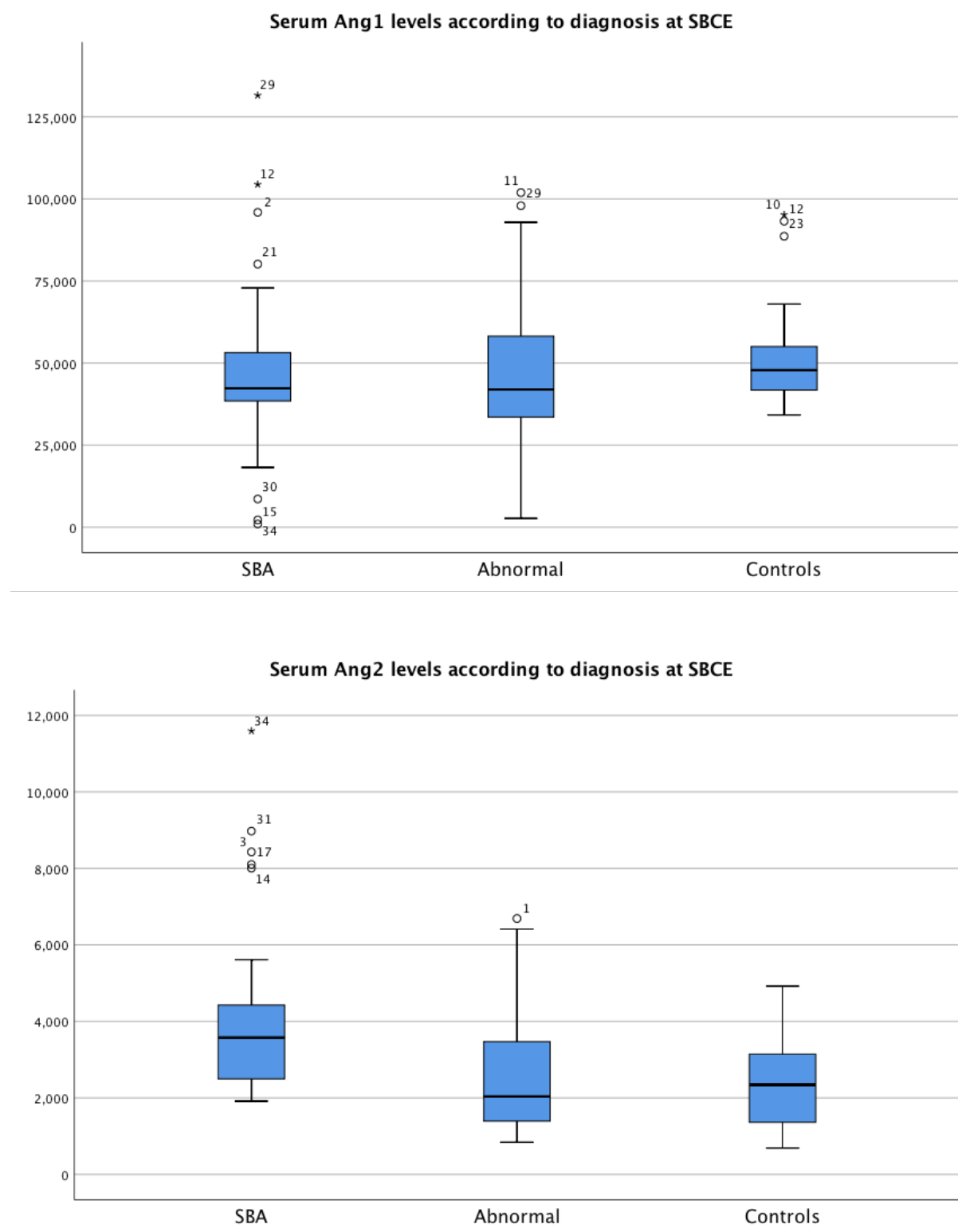
Overall, we found significantly higher levels of Ang2 in SBA patients compared to patients with other causes of bleeding identified and those with a normal CE. There was a trend towards lower Ang1 levels in patients with SBA, however this was only statistically significant when compared to those with a normal CE. Although TNF- α levels did appear to be lower in the SBA group there were no significant differences between

groups. Levels of each factor in the three groups are outlined in Table 17, and figures 16 and 17.

Table 17. Median serum levels of each angiogenic factor according to diagnosis

	SBA n=40	Abnormal n=40	Normal n=40
Ang1 (pg/ml)			
Median	40976	44770	47639
Range	974-97,511	2660-101,930	17,899-95,173
P value compared to SBA		0.33	0.04
Ang2 (pg/ml)			
Median	3759	2261	2620
Range	1915-14,731	842-14,000	686-8,850
P value compared to SBA		<0.004	<0.003
Ratio of Ang1/Ang2			
Median	11.4	20.2	19
P value compared to SBA		<0.006	<0.001
TNF-α (pg/mL)			
Median	5.76	9.76	10.14
Range	0.35 – 40	0.46 – 58	0.35 – 38
P value compared to SBA		0.12	0.13

Figures 16 and 17. Boxplots showing mean serum Ang-1 and Ang-2 levels in patients based on their diagnosis at CE.



Association of Ang2 with other clinical factors:

To determine its use as a predictive marker, serum levels of Ang2 were controlled for the presence of CKD and anaemia, both of which are thought to be associated with higher serum Ang2 levels.

Table 18. Comparison of mean serum levels of Ang-2 in patients with and without CKD, and with and without anaemia in the 3 groups based on diagnosis at CE.

	SBA	Abnormal	Normal
Mean level in CKD	5023 n=7	8142 n=6	3620 n=6
Mean level normal renal function	5140 p=0.95	2775 p=0.0002	3495 p=0.95
Mean level Hb<10g/dL	4237 n=7	6142 n=8	N/A n=2
Mean Hb	4449	2321	
Hb>10g/dL	p=0.86	p=0.0001	

Pearson's correlation testing was used to determine whether anaemia was associated with serum Ang2 levels in the SBA or abnormal bleeding group, and showed no correlation between the two factors ($r=0.3557$, r squared = 0.1265, $p=0.348$). Serum Ang2 levels even when controlled for anaemia and chronic kidney disease were found to be selectively elevated in patients with SBA.

Serum Ang1 or Ang2 as a screening tool for SBA:

As demonstrated in figure 18 we established a cut-off Ang2 level of 2600pg/ml using ROC curve analysis with an area under the curve (AUC) of 0.695, (standard error 0.048,

95% CI 0.601-0.789, $p=0.001$) which gave a sensitivity of 84% and negative predictive value of 87% (table 19).

Figure 18. ROC curve analysis showing a cut-off level for Ang-2 of 2600pg/ml with an AUC of 0.695

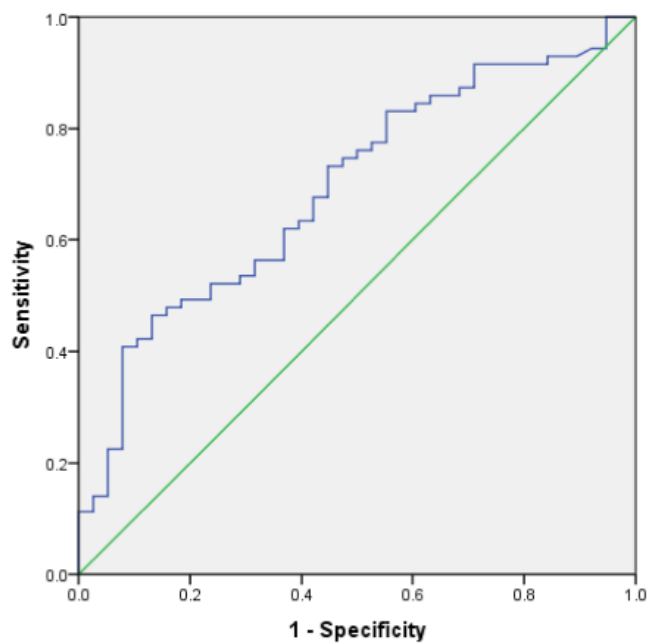


Table 19. Predictive value of a cut-off level of 2600pg/ml of Ang-2 for SBA

True positive n=33	False positive n=40	Sensitivity =85% Specificity =50%
True negative n=7	False negative n=40	Positive predictive value =45% Negative predictive value = 85%

Using the RR model, a serum level of 2600pg/ml had a RR of SBA of 2.22 ($p=0.012$, 95% CI 1.20-4.11). Although the positive predictive value for SBA was only 45%, 53% of the false positive patients did have significant findings (including 2 potential malignancies, 2

Meckel's diverticulae and an actively bleeding Dieulafoy lesion) all of which would warrant an expedited SBCE, making the true false positive rate only 26%. Ang1 could not be used as a predictive marker for SBA or any other cause of OGIB. In addition to Ang2, the ratio of Ang1/Ang2 levels was also significantly different in the Angiodysplasia group, although the ROC curve was similar to the Ang2 alone curve (AUC 0.695 vs 0.692 respectively) thereby conferring no additional benefit to the use of two markers.

Figure 3. ROC for a serum level of 2600pg/ml of Ang-2 as a diagnostic tool for SBA

Discussion:

Both the diagnosis and management of gastrointestinal angiodysplasias have been a longstanding challenge for gastroenterologists, however due to their increasing frequency, particularly in the small bowel; they are now becoming a challenge encountered by all physicians, from primary to tertiary care. There are several reasons for recent increases in the reporting of SBA; firstly, their association with increasing age, in our constantly aging population. Secondly, advances in other medical specialities particularly cardiovascular and stroke medicine have led to an increased dependency of elderly patients on antiplatelet and anticoagulant medications, thereby aggravating bleeding from lesions which would previously have remained asymptomatic or produced mild bleeding only. Finally, access to the small bowel and the ability to diagnose SBAs has only really been widely available since the introduction of CE in 2000. This has led to an increase in the reporting of SBAs and a resultant increased awareness of their significance among physicians. Although reports on the natural history and treatment outcomes of SBA are very limited, mainly by small patient numbers, the overall

prognosis associated with the diagnosis is thought to be relatively poor. No treatment has yet been shown to prevent re-bleeding from SBAs in the longer term, however; there have been suggestions that the earlier diagnosis and intervention of directed treatment prior to respiratory or cardiac decompensation due to anaemia, may improve the longer-term outcome for patients. Although CE is the most sensitive diagnostic tool for SBA and is far less invasive than conventional small bowel endoscopy, it is still not widely available in all centres worldwide, does carry some risks, and remains a relatively expensive test. Thus, the availability of some additional diagnostic and prognostic tools, in the form of predictive clinical or serum factors could prove extremely useful in reducing the dependence on CE and directing treatment for patients at an earlier stage. The aims of this study were to identify either clinical or biochemical tools which may aid in the diagnosis.

Unexplained IDA and OGIB have many causes ranging from physiological and dietary to medication induced, and benign and malignant pathology (195). While all cases warrant thorough investigation as recommended by all International gastroenterology societies, not all cases can be investigated urgently. Unlike the guidelines for the prioritisation for upper and lower endoscopy in the investigation of IDA, there are currently no available guidelines on how to prioritise CE, although it has been noted that the DY is increased the earlier the test is undertaken in overt bleeding (196, 197). As the clinical presentation of small bowel pathology is so varied, the first aim of this study was to identify any clinical factors which may be predictive of a diagnosis of SBA.

Recent publications have reported an increased risk of small bowel malignancies in patients less than 40 years presenting with IDA, highlighting the need for urgent investigation in this group. Although we did find a significant percentage of potential

malignancies in the less than 50 years group, our results still suggest an increased likelihood of all small bowel pathology with increasing age. In addition, we found age to be predictive of a diagnosis of SBA specifically with a RR of 4.9 with age>50 compared to age <50 years.

Our systematic literature review in the first section of this chapter looked at clinical factors predictive of the presence of gastrointestinal angiodysplasias. We identified increasing age, CKD, cardiovascular disease and the use of anticoagulant medications as the main risk factors for the presence of gastrointestinal angiodysplasias, and these factors were found to be heavily represented in our SBA cohort. This was not a surprising finding as our own study in the systematic review was the largest of all included studies. However, although it is recognised that SBA has an association with certain medical co-morbidities, particularly those driven by ischaemia or hypoxia, we found none of these conditions other than CKD to be predictive of a diagnosis of SBA. Which makes it likely that the conditions suggested by the systematic review are likely to be predictive of obscure gastrointestinal bleeding overall and not a specific diagnosis within that. Furthermore, the overall increased bleeding risk in this group may solely be secondary to their need for antiplatelet or anticoagulant therapy rather than due to the pathophysiology of a certain condition.

The systematic review also assessed the use of clinical factors to predict the presence of bleeding from gastrointestinal angiodysplasias, or the prediction of disease severity and suggested that inpatient status and multiplicity of lesions were associated with bleeding and increased severity of the condition. All patients included in our cohort had a history of gastrointestinal bleeding at some point, although not necessarily at the time of collection of clinical data or serum, therefore identifying clinical factors predictive of

bleeding episodes was not possible. However, we did assess the predictive factor of inpatient status for a diagnosis of SBA in our group of patients with OGIB of any type, by performing a subgroup analysis of patients undergoing SBCE within 4 days of presentation, and found no differences in clinical factors or clinical diagnosis in patients admitted with gastrointestinal bleeding. Not surprisingly the mode of bleeding was an important factor in predicting pathology overall, with the DY significantly higher in overt bleeding; however, it was not useful in helping to predict SBA, with no difference in DY between any groups. This is similar to published data on the natural history of the condition and highlights the difficulty in expediting a diagnosis for patients, as many are unaware of any overt bleeding, even after significant haemorrhages. We did not collect data on the number of SBAs detected in each patient, however it would be interesting to assess whether this has an impact on disease severity as suggested by our systematic review. Hb levels were found to be significantly lower in patients with SBA, which suggests that patients with a higher number of SBAs or larger lesions may have an increased disease severity. However, the occurrence of relatively higher Hb levels and a mild anaemia only in patients with potential malignancies obviates its potential use as a selection tool to prioritise patients for urgent investigation.

Overall, these results in addition to a few other small retrospective studies show the inaccuracy of using clinical factors to predict specific small bowel pathology (198-201). Specifically, as the clinical course of SBA is so unpredictable and patients are often unaware of re-bleeding episodes an objective and re-measurable serum diagnostic tool would be of far greater advantage than the dependence on clinical factors or assessing Hb levels, which don't drop until after a bleeding episode. Furthermore, the development of a serum marker specific for SBA would allow patients to be easily

triaged from other centres prior to CE, and could be monitored during known disease to reduce the need for repeated CE after a drop in Hb.

The Angiopoietin pathway plays an important role in angiogenesis and we have previously identified an association between elevated levels of Ang2 and decreased levels of Ang1 in the serum of patients with known SBA compared to non-bleeding controls. Ang1 and Ang2 are present in endothelial cells and act by binding to their receptor, the Tie2 receptor. Once bound to Tie2, Ang1 mediates vessel stabilisation and maturity. Ang2 acts as a competitive inhibitor of Ang1 for Tie2 binding where its action is to prevent vessel maturity, meaning that an excess of Ang2 leads to excessive uncontrolled growth of weakened vessels (202). More recently a Tie2 independent role of Ang2 has been suggested via binding of integrins, again with a vessel destabilising effect (203). The discovery of abnormalities of these factors in the serum of patients with SBA may well offer an explanation for the pathophysiology of the condition, as angiodysplasias are characterised by the presence of tortuous and weakened blood vessels, lacking a basement membrane, in the submucosal layer of the gastrointestinal tract. Much work needs to be done to fully delineate the role of the Angiopoietins in SBA, and the validation of Ang1 and Ang2 as predictive factors of SBA needs to occur prior to their use clinically which was the aim of this study.

One of the concerns raised by the peer reviewers of our publication from chapter 2 describing our initial findings of abnormalities in serum levels of angiogenic factors in SBA patients was that our use of non-bleeding controls may mean that the abnormalities in Angiopoietin levels may have been driven by OGIB or anaemia overall and not specifically by SBA. One of the main objectives of this study therefore was to

measure levels of these factors in groups of patients with OGIB and IDA of varied causes compared to SBA. This assessment firstly validated our previously detected association of elevated Ang2 levels in patients with SBA. In addition, this study has shown that the elevation of serum levels of Ang2 is specifically associated with a diagnosis of SBA, and is not seen in other causes of OGIB. Further to this, after controlling for anaemia, we could not find an association between anaemia and elevated Ang2 levels. This suggests that the response in Ang2 levels in SBA patients is thereby not influenced by a general angiogenic response to gastrointestinal bleeding, and is more likely to be linked to the underlying pathophysiology of the condition.

In contrast to this, our results showed that the decrease in serum levels of Ang1 does not appear to be specific for SBA and may be related to other factors such as OGIB or anaemia overall as there were no significant differences in the mean or median levels of Ang1 between SBA patients and patients with abnormal small bowel findings. Although there was a significant difference in Ang1 levels between SBA patients and patients with normal findings ($p=0.04$) the confidence interval was wide and as seen by the boxplot images the ranges of Ang1 in all groups were extremely similar, making it useless as a diagnostic aid. Interestingly however there was still a significant difference in the ratio of Ang1/Ang2 levels between SBA and non-SBA patients, which likely reflects the interplay of Ang1 and Ang2 and their binding to their receptor Tie2. The finding of a significantly different ratio of Ang1/Ang2 only in SBA patients reaffirms the hypothesis put forward in chapter 2 of a likely role for the Angiopoietins in the pathophysiology of SBA formation, which is not influenced by anaemia or gastrointestinal bleeding overall. No significant differences were found in serum TNF- α levels between any of the groups, although there appeared to be a trend towards lower levels in SBA patients. Of the patients with

abnormal findings, approximately 50% had an enteritis. As circulating TNF- α levels are known to be elevated in intestinal inflammation we might have expected this group to have a higher median level of TNF- α , however levels were comparable to both the SBA and normal small bowel group.

The second aim of our study was to determine whether serum levels of Ang1 or Ang2 could be used as a filter test in patients with OGIB, to predict patients likely to have SBA and prioritise them for SBCE. The difficulty in establishing levels for a diagnostic tool is defining a cut off level with adequate sensitivity and specificity levels to make them clinically useful. Using ROC curve analysis we determined that an Ang2 level of 2600pg/ml with a sensitivity level of 83% and a negative predictive value of 85%, was the most clinically appropriate cut-off level. Although an AUC of 0.7 is not particularly accurate for a diagnostic tool, the purpose of this screening tool is to identify patients likely to have SBA and prioritise them for CE and not to decide whether or not to perform the test, as all patients deemed clinically to require SBCE will undergo the examination. Therefore, the most important aspects of this tool were the sensitivity and negative predictive value for a diagnosis of SBA. Our results showed that Ang2 in isolation could be used as a diagnostic tool, as although the ratio of Ang1/Ang2 differed significantly from other causes of OGIB, we found no diagnostic benefit to measuring both serum factors, making the test more economically feasible if translated to clinical practice.

One of the limitations of this study was the retrospective design of the clinical predictive factors assessment meaning that some information on patient co-morbidities or medication usage may not have been accurately captured. Furthermore, we did not have available information on the interval of bleeding from a SBA to data capture,

meaning that the presence of clinical conditions and medication usage was taken at a single point in time and the bleeding event may have occurred prior to the presentation of a clinical condition or prior to commencement of anticoagulants etc. although this is likely to represent a very small number of cases. Similarly, serum samples were taken at a single point in time from patients undergoing CE, the majority of whom may not have been actively bleeding at the time, which means that we could not assess whether serum Ang2 levels were representative of clinical severity. It was therefore beyond the scope of this study to assess the ability of serum angiogenic factors as biomarkers of disease activity, however an interesting assessment and a necessity in determining whether serum Ang2 levels could be used as a prognostic marker would be to take serial measurements from patients with known SBA and establish whether levels correlate with changes in disease course, around the time of active bleeding, or following a definitive treatment intervention.

A recognised weakness of our study is that the patients with a normal SBCE had lower mean Hb levels than the other groups. All patients at the time of referral were anaemic, however due to significant waiting times for CE in our unit a significant proportion of these patients may have had a spontaneous recovery of their anaemia by the time they underwent CE. This is likely to be the case in clinical practice as patients will receive some empiric treatment with iron or red cell transfusions and future prospective studies may determine whether a Hb level alone could be used as a predictive or prioritisation tool in milder cases of anaemia, with serum Ang2 reserved for currently anaemic patients only. In addition, our study relied on CE being the gold standard for diagnosis of small bowel causes of OGIB, however it must be recognised that the sensitivity of CE is

not 100%, and there is a possibility that some of the patients in the normal will include those with false negative studies which may have impacted Ang2 levels.

Conclusion:

This study has shown that clinical factors are not useful in predicting the presence of SBA in a group of patients with anaemia and OGIB due to the shared clinical characteristics of most patients undergoing CE for this indication, including cardiovascular disease, CKD and anticoagulant use. This study has validated our previous findings showing an elevated serum Ang2 in patients with SBA. In addition, it has shown that this association is specific for SBA and is not driven by bleeding or anaemia of other causes. We have identified the potential use of serum Ang2 as a diagnostic aid in SBA, and developed a basis for further work to examine its use as an indicator of disease activity. In the future these findings may lead to a more timely diagnosis for patients with SBA and provide a more accessible and non-invasive mode of follow up. Further validation of the use of Ang2 at this cut-off level in a larger group of patients with OGIB and IDA are needed to ensure its accuracy as a potential predictive tool, however it offers a potentially cheap and non-invasive marker to aid in the diagnosis of SBA.

Chapter 4 – Multiplex and ELISA study

Publication: submitted to BMC Gastroenterology

Poster Presentations:

1. Irish Society of Gastroenterology Winter meeting 2016. The identification of novel putative angiogenic factors in small bowel angiodysplasia.
2. United European Gastroenterology Week, Vienna, 2018. The determination of novel angiogenic factors in small bowel angiodysplasia.
3. the use of ELISA measurements of factors detected by the multiplex study in a cohort of patients with other causes of obscure gastrointestinal bleeding

Introduction:

Following on from our putative assessment of angiogenic factors in SBA we developed an appreciation for the likely role of the Angiopoietin pathway (Ang 1 and Ang2) in SBA formation. However, we also found conflicting evidence with the previously published, although admittedly limited, literature regarding angiogenic factors in gastrointestinal angiodysplasia overall. Of particular note, our results so far indicated that VEGF did not appear to be related to SBA formation. As VEGF has been one of the most widely studied factors in angiogenesis to date, and has a readily available anti-angiogenic pharmaceutical form available (Bevacizumab), we wanted to gain further clarity and reassurance that we were following the correct line of interest. Over the last decade, and since the beginning of this PhD study, angiogenesis research has rapidly developed and become a more widely studied area. Many studies are now uncovering the complex roles of anti and pro- angiogenic factors, and the concept of factors having dual roles, capable of both inducing and prohibiting angiogenesis, is emerging regularly. This dual

role is now understood to occur at certain threshold levels, often influenced by other up or downstream angiogenic, hypoxic or inflammatory factors.

In this chapter we hoped firstly, to be able to determine whether the angiopoietin pathway may be only the “tip of the iceberg” among other key pathways in SBA formation, and secondly, whether up or downstream factors of VEGF, or alternative forms or subtypes to the one we had tested for, were implicated in SBA, and may account for the contradictory results we had observed. We decided to use a new commercially available multiplex angiogenic array kit to measure the relative expression of 55 angiogenic factors in the serum of SBA patients and controls (Table 20). Although less sensitive than the quantitative ELISA serum assessments we had previously performed, we hoped that this array would identify some prominent factors involved, which could then be confirmed by more precise ELISA measurements.

Activin A	FGF-7/KGF	PD-ECGF
ADAMTS-1	GDNF	PDGF-AA
Angiogenin	GM-CSF	PDGF-AB/PDGF-BB
Angiopoietin-1	HB-EGF	Persephin
Angiopoietin-2	HGF	CXCL4/PF4
Angiostatin/Plasminogen	IGFBP-1	PIGF
Amphiregulin	IGFBP-2	Prolactin
Artemin	IGFBP-3	Serpin B5/Maspin
Tissue Factor/Factor III	IL-1 beta	Serpin E1/PAI-1

CXCL16	CXCL8/IL-8	Serpin F1/PEDF
DPPIV/CD26	LAP (TGF-beta 1)	TIMP-1
EGF	Leptin	TIMP-4
EG-VEGF	CCL2/MCP-1	Thrombospondin-1
Endoglin/CD105	CCL3/MIP-1 alpha	Thrombospondin-2
Endostatin/Collagen XVIII	MMP-8	uPA
Endothelin-1	MMP-9	Vasohibin
FGF acidic	NRG1-beta 1	VEGF
FGF basic	Pentraxin 3	VEGF-C
FGF-4		

Table 20. The 55 angiogenic factors measured by the multiplex angiogenesis array

Background to the 55 angiogenic factors tested and their potential relationship to angiogenesis in angiodysplasia

As angiogenesis is an important process in all cell growth and development, this kit assesses levels of a broad range of factors, and understandably not all factors appeared to be of significant interest in gastrointestinal angiogenesis. We performed a literature review to determine the significance of each factor to gastrointestinal angiogenesis and have briefly summarised the key role of relevant factors below.

Activin appears to be relevant to the reproductive system and fertility.

ADAMTS-1 is a predominantly anti-angiogenic protein which is known to induce downstream changes in VEGF (affecting vascular integrity) and vWF (affecting coagulation) (204-207).

Amphiregulin is a soluble paracrine growth factor, involved in physiological responses required for healthy organ development, however it has also been implicated in neoplastic proliferation of certain cancers including colorectal cancer (208, 209).

Tissue factor is a coagulation factor, sensitive to hypoxia which is usually confined to sub-epithelial cells during homeostasis but can migrate to endothelial cells in induced states, particularly in response to growth of angiogenic malignancies (210, 211).

Epidermal Growth Factor (EGF) is known to be an important factor in controlling the angiogenesis required for tumour growth. It is found to be elevated in most solid organ malignancies and is closely related to VEGF, as it is thought to upregulate VEGF expression (212).

Endocrine-gland-derived (EG)-VEGF, is predominantly confined to the reproductive organs and is thought to be a crucial factor in the development of the placenta (213). However, it has also been shown to have some pro-angiogenic effects in the gastrointestinal tract, particularly in relation to colorectal cancer progression, and although distinct to VEGF, the two factors are often simultaneously elevated in certain malignancies and pathological responses, suggesting a common pathway of action (214, 215).

Endostatin, also referred to as *Collagen XVIII*, is a potent angiogenic inhibitor which has an important role in vessel stabilisation through its action at the level of the basement membrane (216).

Endothelin-1 is a potent vasoconstrictor with a role in vessel growth and proliferation (217). Endothelin-1 has a very close relationship with VEGF, with its release stimulated by inhibition of VEGF (218).

Both acidic and basic forms of *Fibroblast Growth factor (FGF)* have been identified as important pro-angiogenic factors in new vessel growth with an inter-dependent relationship with VEGF (219). FGF is required to stimulate VEGF release, with FGF signalling requiring a threshold level of VEGF to enable its function (220, 221). FGF4 has been shown to have a vessel-stabilising role, similar to that of Ang-1, and has been shown in pre-clinical trials to enhance healing in hypoxic injury when over-expressed simultaneously with VEGF (222). *FGF7/Keratinocyte Growth Factor (KGF)* has been shown to be an important angiogenic factor in several gastrointestinal malignancies. Although the protein itself is not expressed within tumour cells, its receptor (KGFR) is expressed by most cancer cells, and its presence has been shown to induce the expression of certain pro-angiogenic factors, particularly VEGF-A (223).

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is an important regulator of haematopoietic cell differentiation and growth. However, it has also been shown to modulate healing in endothelial cell layers and can be induced by many inflammatory stimuli, including TNF- α (224). GM-CSF has a regulatory role in angiogenesis by controlling VEGF expression and mediating the Ang1/Ang2 ratio (225).

Heparin-Binding Epidermal-like Growth Factor (HB-EGF) has been demonstrated as an important factor in promoting repair in hypoxia induced injury in the intestines, via a vasodilatory effect and a reduction in inflammatory cytokines including TNF- α and a number of interleukins (226, 227).

Hepatocyte Growth Factor (HGF) is found in normal gastrointestinal endothelial cells and plays a role in cell proliferation and growth, however, under pathological conditions, it has been shown to promote angiogenesis via upregulation of VEGF (228).

The *Insulin Growth Factor-Binding Proteins (IGFBPs)* 1-3 are known to have a role in cell growth and are present in normal gastrointestinal cells, however more recently their role in angiogenesis has been reported (229). *IGFBP-2* in particular has been shown to activate VEGF expression, although its role in tumorigenesis in the gastrointestinal tract is not reported (230). *Interleukin-1 beta (IL-1 β)* is a pro-inflammatory cytokine with the ability to exert an angiogenic effect via the induction of several pro-angiogenic proteins, most notably IL-6 and CXCL8, which both cause increased VEGF expression (231, 232).

Transforming Growth Factor (TGF- β) is an important regulator of cell growth and division and is known to be involved in limiting the progression of tumorigenesis, however at certain levels it has also been shown to have a pro-angiogenic and pro-metastatic effect in certain malignancies via an incompletely understood effect on Thrombospondin and SMAD signalling (233, 234).

Leptin is a cytokine with a role in regulating food intake and body composition. Leptin has been implicated in angiogenesis in certain types of cancer via an effect on *Hypoxia inducible factor (HIF)*, *HIF-1 α* and the *Matrix Metalloproteinases MMPs* (235, 236).

The *chemokines CCL2 and CCL3* have a recognised role in the inflammatory process but they have also been shown to initiate angiogenesis in specific malignancies, thought to be through a direct action on VEGF and the MMPs (237, 238).

The main role of the MMPs is in wound healing by degradation of the extra-cellular matrix but they have also been shown to have a role in angiogenesis, and their action appears to be controlled by many cytokines (including TNF- α and VEGF) and TIMPs (239). Both *MMP8* and *MMP9* have been implicated to have a role in gastrointestinal angiogenesis in both malignant and inflammatory conditions (240).

Neuregulin, *NRG1-β-1* is expressed in cardiac tissue and has been shown to have an angiogenic effect there through a likely effect on Ang1 and VEGF, however its effect in the gastrointestinal system has not been reported (241).

Pentraxin 3 has been shown to have an important role in vascular inflammation. It has been shown to have a tissue-specific role in angiogenesis with either a pro or anti-angiogenic effect (242, 243). Elevated serum levels of Pentraxin 3 have been reported in patients with HHT and epistaxis, however it has not yet been associated with gastrointestinal bleeding in this cohort and seems to have a limited role in gastrointestinal angiogenesis (244).

Platelet Derived Endothelial Cell Growth Factor (PD-ECGF) is also known as Thymidine Phosphorylase (TP) and has its angiogenic effect by stimulating endothelial cell migration via the phosphorylation of thymidine in a number of enzymatic reactions (245). Several studies have correlated the overexpression of PD-ECGF/TP with gastrointestinal and other organ malignancies, and an association with over-expression of VEGF has been detected (246-248). *Platelet Derived Growth Factor (PDGF)* is a 30 kDa dimer composed of an A- and/or B-chain, which are encoded by separate genes and regulated independently (249). PDGF is an important factor controlling cell turnover and PDGF signalling has been shown to be highly active in mesenchymal cells including smooth muscles cells, fibroblasts and pericytes (250). Different functions are attributed to the different family members of PDGF, with *PDGF-B* shown to have an analogous and coordinated role to Ang-1, although both factors seem to be required at different stages of cell development (252). The action of PDGF in endothelial cells is closely related to VEGF, although the role of PDGF-B in this regard is more completely understood than

that of *PDGF-A* (252). Studies have shown that increased PDGF signalling leads to upregulation of VEGF-A, and in return VEGF-A enhances PDGF-B expression (253).

Platelet Factor 4 CXCL4/PF4 is a platelet derived chemokine with anti-angiogenic properties via binding to Heparan sulfates, growth factor and integrins (254). PF4 has been shown to have an inflammatory effect on the degradation of endothelial cell integrity in the presence of co-inflammatory stimuli such as TNF- α (255).

Placental Growth Factor (PlGF) is a pro-angiogenic factor related to the VEGF family, which has its effect via binding to the VEGF receptor and attenuating its action (256).

The *Serpins* are a large family of protease inhibitors, recently discovered to have a role in angiogenesis, predominantly as anti-angiogenic factors. *Serpin B5/Maspin* is an angiogenic inhibitor via a direct action on endothelial cells preventing their migration towards BFG and VEGF (257). In contrast, *Serpin E1/ Plasminogen Activator Inhibitor -1 (PAI-1)* has been shown to have both pro- and anti-angiogenic effects, depending on its local concentration levels, and its effect is mainly via effects on VEGF expression (258, 259). *Serpin F1/ Pigment Epithelium Derived Factor (PEDF)* has its anti-angiogenic effects by inhibiting the action of VEGF and binding with certain integrins (260).

A delicate balance between the *Tissue Inhibitor of Metalloproteinase (TIMPs)* and MMPs is required for normal extracellular matrix (ECM) development and integrity, with impaired ECM remodelling in blood vessels leading to disordered and weakened vessels. *TIMP1* has been shown to play a protective role in vascular remodelling, as an anti-angiogenic factor, through its inhibitory action on *MMP-9* (261). The specific mode of action of *TIMP4* as an angiogenic factor is still being defined, however elevated circulating levels of *TIMP4* have been associated with certain angiogenic conditions,

including systemic sclerosis and cardiovascular disease, and certain malignancies (262, 263).

Thrombospondins (TSP) have been shown to affect the ECM structure with an important role in tissue remodelling. *TSP-1* has been demonstrated to be an important inhibitor of tumorigenesis and angiogenesis via downregulation of on TGF- β , FGF and VEGF (264, 265). *TSP-2* has been shown to have similar actions, which are modulated and repressed by increasing levels of HIF-1, a factor which has been demonstrated as a poor prognostic factor in gastrointestinal malignancies (266, 267).

Urokinase Plasminogen Activator (uPA) has been shown to have a central pro-angiogenic role in the migration of endothelial cells during angiogenesis (268). This action arises predominantly via stimulation by VEGF, but also to a lesser degree by FGF, EGF and HGF, with the action inhibited by upregulation of PAI-1 (269, 270).

Vasohibin is an endothelium derived anti-angiogenic factor, with its expression known to be upregulated by VEGF and FGF (271). Its action is thought to be via a negative feedback reaction resulting in suppression of the VEGF receptor (272). *VEGF-C* is mainly thought to be involved in the development of the lymphatic vasculature, however it has been shown to have a role in angiogenesis in hypoxic conditions by increasing vascular permeability (273, 274).

To further simplify the above information the below table (Table 21) outlines which of the 55 angiogenic factors tested for by the multiplex kit and their reported significance in the literature in 4 key areas; gastrointestinal angiodysplasia, gastrointestinal angiogenesis overall, interactions with VEGF, and interactions with the Angiopoietins.

Table 21. A summary of the angiogenic factors included in the multiplex assessment associated in the literature with GIAD, general angiogenesis, interactions with VEGF, and interactions with the Angiopoietins.

Significance in gastrointestinal angiodysplasia	Angiopoietin -1, Angiopoietin -2, Endoglin/CD 105, PDGF-BB, VEGF
Significance in general gastrointestinal angiogenesis	ADAMTS-1, Angiogenin, Amphiregulin, Tissue Factor, DPPIV/CD26, EGF, EG-VEGF, Endoglin/CD105, Endostatin/Collagen XVIII, FGF-acidic, FGF-basic, FGF7/KGF, HB-EGF, HGF, IL-1 β , CXCL8, MMP8, MMP9, PD-ECGF, TSP-1, TSP-2
Known to interact with VEGF	ADAMTS-1, Amphiregulin, TF, EGF, EG-VEGF, Endostatin/Collagen XVIII, Endothelin-1, FGF-acidic, FGF-basic, FGF4, FGF7/KGF, GM-CSF, HGF, IGFBP-2, IL-1 β , CXCL8, CCL2, CCL3, MMP8, MMP9, NRG1- β 1, PD-ECGF, PDGF-BB, PlGF, Serpin B5, Serpin E1, Serpin F1, TSP-1, TSP-2, uPA, Vasohibin, VEGF-C
Known to interact with the Angiopoietins	GM-CSF, NRG1- β 1, PDGF-BB

Aims:

1. To determine whether the angiopoietin pathway may be only one of several other pathways associated with SBA formation.
2. To determine whether up or downstream factors of VEGF, or alternative forms to the one we had previously tested for, were implicated in SBA which could account for the contradictory results we had found to date.

Methods:**Human angiogenesis array**

Blood was collected from patients with a definite diagnosis of SBA and from healthy non-bleeding controls as described in previous chapters. The blood samples were centrifuged at 1000rpm for a minimum of 10 minutes, the resultant serum supernatant was then extracted and stored in aliquots at -80C for later batch analysis. The human angiogenesis assay (ARY007; R&D Systems, Minneapolis, MN) was performed as per the manufacturer's guidelines.

In brief, the human angiogenesis array is a nitrocellulose membrane with 55 selected capture antibodies spotted to it in duplicate.

1. Serum samples (0.5ml each) are diluted and mixed with a cocktail of biotinylated detection antibodies and the resultant sample/antibody mixture is then incubated with the angiogenesis array membrane.
2. Any protein/detection antibody complexes present are then bound to the antibodies spotted on the nitrocellulose membrane during overnight incubation.
3. The membrane is then washed a number of times using distilled water and buffers before being placed in a diluted solution of Streptavidin-horseradish peroxidase.

4. Following a 30-minute incubation period the washing steps are then repeated, prior to the membrane being evenly covered by a prepared chemi-reagent mix and allowed to incubate for 1 minute before being removed.
5. The membranes are then tightly covered in cling film and placed in an autoradiography film cassette and are exposed to x-ray film for a variety of exposure times to ensure adequate signal spots are obtained.
6. Following development of the film the positive signals are identified by placing the transparency overlay on the array image and aligning it with the pairs of reference spots in each array.
7. The pixel densities on the developed film are then collected and analysed using a transmission mode scanner and image analysis software. Densitometric values of duplicate samples were averaged and subtracted from the average densitometric value for each negative control to compensate for background. Values are reported in densitometric units (DU). Molecules that were present at levels below that of the negative control were assigned a value of 0 DU. Relative levels of DU for each angiogenic protein was then collected for each patient and control and mean relative levels were generated for the patient and control groups.
8. Mean relative levels in each group were compared using a Mann Whitney U test with a p value of <0.05 considered statistically significant. All analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL).

Validation of detected factors by ELISA measurement

Significant differences were detected in 5 of the 55 angiogenic factors detected by the multi-detection angiogenesis array kit (Ang-1, Ang-2, Endostatin, TIMP-1 and PDGF-AA)

and precise quantitative measurements of these factors were then performed using commercially available ELISA kits. Blood samples were collected, processed and stored as in the above step from a larger number of both SBA patients and controls with no evidence of gastrointestinal bleeding as described before. Data was also collected regarding patient demographics and Hb levels.

ELISA measurements of the three new factors (Endostatin, TIMP1, and PDGF-AA) identified by the multiplex angiogenesis array were performed using commercially available ELISA antibody array kits (DNSTO, DTM100, DAAOOB; R&D systems, Minneapolis, MN). Repeat measurements of Ang-1 and Ang-2 were not performed. All measurements were performed according to the manufacturer's guidelines. The exact methods for the ELISA measurements were described in detail in chapter 2. Samples were prepared in duplicate and results were read at 450 nm absorbance.

Statistical analysis

Results of all assays and patient demographics were expressed as a mean and/or median and compared between patients and controls using the Student t test or Mann-Whitney test, as appropriate, with a difference of <0.05 considered significant. The presence of confounding variables was determined by using a one way ANOVA test, with a p value of <0.05 considered significant. All analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL).

Results:

Multi-detection angiogenesis array:

An initial assessment using the 55 antibody array assay was performed in 14 samples including 7 SBA patients and 7 controls. There were no differences in age or gender in the two groups with mean ages of 60.3 years (range 52 – 73) and 67.0 years (range 53 – 79) [$p=0.085$] and Male: Female gender ratios of 4:3 and 5:2 [$p=0.306$] for the control and SBA patient groups respectively.

Significantly lower levels of four factors were found in patients with SBA vs controls including: Ang-1, PDGF-AA, Endostatin/Collagen XVIII and TIMP1, with a significantly higher level of Ang-2 in SBA patients compared to controls. The image below (figure 19) shows the developed film with the reference spots for 4 of the tested samples. Table 22 shows the mean DU for the significant factors in SBA patients and controls.

Figure 19. A photograph of one of the developed films showing the reference spots for the 55 angiogenic factors and the control factor in 4 of the examined serum samples.

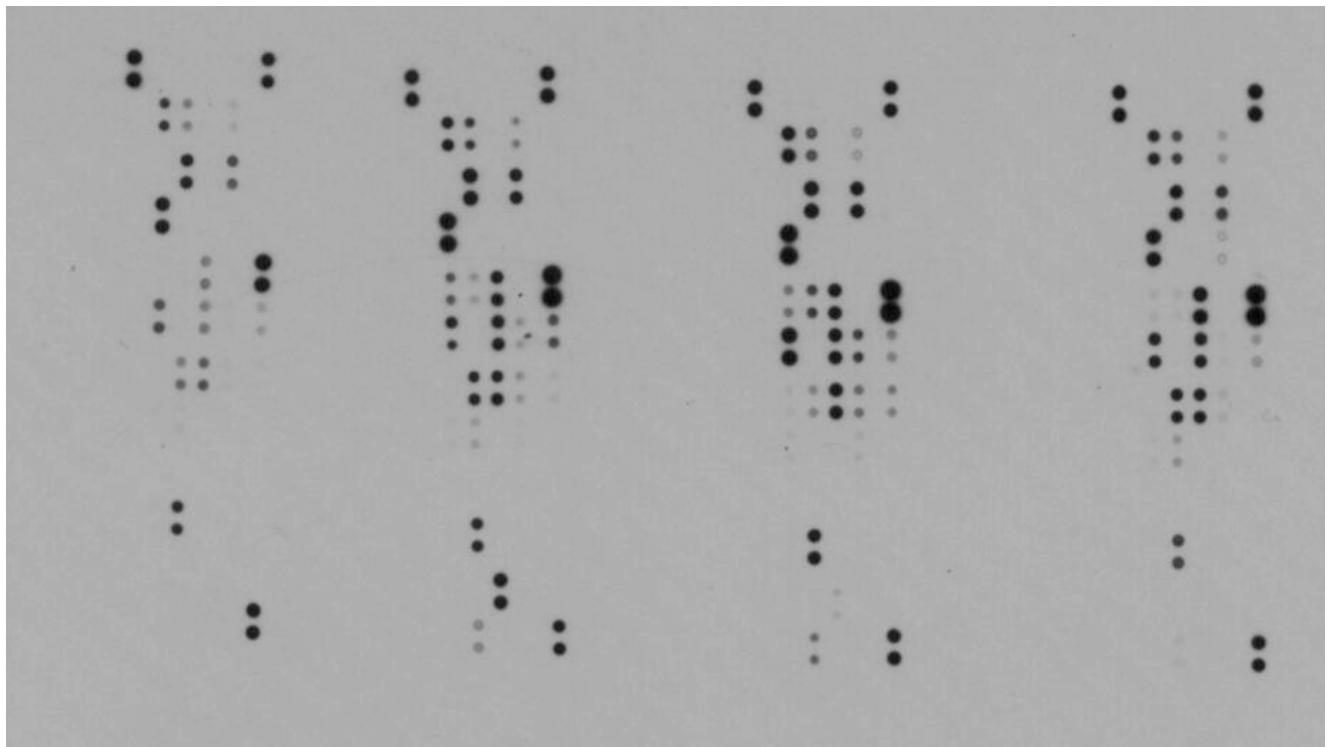


Table 22. The mean DUs for the 5 factors found to be significantly different in SBA patients compared to controls.

	Angiopoietin-1	Angiopoietin-2	Endostatin / CollagenXVIII	PDGF-AA	TIMP-1
Controls					
C1	2049	265	1602.5	3001	5508
C2	3265.5	265	2785.5	1812.5	2274.5
C3	2763.5	295	709.5	431	2096.5
C4	825	82	695.5	1237.5	3546
C5	1068	265.5	546	1244.5	3216.5
C6	413.5	106.5	1057	427.5	3932.5
C7	1375.5	152.5	740.5	823.5	3152
Mean	1680	204.5	1162.357143	1183	3389.428571
SBA patients					
SBA1	640	268	535.5	583	1731
SBA2	462.5	360	385	79.5	458.5
SBA3	818	204.5	638	190.5	3164
SBA4	512.5	374.5	495	349	3188
SBA5	986	410	520	843	1149.5
SBA6	1131	459	581	570.5	3003.5
SBA7	186.5	201	125	804.5	714.5
Mean	676.6428571	325.2857143	468.5	488.5714286	1915.571429
P value	0.0327	0.035	0.044	0.0473	0.0359
95% CI	97.320- 1909.394	10.020- 231.551	21.923- 1365.791	11.151- 1576.707	114.162- 2833.552

Interestingly, in correlation with our findings in the first study to identify putative angiogenic factors in SBA, there were no significant differences in relative expression levels of other factors which had been associated with angiodysplasia in previous publications; including various forms of VEGF, sEnd and PDGF-AB/BB. The relative expression levels (mean DUs) of other factors of interest including VEGF, sEnd are shown in Table 23.

Table 23. The mean DUs of some of the factors suggested to be significant in GIAD in previously published studies in controls and SBA patients.

	EG-VEGF	Endoglin	MMP-9	PDGF-AB / PDGF-BB	TIMP4	VEGF	VEGF-C
Controls							
C1	114	1655.5	6446	520.5	720	208.5	32
C2	82	3060.5	3474.5	1412	1101	157.5	141
C3	87.5	1905.5	4148.5	272.5	165.5	167.5	179.5
C4	124	231.5	2925.5	343	118.5	114	71
C5	102	299.5	4607	179.5	143.5	98.5	52.5
C6	98	288	4066.5	160	199.5	147	81.5
C7	104.5	716	249.5	133.5	2713.5	83	360.5
Mean	101.714	1165.214	3702.5	431.571	737.357	139.428	131.142
SBA patients							
SBA1	115	1591	3446	95	421.5	157	75.5
SBA2	243.5	721.5	351	100	3524	58.5	302
SBA3	105	1040	4580.5	119	403	278	44
SBA4	88	850.5	5454.5	123.5	693.5	301	125.5
SBA5	152	3622.5	3534	517.5	750	123.5	95
SBA6	106.5	1565.5	5199.5	116.5	328	205	168
SBA7	121	99	876.5	1041.5	38	271	79
Mean	133	1355.714	3348.857	301.857	879.714	199.142	127
p value	0.086	0.375	0.370	0.282	0.404	0.075	0.470

Serum ELISA measurements of the additional 3 factors identified by the multiplex

assay:

ELISA measurements were performed in 20 patients with SBA and 20 non-bleeding controls. The patients with SBA were significantly older than the control group with mean ages of 72.75 (51-93) and 45 (18-73) respectively ($p < 0.001$, 95% CI 18.99 to 36.51), however there was no difference in gender, with females accounting for 40% ($n=8$) of the patients with SBA and 55% ($n=11$) of the control group ($p=0.355$). Of the SBA

patients, 85% (n=17) were anaemic, compared to only 30% (n=6) of the controls, with a significant difference in the mean Hb levels between the two groups at 10.79g/dL (9 - 12.5) and 12.59g/dL (9.6 – 15.4) respectively.

Overall significant differences were detected in mean levels of Endostatin/Collagen XVIII and TIMP1 between the two groups, however no differences were observed in levels of PDGF-AA. The mean and median levels of each factor are outlined in Table 24.

Table 24. Mean serums levels of TIMP1, Endostatin and PDGF-AA in SBA patients and controls.

	Endostatin/Collagen		
	TIMP1 (ng/mL)	XVIII (ng/mL)	PDGF-AA (ng/mL)
SBA Patients n=20			
Mean	425.102	556.803	36080.105
Median	392.248	524.521	32573.18
Range	134.34 – 616.3	123.26 – 909.39	376.08 – 81945.71
Controls n=20			
Mean	558.924	387.120	37303.636
Median	520.254	320.957	377769.09
Range	374.2 – 829.94	123.26 – 925.55	478.37 – 70024.52
p value	0.0028	0.0336	0.866
95% CI	(230.993) -(52.299)	13.885 - 325.482	N/A

Effect of age, anaemia and gender on levels of each factor:

One way ANOVA testing was used to determine confounding variables (age >50 years, gender and the presence of anaemia) [table 25]. Of these variable factors, only age >50 years was found to be a confounding factor affecting Endostatin levels.

Table 25. Results of one-way ANOVA testing to determine the significance of age>50 years, gender, and anaemia on mean serum levels of TIMP1, Endostatin and PDGF-AA in SBA patients and controls.

One way ANOVA	Group		TIMP1	Endostatin / Collagen XVIII	PDGF-AA
Age>50 yrs.	SBA patients	f ratio	N/A	N/A	N/A
		p value	N/A	N/A	N/A
	Controls	f ratio	1.204	4.929	0.422
		p value	0.288	0.04	0.524
Gender	SBA patients	f ratio	0.066	1.172	3.398
		p value	0.8	0.294	0.082
	Controls	f ratio	1.072	0.119	1.145
		p value	0.315	0.734	0.299
Anaemia	SBA patients	f ratio	N/A	N/A	N/A
		p value	N/A	N/A	N/A
	Controls	f ratio	3.403	2.924	0.023
		p value	0.083	0.106	0.881

When age >50 or <50 years was considered a binary factor in the control group, we noticed a higher mean Endostatin/Collagen XVII level in the group aged >50 years (n=7) of 523.57ng/mL compared to those aged <50 years (n=13) of 324.14ng/mL (p=0.0403, 95% CI -388.945 - - 9.910). When Endostatin/Collagen XVIII levels in the SBA patient (n=20) and control (n=7) groups were controlled for age >50 years there was no difference in mean levels between the groups at 556.803ng/mL and 523.571 ng/mL respectively (p=0.777), suggesting that age may be a confounding factor associated with Endostatin/Collagen XVIII expression. Age did not seem to be a confounding factor affecting mean levels of TIMP1 or PDGF-AA with no variation in significance levels of either factor when controlled for age <50 or >50 years. When the groups were controlled for gender and for the presence of anaemia, patients with SBA were still found to have significantly lower levels of TIMP1 and higher levels of Endostatin/Collagen XVIII, with no differences in levels of PDGF-AA, suggesting that gender and the presence of anaemia were not confounding variables (Table 26).

Table 26. Mean serum levels of TIMP1, Endostatin and PDGF-AA in SBA patients and controls according to variable factors of age>50 years, gender, and anaemia, compared using a Student's t test.

Factor controlled for		Endostatin		
		TIMP1 ng/mL	/Collagen XVIII ng/mL	PDGF-AA ng/mL
Age >50 years	SBA patients n= 20	425.102	556.804	36080.105
	Controls n=7	602.597	523.571	41706.68
	p value	0.0059	0.777	0.586
	95% CI	(298.295) - (56.695)		
Gender	SBA n=12	418.264	525.036	31299.176
	Controls n=9	521.881	349.188	43397.475
	p value	0.03	0.049	0.243
	95% CI	11.144 - 196.089	(415.449) - (2.117)	
Presence of anaemia				
	SBA patients n=17	410.767	525.896	37684.034
	Controls n=6	647.567	260.201	38514.865
	p value	0.003	0.019	0.464
	95% CI	(384.191) - (89.409)	47.553 - 483.836	

Discussion:

The initial assessment of 55 angiogenic factors identified 5 factors with significant differences in serum levels between SBA patients and non-bleeding controls, Ang-1, Ang-2, TIMP-1, Endostatin/Collagen XVIII and PDGF-AA. Although the number of patients (n=7) and controls (n=7) in this group were small, we still managed to find statistically significant results which correlated with our initial finding of an association with Ang-1 and Ang-2, thereby further strengthening this association. Reassuringly, there were no significant differences in the mean expression levels of the remaining 50 angiogenic factors, suggesting that the Angiopoietin pathway is likely to be the key pathway in SBA formation. Furthermore, our previous negative findings of any association of SBA with VEGF, sEnd, or PDGF-AB/BB (in chapter 2), suggested in the literature to be associated with colonic angiodysplasia and a hereditary form of gastrointestinal angiodysplasia (HHT), was further supported by negative findings in this assessment. During our literature review of the specific role of each of the 55 factors tested for by the multiplex kit, we found that approximately half of them had reported evidence of a significant role in angiogenesis within the gastrointestinal tract, ranging from normal blood vessel development to disordered pro-neoplastic angiogenesis (Table 21). Furthermore, as VEGF is a critical mediator of angiogenesis overall, we performed a review to determine factors which specifically interact with VEGF to exert their angiogenic effect, either at an upstream or downstream level. This identified an even greater proportion of the 55 factors, which we felt made the multiplex assessment a worthwhile and relevant assessment tool in our work. There was a suggestion from the multiplex study of a trend towards higher levels of both VEGF and EG-VEGF (p levels of 0.075 and 0.086 respectively), which could in theory become significant if tested in a larger cohort,

however, our putative ELISA assessment of quantitative serum levels of VEGF in SBA patients and controls allowed us to disregard these trends with reasonable confidence and focus on the significant results we obtained to direct our next steps. Additionally, although the great majority of the 55 factors assessed are known to interact with VEGF, only Endostatin was found to be associated with SBA, which further strengthens our conclusion that VEGF is not a key mediator of SBA development. The specific findings of a decreased serum level of Ang-1 and increased serum level of Ang-2 fit with our hypothesis of an alteration in the ratio of Ang-2/Ang-1 levels with the disordered vessel growth associated with excess Ang-2 winning out over the protective vessel stabilising role of Ang-1. However, the identification of reduced serum levels of three new factors – TIMP-1, Endostatin/Collagen XVIII and PDGF-AA in patients with SBA compared to non-bleeding controls was an interesting new observation.

In the second part of this study we performed a quantitative serum assessment of the factors identified by the multiplex study in SBA patients compared to non-bleeding controls, in a larger cohort which we hoped would either confirm or exclude the association detected. As we had already performed quantitative ELISA measurement of Ang-1 and Ang-2 in this cohort, and in a further prospective cohort, which were in fact validated by the multiplex assessment, we elected not to repeat these measurements and performed ELISA assessments of the three new factors only, TIMP-1, Endostatin and PDGF-AA. This assessment confirmed a statistically significant difference in mean serum levels of TIMP-1 and Endostatin between SBA patients and non-bleeding controls, however it rejected any difference in mean levels of PDGF-AA between the two groups. An unexpected finding however, was that although this assessment detected a

significant difference in Endostatin between the groups, this difference was contradictory to that of the multiplex study. In fact, the quantitative ELISA assessment determined that patients with SBA had higher mean circulating levels of Endostatin than non-bleeding controls. The lower levels of TIMP-1 in SBA patients compared to controls was confirmed by the quantitative ELISA measurement.

Endostatin, an anti-angiogenic factor, is found in only a few organs in humans, of which the small intestine is one. It is thought to have an important role in tumour angiogenesis, and has a currently available recombinant human pharmaceutical form, Endostar, used predominantly in lung cancer, but also in trials of colorectal and gastric cancer. Studies have shown that Endostatin has its effect by directly binding to VEGF receptors 1 and 2 to prevent the downstream angiogenic action of VEGF tyrosine-kinase-induced effects, following binding to both receptors. It also causes downregulation of Hypoxia-Inducible Factor (HIF)-1 α , Matrix Metalloproteinase (MMPs), Fibroblast-Growth Factor (FGF) and binds with a high affinity to certain integrins (277-280). Elevated circulating levels of Endostatin have been associated with many diseases, including metastatic cancers, Diabetes Mellitus, Chronic Kidney Disease (CKD) Alzheimer's dementia and Coronary Artery Disease (281). Studies have concluded that the upregulation of Endostatin is likely to be triggered by hypoxia or ischaemia, which would be in keeping with our observation in patients with SBA, a condition which is accepted to be closely related to ischaemia (282-284). The seemingly paradoxical association of elevated levels of Endostatin, an anti-angiogenic factor in what are thought to be angiogenic-dependent conditions, has been investigated by several different groups. Early hypotheses suggested that Endostatin may be a by-product or a factor required for

the mobilisation of other promoters of angiogenesis, rather than a direct acting factor itself (285). More recently, a regulatory role in pathological angiogenesis has been attributed to Endostatin, with a disruption in the balance between other pro- and anti-angiogenic factors in favour of uncontrolled angiogenesis (286).

Another potentially relevant observation is that anti-angiogenic factors have been shown to have longer half-lives than pro-angiogenic factors, which suggests that their detection may not actually reflect the real-time manifestation of their angiogenic effect (287). This theory may apply to our results and possibly explain the discrepancy between the multiplex and ELISA results, as serum from patients with a diagnosis of SBA was taken at a single point in time. Although all patients with SBA had a history of bleeding at some point in time, their blood was not always collected at a bleeding episode, and the detection of elevated Endostatin in these patients may reflect the concept of a long half-life of Endostatin, rather than over-expression due to its current activity. An interesting observation in patients with CKD was that plasma levels of Endostatin demonstrated a concentration-dependent relationship with disease severity (288). This may also be relevant to our results, particularly the variability in the range of Endostatin levels, even among patients. We did not classify the severity or burden of disease in our SBA patient group but it is possible that this may influence serum levels of Endostatin and would be an interesting future assessment.

Interestingly, a few studies have measured the relationship of combined circulating levels of Endostatin and VEGF in angiogenic-related conditions, and found an inverse correlation between the two factors at different time intervals depending on disease activity (289, 290). Hypothetically, this could explain why we have not observed any

differences in VEGF levels associated with SBA to date, as elevated levels of Endostatin may reflect a point-in-time decrease in VEGF activity, rather than ruling out any role for VEGF in SBA pathophysiology. Similarly, Ang1 and Ang2 expression is known to be affected by certain threshold levels of VEGF (291). Our findings to date suggest that VEGF is not the key mediator of angiogenesis in SBA, however it may have an important role in coordinating the expression of other key factors, and future prospective studies including serial VEGF levels may confirm this hypothesis.

TIMP-1 is an anti-angiogenic factor, which binds with high affinity to (MMP)-9 (292). TIMP-1 expression is induced by several pro-inflammatory cytokines, particularly the Interleukins, Tumour Necrosis Factor (TNF)- α , and Transforming Growth Factor (TGF)- β . TIMP-1 is thought to exhibit two separate modes of action in cell growth and turnover, the first of which is related to its inhibitory action on the MMPs. MMPs cause degradation of the ECM by affecting collagen, elastin and other basement membrane proteins which is a necessary step in coordinated vessel development (293). TIMPs inhibit this ECM breakdown by preventing their conversion from a latent to an active form. More recently a mode of action of TIMP-1 independent from the MMPs has been discovered, with an ability to potentiate erythroid cells, and to inhibit apoptosis in B cells (294, 295). TIMP-1 has been shown to play a protective role in vascular remodelling, and in an over-simplified hypothesis, a deficiency of TIMP-1 in patients with SBA, may have a role in the development of disordered blood vessel development in the small intestine. The ELISA assessment of serum levels of TIMP-1 confirmed the initial multiplex results, that patients with SBA have lower circulating TIMP-1 levels than non-bleeding controls. Many studies have been performed looking at the effects of TIMP-1, particularly in

cancer angiogenesis, and it has been determined that TIMP-1 can exert either a pro- or anti-angiogenic effect, determined by circulating levels at the time of vessel development (296,297). Elevated circulating TIMP-1 levels have been reported by many studies as a poor prognostic indicator of disease activity in inflammatory conditions, and of a higher burden of disease and reduced likelihood of response to systemic treatments in malignancies (298-301). A deficiency of circulating TIMP-1 has been associated with cardiovascular disease, however, few studies to date have looked at the angiogenic effects of low circulating levels of TIMP-1 to compare our results to (302). Based on suggestions of a protective role of TIMP-1 in blood vessel development and cell turnover and repair, it could be hypothesised that the disordered blood vessel development in SBA could be attributed to the loss of this protective factor, although the explanation for our observed association is likely to be far more complex than this. The reported effect of TNF- α on TIMP-1 expression is an interesting observation also, as we have previously demonstrated that SBA patients have decreased serum levels of TNF- α , and further assessments of the two factors together at different points in disease activity may yield further information into their role in the pathophysiology of SBA formation.

We performed statistical analysis to determine confounding variables in our cohort which may have influenced serum levels of Endostatin or TIMP-1, and found that age over 50 years was associated with elevated Endostatin levels irrespective of the presence of SBA, although no confounding variables were detected for TIMP-1 expression. This observation may be explained by the likelihood of increasing co-morbidities of both SBA patients and controls in the presence of advancing age, particularly conditions associated with ischaemia, which occur predominantly in more elderly patients. We did not have adequate clinical data to perform a sub-group analysis

of co-morbidities to prove this hypothesis, however given that SBA is a condition almost exclusively present in elderly patients, the detection of age as a confounding factor would make its use in clinical practice as a diagnostic or prognostic factor less likely to be of use.

A major limitation of this study were the small cohorts used, particularly in the multiplex section of the study. Small cohort sizes and inadequate clinical data in certain areas in both sections of the study presented a number of issues when performing statistical analysis of the results, and multiple statistical tests were performed to allow us to undertake some form of analysis in cases where adequate data was not available. This will have resulted in less robust statistical data, with the risk of type I error due to multiple testing, and type II error due to small patient cohorts, and the study being underpowered. Notwithstanding this, the studies allowed us to further investigate the role of angiogenic factors in SBA serum and tissue, and provide a basis for further more robust studies, while also confirming our initial results detected in earlier chapters, despite a small sample size.

Conclusion:

The multiplex assessment yielded three main pieces of information regarding the likely important angiogenic factors in SBA formation. Firstly, it confirmed our previously determined results of elevated serum levels of Ang-2 and decreased serum levels of Ang-1 in SBA patients compared to controls. Secondly, it identified three new angiogenic factors which may be of importance in SBA formation, TIMP1, Endostatin, and PDGF-AA, which warranted further assessment. Thirdly, in support of our previous findings, it determined no differences in serum VEGF levels (including assessments of VEGF, VEGF-C

and EG-VEGF) between SBA patients and controls, giving us further reassurance that VEGF is unlikely to be the driving force behind SBA formation. We went on to measure quantitative serum levels of TIMP1, Endostatin and PDGF-AA in patients and controls using ELISA measurements and determined significant differences in TIMP1 and Endostatin, but no differences in PDGF-AA. SBA patients were found to have elevated levels of Endostatin, an anti-angiogenic factor, known to be induced by hypoxia or ischaemia and to have complex interactions with VEGF. SBA patients were found to have lower levels of TIMP-1, an anti-angiogenic factor with a protective role in vascular remodelling. Both Endostatin and TIMP-1 levels have been shown to be associated with other clinical conditions, where they may be predictive of disease severity, meaning that their further assessment in SBA patients may yield important information both regarding the pathophysiology of the condition, but also their potential use as diagnostic or prognostic markers.

The next step: assessment of TIMP1 and Endostatin levels in patients with other causes of OGIB.

In keeping with our subsequent biomarker assessment study described in chapter 3 we decided to perform quantitative assessments of TIMP1 and Endostatin in the serum of patients with other causes of gastrointestinal bleeding to determine whether the observed differences that we detected in our SBA cohort were specific for the condition, or whether they were driven by an angiogenic process stimulated by gastrointestinal bleeding of any type. We retrieved frozen serum samples from 40 patients who

underwent SBCE for the indication of IDA or OGIB, who went on to be diagnosed with any cause of small bowel bleeding which was not felt to be SBA. The samples were collected as detailed in chapter 3 and stored at -80°C. ELISA measurements of TIMP1 and Endostatin were performed using commercially available ELISA kits as detailed earlier, and statistical analysis was performed in a similar fashion.

Results:

Of the 40 patients included, 47.5% (n=19) were Male, and the mean age was 53.15 years (17-81). The mean overall Hb level was 12.2g/dL, with a mean Hb for Males of 12.65g/dL (7.2-13) and for Females of 11.7g/dL (6.2-14.6). Of the group overall, 45% (n=18) were anaemic at the time of blood collection and 66.67% (n=12) of these were Male. There were significant differences in age and rates of anaemia between this group and the SBA group, who had a mean age of 72.75 ($p<0.0001$, 95% CI (28.73) – (10.47) and rate of anaemia of 85% ($p<0.008$, 95% CI 0.12 – 0.68). The specific diagnoses made at SBCE included; non-specific enteritis n = 20, active bleeding n = 6, polyp/possible malignancy n = 5, denuded coeliac mucosa n = 2, Meckel's diverticulum n = 2, dieulafoy lesions n = 2, non-specific mucosal erythema not consistent with SBA n = 3.

The mean and median levels of TIMP1 and Endostatin for the group overall were 319.54ng/mL and 309.91ng/mL and 248.90ng/mL and 206.33ng/mL respectively (figure 20). Both TIMP1 and Endostatin levels were significantly lower in the group with other causes of bleeding than either the SBA or the non-bleeding control group.

Figure 20. Boxplots showing mean serum TIMP1 and Endostatin levels in patients undergoing CE and diagnosed with SBA, any other cause of GI bleeding, and a normal small bowel.

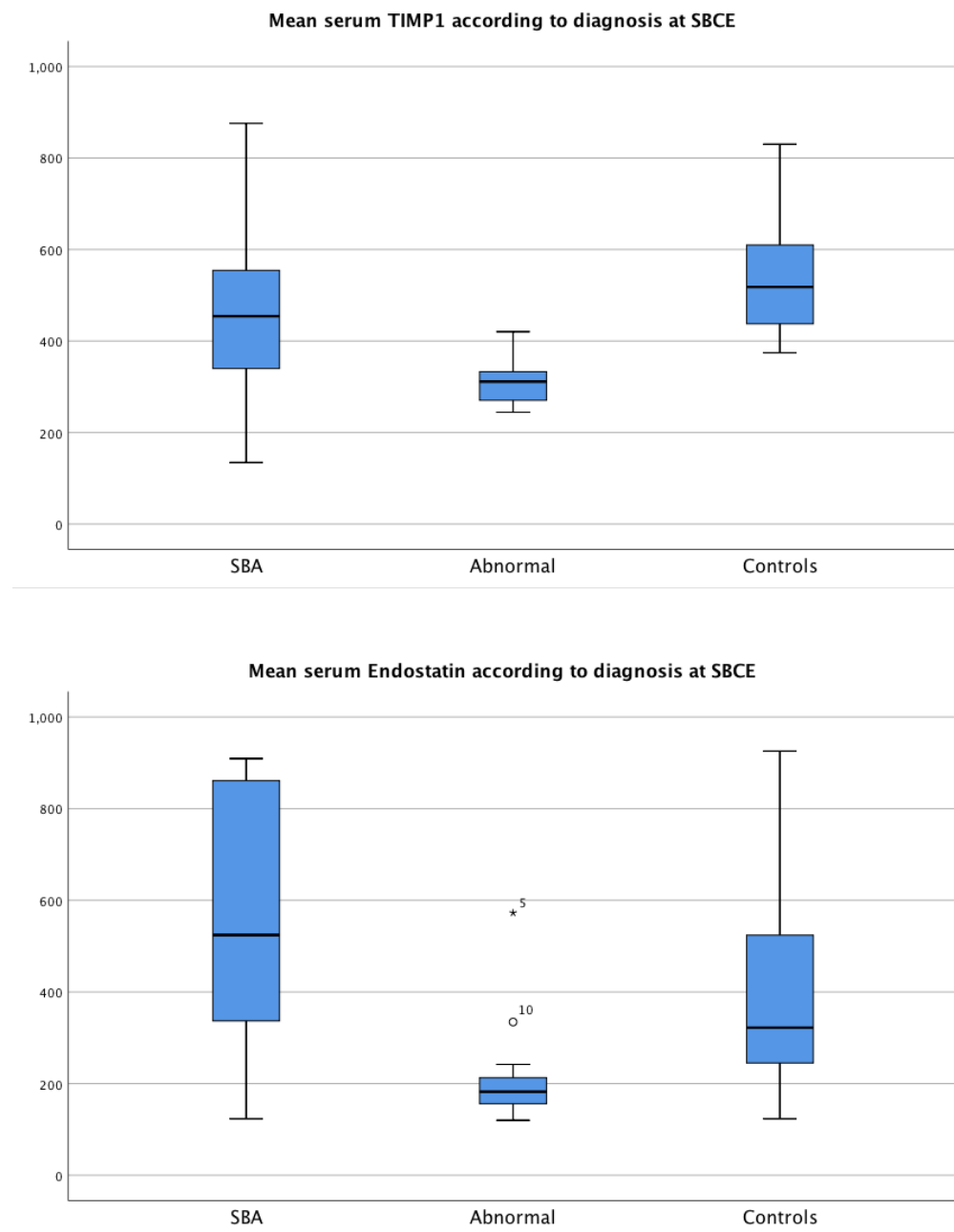


Table 27. Mean serum levels of TIMP1 and Endostatin in patients based on their CE diagnosis of abnormal bleeding, SBA or normal small bowel.

	Mean level in abnormal bleeding N=40	Mean level in SBA N=20	Mean level in controls N=20
TIMP1	319.54	467.17	550.77
P value		<0.0001	<0.0001
95% CI		(207.62) – (87.64)	180.79 - 281.65
Endostatin	248.90	556.80	387.12
P value		<0.0001	0.0062
95% CI		196.10 - 419.72	40.91 -235.54

Using a one way anova test we controlled for age >50 years, gender and the presence of anaemia in the abnormal bleeding group. Age >50 years and the presence of anaemia appeared to be confounding factors for TIMP1 expression, and Gender appeared to be a confounding factor in Endostatin expression.

Table 28. One-way Anova testing to determine whether age>50 years, gender or anaemia were confounding factors affecting serum TIMP1 or Endostatin levels.

		TIMP1	Endostatin
Age >50 years	F ratio	6.81	3.12
	P value	0.013	0.085
Gender	F ratio	2.88	5.41
	P value	0.098	0.025
Anaemia	F ratio	11.66	4.09
	P value	0.003	0.058

Discussion:

This assessment of serum levels of TIMP1 and Endostatin in patients with other causes of small bowel bleeding yielded some important findings which helped us to determine the significance of our detected abnormalities of both factors in patients with SBA. Our initial findings of decreased levels of TIMP1 and increased levels of Endostatin in SBA patients suggested that these factors could potentially be useful in either diagnosing or assessing disease activity in SBA patients, or could act as targets for treatment development down the line. However, the assessment of these factors in patients with other causes of small bowel bleeding has made these presumptions less clear cut. We found significant differences in the levels of both factors in all 3 groups which was a very interesting observation and supports our earlier findings in this chapter, that both factors may have an important role in angiogenesis in SBA. Their hypothetical roles based on elevations or reductions in levels of either factor in SBA or other causes of bleeding can be interpreted based on the available literature, as described in the discussion section above, as each factor seems to have a dual role in angiogenesis, and

this may be dictated based on the specific cause of bleeding. However, in terms of evaluating their use as diagnostic or prognostic aids for SBA, this study has identified some problems. For TIMP1, the mean value for SBA patients lies somewhere in the middle between patients with other causes of bleeding and patients with no gastrointestinal bleeding, meaning that it has no specificity for SBA, and would be unlikely to be of any value in aiding diagnosis in a cohort of patients with OGIB. In contrast, for Endostatin, the mean value for SBA patients was significantly higher than for either the abnormal bleeding or normal small bowel cohorts, suggesting that it has specificity for a diagnosis of SBA. However, in our earlier assessment we determined that age over 50 years was a confounding variable in Endostatin levels. In this assessment also, we observed a trend towards higher levels of Endostatin in patients >50 years of age, although this did not reach statistical significance ($p=0.085$). However, in view of the likely older age of SBA patients to begin with, age as a confounding variable would not be helpful in predicting the disease, and would be no more sensitive than using demographic or clinical predictive factors. Although perhaps not useful as diagnostic aids, these assessments have demonstrated significant differences in expression levels of both TIMP1 and Endostatin in patients with SBA compared to other causes of bleeding and their inclusion in further experiments may be of value in delineating the role of angiogenesis in SBA pathophysiology.

Chapter 5

Further assessment of angiogenic factors in SBA tissue

Introduction

The work in the previous chapters of this thesis have identified an association between an alteration in circulating serum levels of several angiogenic factors with SBA. Further analysis of these factors in our cohort managed to out rule potential confounding variables including age, gender, anaemia, or certain common co-morbidities, as the driving factor behind the association. However, several other variable factors may potentially induce changes in circulating levels of angiogenic factors. In order to progress the relevance of our initial findings from bench to bedside and to prove the theory of their involvement in the pathophysiology of SBA formation, the detection of these angiogenic factors in actual SBA tissue is required. This is a crucial step before we can identify potential diagnostic or treatment targets for SBA. Options for assessing angiogenic factors in SBA tissue include measuring relative quantitative gene expression levels, or direct visualisation and relative quantification using immunohistochemistry.

Throughout our tissue based experiments we encountered a few difficulties in obtaining SBA tissue. Firstly, SBAs are usually very small (often <5mm) areas of mucosal abnormalities which can only be obtained via small bowel endoscopy. Small bowel endoscopy is generally performed in patients with bleeding SBAs, with a view to applying a form of endoscopic treatment to control bleeding. Therefore, obtaining the tissue by mucosal biopsy incurs an inevitable risk of inducing bleeding. Taking mucosal

biopsies from areas of abnormal appearing vascular lesions in the gastrointestinal tract is relatively commonly performed in clinical practice by gastroenterologists to acquire histological confirmation of a suspected diagnosis. Biopsies are generally only taken in the absence of significant active bleeding, and when the suspected diagnosis is thought to be due to a low pressure feeding vessel. For example, a histological diagnosis of Gastric Antral Vascular Ectasia (GAVE) is clinically useful prior to committing a patient to therapeutic endoscopic regime requiring multiple repeat procedures (303). In contrast, mucosal biopsies would not commonly be taken from a suspected Dieulafoy or variceal lesion. Taking biopsies from SBAs in our institution to date has generally not been clinically useful, in terms of confirming a histological diagnosis of angiodysplasia due to non-specific and subtle histological changes. However, we have not noted any difficulties in controlling bleeding following mucosal biopsy and generally apply two forms of endoscopic treatment to a lesion if biopsy has been performed, including a combination of APC with either the subsequent application of an endoclip or injection of adrenaline diluted 1:10,000 in normal saline. This experience allowed us to overcome initial difficulties in obtaining ethical approval to collect mucosal biopsies from SBA lesions for assessment of tissue levels of angiogenic factors. A further difficulty we have encountered in obtaining biopsies from SBAs, has been the size of the tissue obtained. The DBE endoscope used in our unit during tissue collection for these experiments had a working channel diameter of only 2.2 mm which permitted only a single-use biopsy forceps, meaning that only one biopsy could be taken from the SBA prior to inducing bleeding, at which point endoscopic haemostasis was required. This meant that the tissue obtained was only 1-2mm in size for each patient. The size of the tissue obtained from mucosal biopsies from SBAs is likely to explain the difficulties in histological

diagnosis of the condition, as in our experience a diagnosis of angiodysplasia appears to be readily made in the resected tissue of larger lesions which have required surgical resection in our institution.

In order to overcome these limitations in acquiring SBA tissue, we wanted to use a laboratory technique which would allow us to identify the highest number of angiogenic factors from the smallest amount of SBA tissue, and allow us to calculate a reproducible relative quantification of each factor. Direct visualisation by immunohistochemistry (IHC) is the most reliable technique, providing information regarding the exact location and quantification of factors. However, the number of factors measured would be significantly limited by the need for antibody staining, meaning that realistically only two factors could be assessed for each IHC slide examined. Quantitative real-time PCR (qRT-PCR) on the other hand, using commercially available gene assays gave us the option of measuring relative expression levels of several angiogenic factors at the same time, from the same piece of SBA tissue, as the technique requires only a very small amount of RNA to be obtained from a mucosal biopsy. Based on our experience using gene expression measurement in our putative assessment work in chapter 2, we decided to employ the use of gene expression detection as a first step in our tissue assessments, with a view to employing IHC to confirm or validate any significant factors detected by gene expression measurement. This step-wise process has been used in a few studies attempting to uncover the angiogenic process of many malignancies with good results.

Gene expression assessment:

In chapter 2 we measured gene expression levels of Ang-1, Ang-2, Tie2, and TNF- α , as these factors were found to be abnormally represented in the serum of SBA patients

compared to controls. We also measured VEGF expression levels as VEGF has repeatedly been associated with gastrointestinal angiodysplasia at both a serum and tissue level in the literature. Using this assessment we found differences in gene expression levels of only Ang-1, Ang-2 and Tie2 to be differ between SBA tissue and controls. We also found no differences between gene expression levels of any factor between tissue taken from controls and that taken from macroscopically normal mucosa in SBA patients, which would allow us to use patients as their own controls moving forward. On this occasion, we decided to repeat the measurement of genes encoding Ang-1, Ang-2 and Tie2, in a further cohort of SBA patients as a benchmark, and to additionally measure relative expression levels of genes encoding TIMP1 and Endostatin, which had been identified as potentially important factors in SBA formation in the previous chapter (chapter 4). Following our negative findings with VEGF in multiple serum assessments and the previous gene expression measurement, we elected not to repeat VEGF measurement in this section.

Since our initial gene expression measurement work in chapter 2, we have learned a lot about the variation in levels of circulating angiogenic factors between patients with SBA, due to a number of potential confounding factors, including the presence of co-morbidities, age, anaemia and most likely although not yet proven – the time interval between a recent bleeding event and the acquisition of the serum from the patient. In chapter 2, we did not have matching macroscopically normal tissue available for comparative analysis in all SBA patients. However, with this information in mind we decided that moving forward, measuring and comparing gene expression levels between patients would be more specific if we included only patients where an adequate tissue

sample survived processing from both an area of SBA and macroscopically normal tissue which would hopefully control for any variable confounding factors.

Aims:

To determine whether the detected abnormalities in circulating levels of angiogenic factors including Ang-1, Ang-2, Tie2, TIMP1 and Endostatin in patients with SBA were associated with abnormalities in relative gene expression levels of these factors in SBA tissue.

Methods for gene expression assessment

Sample collection

Based on our initial experience with measurement of gene expression levels of angiogenic factors in patients with SBA, finding that macroscopically normal patient small bowel tissue was comparable to that of healthy controls, we elected to perform the measurements in SBA patients only on this occasion, and omitted the need for control tissue. Biopsies were taken from the small bowel during DBE which was performed for either diagnosis or endoscopic treatment of SBA. One standard biopsy (1-2mm in size) was taken from a single angiodysplasia lesion, and a further biopsy was taken from macroscopically normal adjacent mucosa, at least 5 cm from the angiodysplasia. Biopsy samples were immediately placed in individual aliquots of RNeasy lysis solution and stored in a fridge overnight before being stored in a -80°C freezer for later batch analysis.

Selection of angiogenic factors for gene expression analysis

Based on the results of the measurements of serum angiogenic factors as described in chapter 2, we proceeded to measure gene expression levels of Ang-1, Ang-2 and their receptor Tie2, and TIMP1 and Endostatin, which were identified as potentially new angiogenic factors in SBA by chapter 4. As we had previously measured gene expression levels of VEGF and TNF- α , and found no differences between angiodysplasia and controls or unaffected patient tissue, we elected not to repeat these assays.

Measurement of gene expression

Relative gene expression of Ang-1, Ang-2, Tie2, TIMP1 and Endostatin were measured using commercially available kits in the steps described in chapter 2.

Results

Mucosal biopsies were collected from an area of SBA and adjacent normal mucosa in 15 patients (30 samples). Adequate RNA concentration levels of at least 60ng/ml (mean 169.4ng/ml, range 61-370) were detected in 23 samples including at least one sample from each patient. However, only 8 patients had adequate RNA concentrations in both an area of SBA and normal mucosa to allow comparative analysis of RNA expression levels. Of these 8 patients 75% (n=6) were male and the mean age was 71 years (range 63-82).

Table 29 shows the average relative expression levels of Ang1, Ang2, TIMP1, Endostatin and Tie2 in SBA and adjacent macroscopically normal tissue for the 8 patients when normalised against levels measured in the macroscopically normal tissue of patient 2.

Patient number	Tissue type	ANG1	ANG2	TIMP1	ENDO	TIE2
1	Normal	1.545273951	0.71680348	1.14752656	0.982304049	0.418444722
	SBA	1.890993697	1.373170574	1.458433398	1.272977738	1.035406631
2	Normal	1	1	1	1	1
	SBA	1.423048152	1.626267542	1.304987023	1.145154122	1.10165418
3	Normal	1.871884399	1.864446451	1.496106933	1.62852916	1.11560774
	SBA	1.542877021	2.603798638	1.609963798	1.636379337	1.082675199
4	Normal	1.484552916	1.120329193	1.388223469	0.960059619	0.742242515
	SBA	1.795548318	0.990113718	2.021004757	1.319051392	1.367887222
5	Normal	2.971679197	4.04069643	1.806343071	2.438353106	2.1903617
	SBA	1.842837465	2.523058866	3.293580703	2.623046668	2.097608067
6	Normal	1.848354666	1.014981419	1.002128993	1.866329276	0.620077775
	SBA	2.204852348	2.393838702	1.586804944	2.003759891	0.983108642
7	Normal	1.067497156	0.885357006	1.443936921	1.658966889	0.397356925
	SBA	1.692215186	1.883964082	1.55179855	1.274702006	0.792470978
8	Normal	1.953817477	1.206971053	0.960282168	1.551225775	0.912908895
	SBA	2.340705318	2.474663596	1.843838833	2.102919503	1.433026461

Table 30 shows the average relative expression levels of each factor in the macroscopically normal tissue compared to the SBA tissue. The SBA tissue was found to have significantly higher levels of Ang1, Ang2 and the Tie2 receptor than the macroscopically normal tissue but there were no differences in the levels of TIMP1 or Endostatin expression.

Patient number	ANG1	ANG2	TIMP1	ENDO	TIE2
Normal tissue					
1	1.545273951	0.71680348	1.14752656	0.982304049	0.418444722
2	1	1	1	1	1
3	1.871884399	1.864446451	1.496106933	1.62852916	1.11560774
4	1.484552916	1.120329193	1.388223469	0.960059619	0.742242515
5	1.842837465	2.523058866	3.293580703	2.623046668	0.976080671
6	1.848354666	1.014981419	1.002128993	1.866329276	0.620077775
7	1.067497156	0.885357006	1.443936921	1.658966889	0.397356925
8	1.953817477	1.206971053	0.960282168	1.551225775	0.912908895
SBA tissue					
1	1.890993697	1.373170574	1.458433398	1.272977738	1.035406631
2	1.423048152	1.626267542	1.304987023	1.145154122	1.10165418
3	1.542877021	2.603798638	1.609963798	1.636379337	1.082675199
4	1.795548318	0.990113718	2.021004757	1.319051392	1.367887222
5	2.971679197	4.04069643	1.806343071	2.438353106	2.1903617
6	2.204852348	2.393838702	1.586804944	2.003759891	0.983108642
7	1.692215186	1.883964082	1.55179855	1.274702006	0.792470978
8	2.340705318	2.474663596	1.843838833	2.102919503	1.433026461
	ANG1	ANG2	TIMP1	ENDO	TIE2

Mean normal	1.576777254	1.291493433	1.466473218	1.53380768	0.772839905
Mean SBA	1.982739905	2.17331416	1.647896797	1.649162137	1.248323877
p value	0.0243	0.0022	0.269940267	0.33322851	0.036
95% CI	(0.741) – (0.071)	(1.326) – (0.438)	0.502	0.314	(0.475) – (0.043)

Table 31. The ratio of relative expression levels of genes encoding Ang1/Ang2 in normal tissue and from an area of SBA in patients.

Ratio of Ang1/Ang2	Normal tissue	SBA tissue
Patient number		
1	2.155784667	1.377100364
2	1	0.875039386
3	1.00398936	0.592548517
4	1.325104198	1.813476861
5	0.730398125	0.73543738
6	1.821072418	0.921053012
7	1.205725091	0.898220514
8	1.618777411	0.945868086
Mean ratio	1.357606409	1.019843015
p value	0.076	
95% CI	(0.04) - 0.722	

Discussion:

This study yields two important findings. Firstly, our initial findings of elevated relative expression levels of genes encoding Ang1, Ang2, and their receptor Tie2, in SBA were confirmed when measured in a larger number of matched SBA and macroscopically normal tissue from individual patients. Furthermore, we found no differences in gene expression levels of either TIMP1 or Endostatin in SBA tissue compared to the macroscopically normal surrounding tissue. These findings validate the findings so far in the thesis, that the Angiopoietin pathway is likely to have a significant role in the pathophysiology of SBA formation. Although the detection of abnormal circulating levels of Ang1 and Ang2 in patients affected by SBA appeared to be a specific finding, their detection in abnormal quantities within the actual SBA tissue confirms their relevance in the actual pathophysiology of the condition and cannot be explained by confounding variables. This conclusion is further supported by our decision to process only samples with adequate RNA from both an SBA area and normal surrounding tissue, as a variance in circulating levels of angiogenic factors could hypothetically lead to alterations in tissue levels between individual patients. Furthermore, the trend of a decreased ratio of Ang1/Ang2 in SBA tissue compared to normal tissue, although not quite statistically significant, supports the hypothesis of a balanced interaction between the two factors required for SBA formation.

Previous criticism from peer-reviewers when presenting our initial data in chapter 2, was the suggestion that the angiogenic factors are simply elevated as they are taken from a vascular lesion, and compared to normal appearing mucosa which would be presumed to have a lower level of baseline angiogenesis. This suggestion however is not accurate,

as in that case we would expect to see a broad increase in all angiogenic factors from SBA tissue, in particular VEGF, which is a crucial factor in blood vessel development. In fact, we could show no difference in VEGF, TNF- α , TIMP1, or Endostatin levels in SBA tissue compared to normal background mucosa, but significant differences in Ang1, Ang2 and the Tie2 receptor, supporting their specificity in SBA development. These findings also go against the hypothesis of a field effect change in angiogenic factors in the background normal appearing mucosa of patients with SBA, which is interesting, given the high rate of spontaneous new lesion development throughout the natural history of the condition. It could be hypothesised that this new lesion development is therefore fostered by abnormal circulating levels of the factors, rather than tissue abnormalities, although this would be difficult to prove given the unpredictable time course and location of new lesion development.

At initial glance the finding of elevations of all 3 Angiopoietin factors in SBA tissue compared to normal appeared to contradict our serum findings, which identified decreased levels of Ang1, and increased levels of Ang2 in SBA patients. However, this was also the pattern of gene expression that we observed in our first gene assessment study, and can be interpreted rationally. Firstly, gene expression levels are a relative comparison and not a precise quantitative assessment, therefore their relative expression could potentially be influenced by the volume of tissue obtained.

Furthermore, when we assessed serum levels of Ang1 and Ang2 in SBA patients and controls, although Ang1 levels were significantly lower in SBA patients, the numerical quantity of Ang1 compared to Ang2 was much higher with mean levels of Ang1 averaging ten times that of Ang2 levels. Therefore, the detection of increased relative expression of the gene encoding Ang1 is reflective only of increased activity of the

pathway in SBA tissue compared to normal mucosa and the levels themselves cannot be presumed to be a sensitive indicator. In contrast, the ratio of Ang1/Ang2 in individual patients is a more sensitive indicator of relative activity and reassuringly this showed a trend towards a lower ratio in SBA tissue than normal looking mucosa, comparable to our finding of a decreased serum ratio of Ang1/Ang2 in the SBA patient cohort of our biomarker assessment study. It also suggests the likely role of additional angiogenic factors identified in abnormal levels in the serum of SBA patients but not confirmed in the tissue assessment. Each of the identified factors, VEGF, TNF- α , TIMP1 and Endostatin have been shown in other studies to induce up or down-stream regulation of other angiogenic factors at a cellular level and the abnormalities in the serum levels of these factors detected in SBA patients may well contribute to the interaction of Ang1 and Ang2 with the Tie2 receptor for example.

A major limitation of this study is the small patient sample size (n=8), which reflects the difficulty in obtaining tissue from SBAs. Firstly, access to the lesions within the small intestine is limited to patients undergoing DBE, which is performed on a relatively infrequent basis compared to conventional endoscopy. In addition, patients that undergo DBE for SBA, generally do so for treatment with APC. In which case a significant proportion of patients have refractory anaemia, and are frail, meaning that the risk of inducing further bleeding from taking a biopsy from a sample needs to be taken into account prior to recruitment, and not all patients were suitable candidates.

Furthermore, despite their ability to cause large volumes of blood loss, SBA lesions are generally small (<5mm), meaning that a single biopsy is all that can be taken from any individual lesion. Due to the small volume of tissue obtained this led to almost half of our initially recruited patients (n=7) being excluded from the PCR stage, as adequate

RNA levels were not present in both an SBA and a normal mucosa sample. However, despite the small numbers analysed, we still managed to obtain significant results, which further strengthened our original hypothesis and were more precise than our initial gene expression assessment. Importantly, this assessment provided the basis for further tissue work using IHC by filtering out the relevant factors requiring exact quantification and location assessment, meaning that the limited SBA tissue could be examined more efficiently in future work.

Immunohistochemistry:

Introduction:

Based on the above results from the measurement of gene expression levels we decided to carry out IHC on SBA tissue using antibodies against the three factors which were found by qPCR to have significantly different relative expression levels; Ang-1, Ang-2 and Tie2. IHC is a method for demonstrating the presence and location of proteins in tissue sections by the detection of antigens (the proteins) which bind to a specific target antibody introduced during the assessment. Though, less sensitive quantitatively than immunoassays such as Western blotting or ELISA, it allows the study of the distribution and localisation of specific cellular components. IHC is a method of visualising a protein within tissue, and is often used in research to confirm results from other methods, such as qPCR.

Histologically, mucosal biopsies from areas of gastrointestinal angiodysplasias show a relatively non-specific increase in ectatic, or weakened blood vessels in the mucosa. To date, there are no known diagnostic aids in clinical practice which are used by

histopathologists to confirm a diagnosis of angiodysplasia, and they are more commonly diagnosed endoscopically by their characteristic appearances. The first report in the literature describing the use of IHC to detect antibodies to angiogenic proteins was in 1999, when Junquera et al reported the detection of strong levels of VEGF in colonic angiodysplasia tissue in 89% of the 18 patients assessed (304). Their findings formed the basis for the focus on VEGF as a key factor in the pathophysiology of gastrointestinal angiodysplasia pathophysiology, and Tan et al subsequently reported moderate expression of VEGF detected by IHC in 75% of samples of colonic angiodysplasias from 12 patients in 2011 (305). To our knowledge these are the only studies available describing the use of IHC in gastrointestinal angiodysplasias available for comparison. The successful use of IHC to detect tissue levels and location of Ang-1, Ang-2, and Tie2 has been described in the literature in a number of different angiogenic-driven disease models including malaria, Kaposi sarcoma, colorectal cancer, and oral squamous cell carcinoma (306-309). Each of these studies reported the use of various commercially available antibodies and the combination of the results of intensity of IHC staining with clinical and biochemical parameters to determine clinically useful information including disease pathophysiology and severity, response to treatment and longer term prognosis.

Aims:

1. To determine whether IHC using commercially available antibodies to Ang-1, Ang-2 and Tie2 could be used to assess the expression of these angiogenic factors in mucosal biopsy samples taken from an area of SBA.
2. To determine quantitative assessments of expression levels of Ang-1, Ang-2 and Tie2 in mucosal biopsies from an area of SBA.

3. To determine whether the identified serum circulating abnormalities in levels of Ang-1, Ang-1 and the Tie2 receptor, and elevated relative expression levels of the three factors identified by PCR gene expression assessment correlated with abnormalities in tissue expression at IHC.

Sample collection and preparation:

Endoscopic mucosal biopsy samples were taken from an SBA at DBE in a similar fashion to that described above for the gene expression levels assessment. For this assessment however, the resultant sample (1-2mm) was immediately placed floating in a container of 10% formalin solution and sent to the pathology laboratory.

Immunohistochemistry technique:

- 1) On arrival to the laboratory, samples were immediately embedded in paraffin, and formalin-fixed paraffin-embedded (FFPE) sections were cut at 4µm onto Superfrost®Plus slides and baked overnight at 60°C.
- 2) Commercially available antibodies (Abcam) were used for this experiment with an adapted IHC protocol for use with an automated system, the Ventana Benchmark Ultra automated system (Ventana Medical Systems, Tucson, AZ).
- 3) Antigen retrieval was carried out by Heat Induced Epitope Retrieval (HIER) in Tris-EDTA buffer pH 7.8 at 95°C for 44 min using a calcium chloride stock solution (Ultra CC1 cell conditioning solution).
- 4) Endogenous peroxides and proteins were blocked using Inhibitor CM.

- 5) The slides were then incubated at various dilutions in the primary rabbit polyclonal antibodies [anti-Ang-1 (abcam ab8451), anti-Ang-2 (abcam ab56301) and anti-Tie2 (ab24859)] for 32minutes at 36°C.
- 6) Previously collected and archived Kidney biopsies were used as positive control tissue to determine the appropriate dilutions for each antibody, as recommended by the antibody manufacturers (Abcam). Based on the available literature for their use, we tried primary antibody dilutions ranging from 1/100 to 1/1000 and found the ideal dilution to be 1/500.
- 7) Direct antibody binding was detected using the Ventana optiView DAB detection kit. Slides were counterstained using Ventana Haematoxylin II, dehydrated, cleared and mounted in Pertex.
- 8) Staining localisation was assessed by a consultant histopathologist and senior medical scientist.
- 9) All staining intensity was assessed using a semi quantitative grading system as previously described (0 - no staining, 1 - weak staining, 2 - moderate staining, and 3 - strong staining) (310).
- 10) Trouble-shooting techniques used for non-specific staining were employed including: Decreasing antibody concentration and decreasing the incubation period, and sections were stored at high humidity to prevent them from drying out.

Results:

16 mucosal biopsy samples from an area of SBA identified at DBE were collected for IHC. This included biopsies from 12 Males and 4 Females. The mean age of patients was 71.3

years (range 62-84), and mean serum Ang-1 and Ang-2 levels were 49751.93pg/ml (range 34310 – 80147) and 3203.79pg/ml (range 2335 – 4420) respectively.

Of the 16 samples analysed in the IHC study, 6 of these had undergone measurement of RNA levels of Ang-1, Ang-2 and Tie2 also, with mean relative expression levels of 2.105 (range 1.54-2.97), 2.458 (range 1.37 – 4.04) and 1.185 (range 0.62 – 2.19) respectively.

Of these 6 samples, 5 showed higher elevated relative expression levels for genes encoding Ang-1, Ang-2, and Tie2 than the mean levels in the overall SBA group, and the 6th sample showed elevated relative expression levels of Ang-1 and Ang-2 but a slightly lower level for Tie2.

Immunohistochemistry:

Positive controls:

Adequate staining of all three antibodies was observed using kidney biopsy tissue as a positive control, although the staining appeared to be consistently weaker for Ang-2 and Tie2 antibodies despite multiple adjustments to the protocol and use of the troubleshooting steps outlined by the manufacturers.

SBA samples:

Ang-2 and Tie2 antibodies:

Insufficient staining was observed in SBA samples using the Ang-2 and Tie2 antibodies, which meant that an intensity grading score could not be applied to any samples.

Ang-1 antibody:

Adequate staining was observed in only 5 (31.25%) of the 16 samples using the Ang-1 antibody, however there was significant variance in the degree of staining observed

between samples and between areas within the same tissue, as demonstrated in the figures below (figures 21-25).

Figure 21. Non-specific staining of Ang-1 antibody (brown colour) in mucosal biopsy from an area of SBA in patient 1

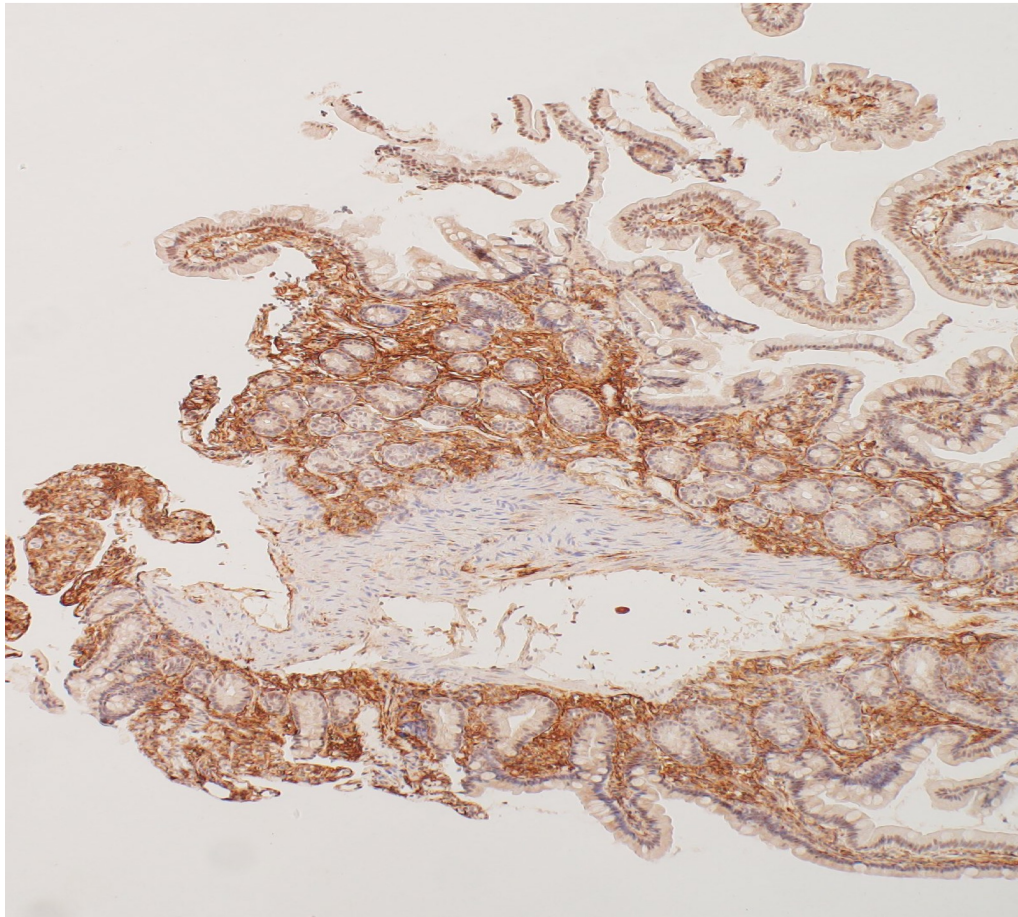


Figure 22. Staining of Ang-1 antibody (brown colour) around a blood vessel (top area) and nerve bundle (lower area) in mucosal biopsy from an area of SBA from patient 16 showing a higher intensity of staining within the blood vessel, however on magnification the staining is widespread and non-specific.

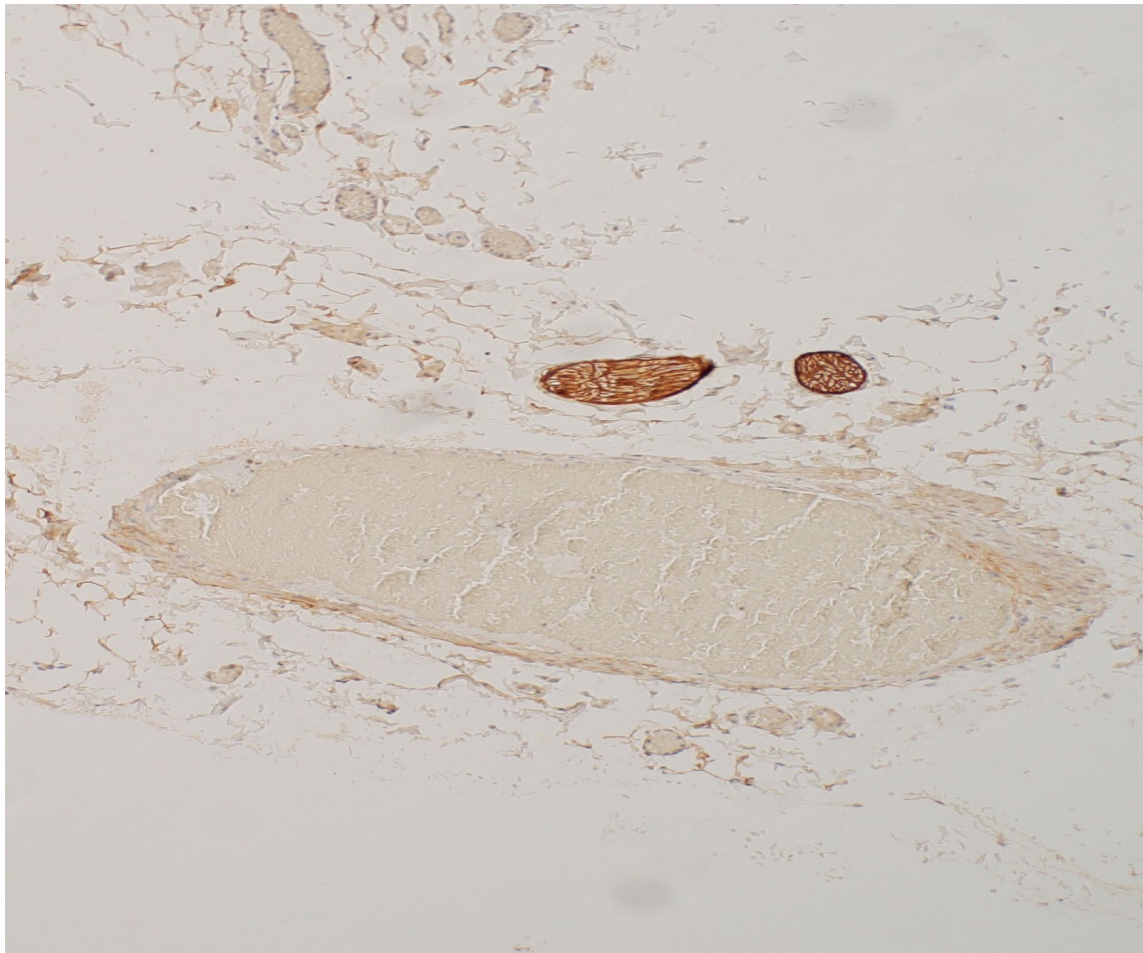


Figure 23. Ang-1 staining (brown colour) in mucosa (bottom area) and a blood vessel (top area) in mucosal biopsy from an area of angiodysplasia from patient 7.

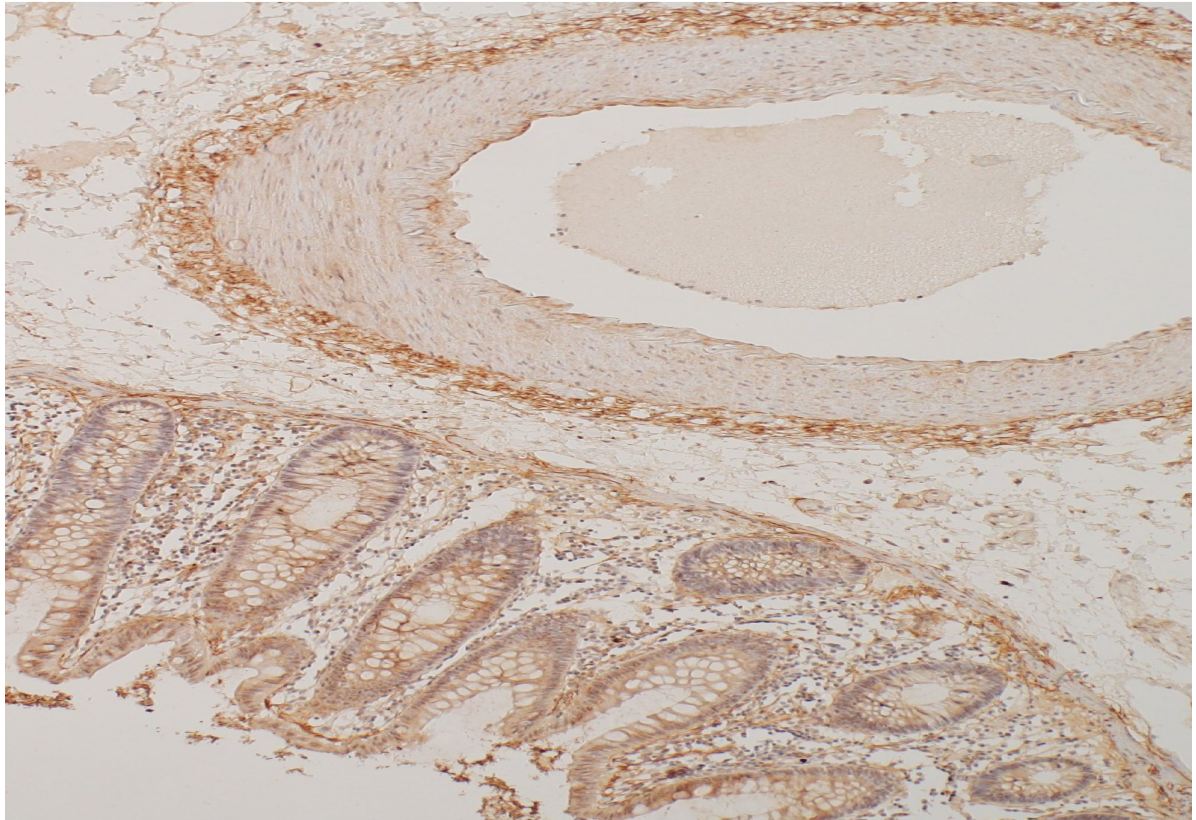


Figure 24. High powered magnification of a blood vessel (lower area) seen in a mucosal biopsy from an area of SBA from patient 15 which shows non-specific low intensity staining.

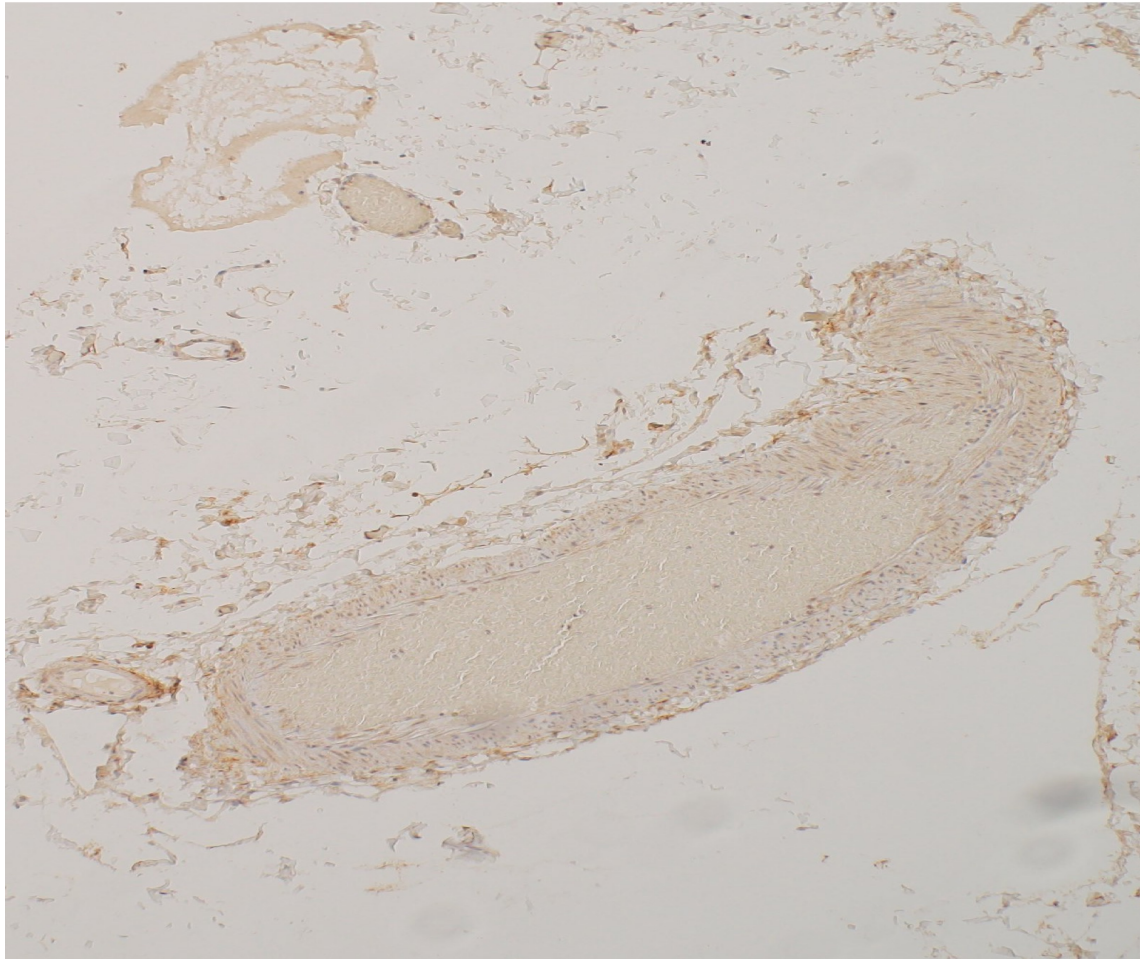
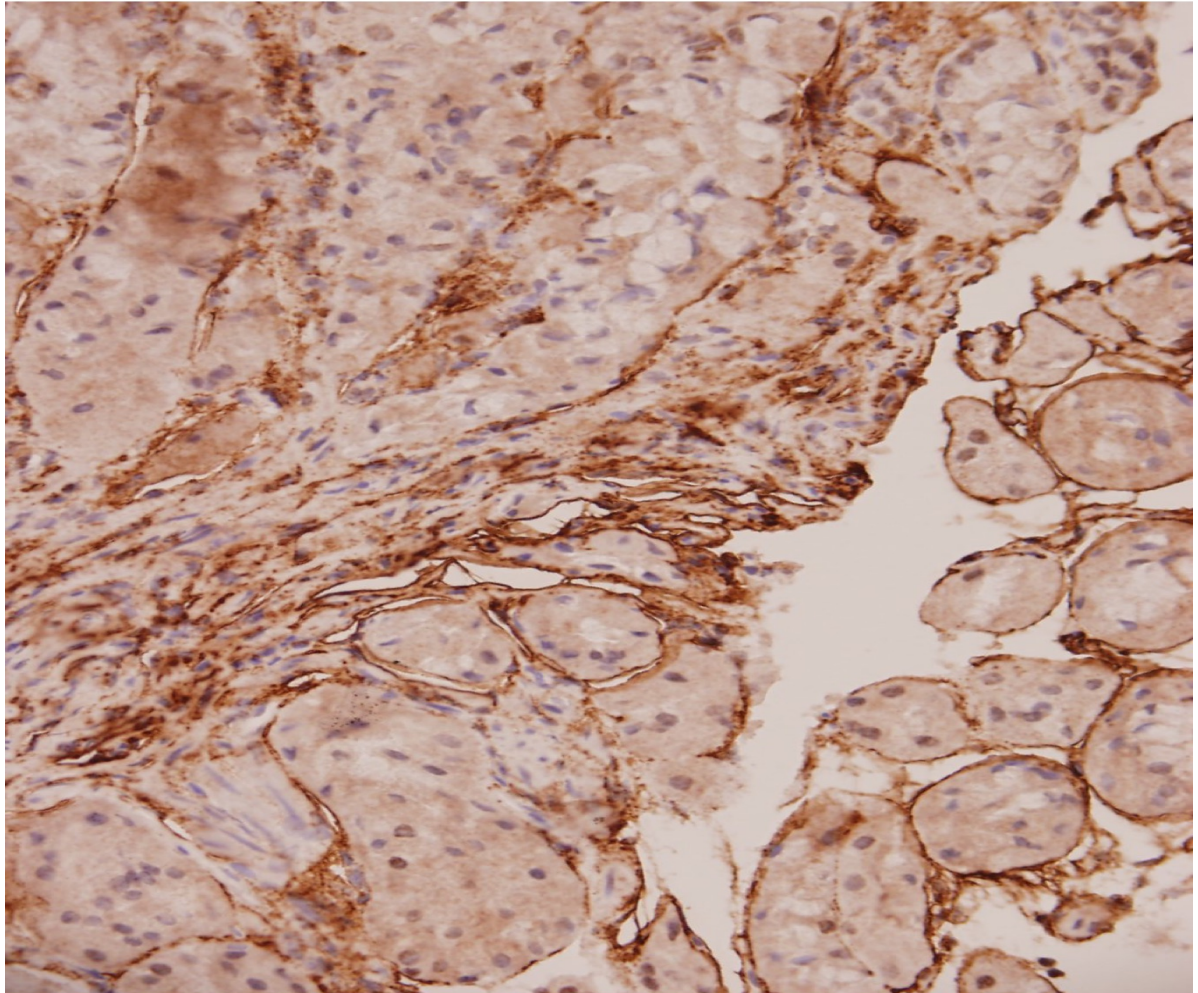


Figure 25. Mucosal biopsy from an area of SBA from patient 5 showing non-specific staining.



Overall, staining appeared to be most intense around blood vessels, however due to the non-specific nature of staining it was not possible to determine the elements of the blood vessel within the stained section. Furthermore, not all blood vessels stained even within the same tissue sample. Comment was made regarding tissue fragility, and many of the samples had very little diagnostic material for immunohistochemistry use following processing and preparation.

Overall, the results were inconclusive and insufficient to assign an intensity grading score, or determine any useful information regarding specific location of antibodies within SBA tissue. Following multiple attempts at protocol adjustment and troubleshooting steps there remained inadequate diagnostic material to proceed using an alternative antibody.

Discussion:

The initial objective of this study was to assess the feasibility of using IHC with commercially available antibodies against Ang-1, Ang-2 and their receptor, Tie2, to assess levels of each of the factors within mucosal biopsies from SBA tissue. Our work in earlier chapters of the study has consistently identified abnormalities of circulating levels of both Ang-1 and Ang-2, however, confirmation of abnormalities of these factors at a tissue level is a crucial factor before further conclusions can be drawn on their definitive role in the pathophysiology of SBA. Using PCR and relative gene expression analysis in earlier studies we were able to confirm that the expression of the genes encoding Ang-1, Ang-2 and Tie2 were significantly increased in mucosal biopsies from areas of SBA, compared to macroscopically normal adjacent patient tissue and also to

macroscopically normal small intestinal mucosal tissue from patients without SBA.

However, the detection of genes encoding these angiogenic factors does not translate to the actual expression of the proteins in the tissue, therefore IHC was a key further step to our work to confirm our hypothesis. Unfortunately, we were unable to detect significant expression of any of the three factors in our collected SBA mucosal biopsies despite using reliable and exhaustive laboratory techniques, and the expertise of a senior medical scientist and consultant histopathologist. Our inability to accurately detect expression levels of any of the three angiogenic factors meant that we were unable to complete the second and third aims of the study also.

We reviewed and altered our experimental technique at a number of different stages without any improvement in outcome, however there are a number of areas for potential protocol adjustment in future which we will discuss. Firstly, we chose to use abcam antibodies as they were compatible with the automated system (Ventana) for IHC in our hospital laboratory, and our medical scientist and histopathologist were both familiar with the use of this brand. Furthermore, there are many publications detailing successful use of these antibodies in human tissue (311-314). We did not have adequate amounts of tissue left to consider using an alternative antibody, and changes to the consent process for research projects due to the implementation of the European Union general data protection regulation (GDPR) during our study meant that we did not have adequate consent to take further biopsy samples from patients, and unfortunately did not have enough time to complete a new application for ethical approval in line with the GDPR consent requirements. However, there are a number of other commercially available antibodies against each of the three factors which have been reported successfully in the literature and it may be useful to repeat the experiment using

alternative antibodies. We attempted several trouble-shooting alterations to our experiment protocol including adjusting antibody dilution concentrations and decreasing the antibody incubation period without any improvements in outcome. A further consideration to future studies may be to employ a secondary antibody, which would allow amplification of the signal intensity and the detection of the antibody in a more diluted form. However, given that we could successfully detect the antibody in control tissue, it is likely that our technique was robust and further attempts using altered protocols with the same antibody may be futile.

A further difficulty we encountered was the quality and size of the actual tissue specimens obtained via endoscopy. As mentioned earlier, the DBE scope in use at the time of sample collection allowed a single biopsy of 1-2mm to be taken and comment was made by the medical scientist and histopathologist that the biopsy size was small and the tissue volume was substantially reduced, and became friable following processing. When we examined the literature for IHC of Ang-1, Ang-2 and Tie2, we found that almost all studies reported the use of surgically excised human tissue, and no studies reported the successful detection of these antibodies using limited mucosal biopsies. In fact, on further examination of the available literature detailing the use of IHC to detect VEGF in gastrointestinal angiodysplasia tissue, we found that all but one study described the use of surgically excised intestinal samples rather than endoscopic angiodysplasia studies, including the regularly cited study by Junquera et al (315). These studies were conducted using resected specimens from colonic angiodysplasias with refractory bleeding. Colonic angiodysplasias can be very large and surgical resection may be more frequent in this cohort, however for our SBA cohort, surgery is generally a last resort, so although it may result in larger specimens for IHC, it would not be a regular

enough occurrence to allow adequate patient recruitment for future studies. The endoscopy unit in the hospital has recently purchased a new DBE enteroscope with a larger working channel which will allow the use of a standard biopsy forceps for future work. This means that potentially larger biopsies could be taken, or more than one biopsy could be taken from a lesion prior to the need to achieve haemostasis, which may make IHC more successful in future work. Of interest also, is the histological findings reported in other studies compared to our own. Both the studies by Junquera et al and by Tan et al commented on abnormal histological assessments of the specimens even without the use of IHC, reporting the detection of an increased number of dilated vessels in the mucosa and submucosa, although it is not stated whether the initial histology reports were made by the same people who performed the IHC, which would obviously introduce bias to the information. All of the mucosal biopsies from areas of SBA used in our study were reported to be “within normal limits”, although comment was made on increased vascularity in 6 of the 16 samples examined. This discrepancy in histology reporting may reflect the biopsy size from previous samples and it will be interesting to see whether histology reports differ with the future use of increased size or number of biopsy samples. Further interesting alterations to the laboratory technique may be to attempt different methods of sample processing, or attempt IHC on fresh samples, if tissue friability following storage appeared to be a significant issue. We were surprised not to be able to detect adequate levels of protein expression of any of the three factors assessed by IHC, given the significantly elevated relative expression levels of gene expression for each factor that we detected earlier in this chapter. Following the detection of elevated gene expression levels by qPCR, confirmation of a correlation of increased expression of the protein in tissue is then recommended, and

there are two main laboratory techniques which do this, IHC and western blot. Western blot is a laboratory technique which detects quantitative levels of a target protein antigen from a denatured tissue sample via electrophoresis, and the addition of a specific antibody. Reports suggest that western blot gives a more accurate quantitative assessment of protein levels than immunohistochemistry, although immunohistochemistry allows a detailed assessment of the location of the target protein (316). After reviewing the literature and discussing our aims with our histopathology colleagues, we felt that the use of IHC to confirm our qPCR findings would result in more clinically useful information regarding the pathophysiology of SBA formation. Furthermore, our collaboration with a consultant histopathologist and senior medical scientist meant that we could use the hospital laboratory equipment for the experiment, meaning that the experiment would be clinically reproducible if successful. Following the failure of our IHC study we have become aware of many other studies where the detection of elevated gene expression levels of proteins was not confirmed by IHC, and that the correlation between the two findings can be as low as 40% (317). This can be explained by several factors. Firstly, qPCR measures levels of mRNA, genetic data required for the expression of target proteins, while western blot and IHC measure the presence of the actual protein in tissue. There are a number of different steps at which the presence of elevated mRNA levels can be prevented from resulting in elevated protein expression levels, including transcriptional, translational and protein stability factors (318). As referenced before, angiogenesis and the Angiopoietin pathway are currently undergoing substantial amounts of research and new developments of the understanding of the process occur regularly. Antibodies to the Angiopoietins have been available only in the last two decades and are in use in a research capacity only,

therefore limited data is available regarding the use of each commercially available antibody. Many researchers now recommend the use of two methods to confirm the correlation of gene expression with protein expression in tissue, and a combination of western blot and IHC to determine the sensitivity of the commercial antibody used in future work may be complementary.

Conclusions:

Despite the significant elevations in relative gene expression levels of Ang-1, Ang-2 and Tie2 in mucosal biopsies from SBA lesions in the earlier study of this chapter, we were unable to detect adequate levels of protein expression of any of the three factors using commercially available antibodies assessed by IHC. After a number of trouble-shooting steps and protocol adjustments, with the expertise of a senior medical scientist and consultant histopathologist we determined that the two most likely explanations for the failure of this experiment were the commercially available antibodies chosen, and the size of the SBA tissue obtained via endoscopic biopsy. Although we did not have the time or resources to repeat the experiment, future trials using alternative antibodies, or by employing western blot technique as an additional aid, may yield the information we desired. Furthermore, our unit has upgraded the double balloon enteroscope since our study took place, meaning that future biopsies taken for IHC assessment are likely to be larger and more robust to survive the processing involved prior to IHC assessment.

Chapter 6

Overall summary and future directions

Summary of thesis and results from each chapter

The over-arching aim of this thesis was to investigate the underlying pathophysiology of SBA formation. SBA is a longstanding condition, becoming a more commonly encountered clinical problem due to our aging population, the increased use of anticoagulants and antiplatelets, and advancements in small bowel endoscopic modalities. However, despite being more commonly encountered, there are still a number of difficulties including; diagnostic delays due to lack of access to small bowel endoscopy, the unknown natural history and prognosis of the condition, the currently poor management options for affected patients, and their resultant poor quality of life. These difficulties all stem from the same issue, significant limitations in our knowledge of SBA pathophysiology. The results from each section of this thesis will hopefully contribute to the knowledge base of SBA as a condition and allow us to address the major challenges faced by SBA patients in the future.

In chapter 2, we attempted to determine which angiogenic pathway might be of most importance in SBA formation, through our putative factor assessment. We used the albeit very limited information from the literature regarding angiogenesis in GIAD to guide this initial assessment, and broadened our inclusion of other factors based on our results. The most unexpected finding from this assessment was that we found no association between serum levels of VEGF and the presence of SBA in patients, despite the published findings of both serum and tissue associations with VEGF previously, and the fact that the VEGF pathway is the most commonly understood of the angiogenic

pathways (6, 107). Understandably, despite having comparable patient numbers to both of the references studies, we were reluctant to entirely base our future work around this negative association as it contradicted the only available literature regarding angiogenic factors in GIAD, and we continued to include assessments of VEGF in our tissue based assessments in chapters 2 and 5, and also broadened our exploration of serum VEGF levels by including assessments of alternative VEGF forms including EG-VEGF and VEGF-C, in chapter 4. All of these further assessments of an association with VEGF were negative, which allowed us to disregard a major association of VEGF as a key factor in the angiogenic pathway behind SBA formation with confidence. However, we acknowledge that VEGF is a key factor in angiogenesis overall, influencing up and downstream factors affecting all other major angiogenic pathways, and it is likely to have a crucial part to play in SBA formation at some level still. Interestingly, we detected a positive association with serum Ang-2 in SBA patients, which was directed by a publication by Ojeda-Fernandez et al in HHT patients (85). In contrast to their findings of a reduced serum level of Ang-2, we found that SBA patients had elevated levels compared to controls. However, we found no association with sEnd which was also shown to be lower in HHT patients, therefore suggesting that although both HHT and SBA pathophysiology share an alteration in the Angiopoietin pathway in their background pathophysiology, the specific differences in these alterations are likely to result in the characteristic presentations and clinical behaviour of each condition. Further assessments performed in this chapter on serum and tissue samples allowed us to conclude that the Angiopoietin pathway and specifically Ang-1, Ang-2, and their common receptor Tie2 were likely to be key factors in the pathophysiology of SBA formation. However, these findings were a rudimentary step in determining whether

this knowledge could be translated into a clinically useful application in the management of SBA, which we attempted to investigate in further chapters.

In chapter 3 we assessed whether the use of clinical factors, or our recently discovered serum factors (Ang-1 or Ang-2), might be useful in predicting diagnosis, clinical severity, or prognosis, in affected patients. We had previously published studies looking at the clinical characteristics and natural history of our own cohort of patients with SBA, and although we had identified certain factors, including advancing age, IHD, CKD and the use of anti-thrombotics and anticoagulants with the presence of SBA, we had not identified any clinically useful information which would aid diagnosis or assessments of prognosis, as the majority of SBA patients were already affected by these common clinical factors. In order to perform a broader assessment in a more heterogeneous GIAD cohort, we collaborated with a group of researchers in Radboud University in the Netherlands, who were carrying out similar clinical research in their SBA and GIAD cohorts. Collectively, we performed a systematic literature review assessing all published studies of GIAD to determine whether clinical factors could be used to aid diagnosis, classify disease severity, or predict prognosis. Although we initially included 66 studies in our quantitative assessment, only 23 were eligible for a qualitative assessment, and none were suitable for a meta-analysis. In essence, the literature review didn't yield any incremental clinical information regarding risk assessment or predictive factors to our own respective cohort studies, which although a reassuring validation of our own research findings, was not clinically helpful. The second study in this chapter assessed the use of serum measurements of Ang-1 and Ang-2 as a diagnostic aid in a group of patients undergoing CE for the investigation of suspected small bowel bleeding. We prospectively collected serum samples from all patients

undergoing CE, and then performed quantitative ELISA measurements of Ang-1 and Ang-2 to determine whether there were any differences in the levels of these factors based on subsequent diagnosis by CE. This assessment allowed us to address the issues around confounding factors affecting serum Ang-1 and Ang-2 levels which were raised by reviewers of our published findings in Chapter 2. By measuring both factors in a group of 120 patients all with suspected small bowel bleeding, and the majority with anaemia, we were able to out rule both factors as confounding variables. Furthermore, the larger cohort size allowed us to further assess the role of age, gender and CKD as confounding factors, and none appeared to significantly affect serum levels. Through this study, we found that the pattern of increased serum Ang-2 and decreased serum Ang-1 was specific for SBA, and was not just reflective of anaemia or an angiogenic effect of small bowel bleeding of any cause. We found that either serum Ang-2 alone, or a combination of the factors in an Ang-1/Ang-2 ratio produced a clinically acceptable negative and positive predictive value for a diagnosis of SBA, when assessed in a cohort of patients with suspected small bowel bleeding. Furthermore, we established a cut-off serum Ang-2 level of 2600 pg/ml to be predictive of a diagnosis of SBA in this cohort with an AUC of 0.695. Although this information represented a very welcome and relatively major discovery in our SBA research, it is not yet ready for application into clinical practice, and its position in the management of suspected small bowel bleeding will need to be fully assessed. It does however open up a number of different avenues for future research in the area, which I will discuss in detail in the future directions section of this chapter.

Chapter 4 used a commercially available multiplex assessment array which measured serum levels of 55 angiogenic factors. Although similar kits were commercially available at the outset of our work, they were very expensive and at that point we opted to

perform our putative assessment based on the available GIAD literature instead.

However, when R&D systems released a more affordable multiplex array we felt it may be of further value at that point in our research, both to hopefully validate the positive and negative associations we had already made with certain angiogenic factors, and to potentially identify other crucial factors which we hadn't yet tested for. Thankfully, the results from the multiplex assessment were reassuring, as they validated our already detected association of SBA with Ang-1 and Ang-2, and further out ruled any significant association with the factors suggested by the literature, including VEGF and sEnd. The multiplex study also identified two new factors for assessment, TIMP1 and Endostatin, which had not been associated with GIAD in the literature previously. We assessed the significance of both factors in the same way we had examined Ang-1 and Ang-2, by firstly using commercially available ELISA kits to measure serum levels in SBA patients and healthy controls, and secondly by assessing their use as diagnostic aids or discriminative factors by measuring serum levels in patients with suspected small bowel bleeding undergoing CE, subsequently grouped into three diagnoses, SBA, other small bowel bleeding source, and normal small bowel. In the first assessment, both factors were found to be significantly associated with SBA, with decreased levels of TIMP1 and increased levels of Endostatin, both anti-angiogenic factors, found in patients with SBA compared to controls. In our assessment of both factors as diagnostic aids, although our findings were interesting, with significantly different serum levels of both factors in each of the three diagnostic groups, they were deemed to be less clinically useful as potential biomarkers than Ang-1 or Ang-2, as there was significant overlap in the ranges of each factor across each diagnosis, making the determination of a cut-off level for diagnosis

impossible. However, the observed differences in serum levels of each factor should not be overlooked, and future assessments in different clinical scenarios may be of value. The initial chapters of this thesis focused on identifying several angiogenic factors likely to have a role in SBA pathophysiology, mainly through measurement of serum levels. However, the process of angiogenesis is highly complex, dynamic and constantly evolving, which meant that in order to prove a causative role rather than a clinical association, we needed to determine significant levels of these factors in SBA tissue rather than just serum. We initially decided to do this by measuring relative gene expression levels of each factor by qPCR. Although not a reliable quantitative assessment modality, it gave us very important information about whether or not the abnormalities in serum were also reflected in tissue, with the major advantage of being able to measure a large number of factors in a small amount of tissue. The results from this assessment showed elevated relative levels genes encoding Ang-1, Ang-2, and Tie2 in SBA tissue compared to the background patient control tissue, while levels of TIMP1 and Endostatin were not significantly different to background tissue levels. This suggests that although TIMP1 and Endostatin are still likely to have a role in SBA pathophysiology, they are more likely to have an indirect effect at a tissue level, possibly through a stimulatory or inhibitory effect on up or downstream markers of the Angiopoietins, which may also be the case with VEGF, and there are a number of ways to potentially explore this in future assessments. We went on to use IHC in an attempt to more accurately quantify tissue levels of Ang-1, Ang-2, and Tie2 in SBA tissue, but unfortunately, we were unable to get any clinically useful results, despite many protocol adjustments and the expertise of a senior medical scientist and consultant pathologist. We felt that the failure of this experiment was likely due to two main factors, tissue size

or quality, and antibody selection. At the time of tissue collection for this experiment, the DBE scope allowed only a single biopsy of 1-2mm from an SBA lesion. Anecdotally, we have noticed in our unit that similar biopsies we have sent for histological assessment in our institution were generally reported as normal, or with mildly increased vasculature, but very rarely assigned a definitive diagnosis of SBA. This likely reflects the fragility of a single small sample which requires significant processing even before taking into account the preparation required for IHC using antibodies. This issue will hopefully be quite easily overcome moving forward due to the acquisition of a new DBE scope in the unit, with a channel which permits use of a larger biopsy forceps, meaning that samples will be almost double the size in future. This may also be helpful for future qPCR work, as we had difficulties obtaining enough DNA in matched SBA and control tissue also, likely due to tissue size. Secondly, we may have been unlucky with the antibodies we selected for use in IHC on this occasion. We chose the brand of antibody based on its compatibility with our pathology department's IHC machinery in order to make the experiment more standardised. However, research involving Angiopoietins, particularly in an IHC model is at the very early stages internationally, and although several companies have developed commercially available antibodies, there are a very limited number of researcher reviews for each antibody, meaning that negative results or failed studies are probably not too surprising at this stage. There do appear to be slightly more publications using the western blot technique of protein quantification with the same antibodies, which may be a viable option in the future, however; the limiting factor in our case was availability of tissue.

Future Directions

The overall focus of this thesis was to learn more about the angiogenic factors driving the pathophysiology of SBA formation so that they might be identified as potentially useful targets to improve diagnosis, allow us to assess prognosis of the condition, and as molecular treatment targets for specific anti-angiogenic treatments to be developed. We have successfully identified the Angiopoietin pathway as a key angiogenic pathway in SBA pathophysiology, primarily through changes in levels of Ang-1, Ang-2 and their receptor Tie2, both in serum and at a tissue level. Furthermore, we have identified TIMP1 and Endostatin as angiogenic factors associated specifically with SBA, and likely to be closely involved in the pathophysiology of SBA at least at a serum level, perhaps through their interaction with the Angiopoietins, or other angiogenic stimuli, or inhibitors. This information, although currently at the basic science level, represents a number of exciting areas for future research in the area of SBA, and is a fundamental step towards advancing our understanding of the condition, which will hopefully translate towards improved patient care and outcomes in the not so distant future. A major challenge at present for SBA patients is an often-delayed diagnosis of the condition. Although IDA is easily picked up and can be treated empirically by physicians, the specific diagnosis of SBA is delayed by the need for small bowel CE, with a median waiting time of over 18 months, generally after a not insubstantial waiting time for initial upper and lower endoscopy, depending on the severity of their anaemia. One of the objectives of this thesis was to determine any useful patient factors which could aid diagnosis. Although we were able to associate a number of clinical factors, including advanced age, cardiovascular disease, CKD and COPD, with the presence of SBA, the high prevalence of these conditions among elderly patients in Ireland and Internationally, means that they are not useful in triaging patients with IDA referred for CE. One of the

factors found to be associated with a positive diagnosis of SBA by CE was the presence of overt bleeding, or the completion of the examination within 4 days of a significant drop in Hb. However, as these are already factors used to prioritise CE, they were again not specific for SBA and therefore not found to be useful as a specific or incremental diagnostic aid. Once we had established an association of a specific alteration in serum levels of Ang-1 and Ang-2 with a diagnosis of SBA we attempted to assess their use in clinical practice as a filter or triage test prior to CE. The results of this assessment in 120 patients undergoing CE for assessment of IDA or suspected small bowel bleeding showed that serum levels of Ang-2 could be used alone to predict a diagnosis of SBA. Using a determined cut-off level of Ang-2 of 2600 pg/ml, the sensitivity and negative predictive value for a diagnosis of SBA were both found to be 85%. Although the positive predictive value and specificity were significantly lower at 45% and 50% respectively, the majority of patients with a false positive result were found to have a clinically relevant diagnosis, meaning that an urgent prioritisation for the examination was correct, although not specific for SBA. Despite a worldwide increased uptake in the use of CE, and its inclusion as the first second line investigation for suspected small bowel bleeding, the provision of a CE service in Ireland within the Health Service Executive (HSE) remains limited to one centre nationally, due to a combination of available expertise and issues around financial reimbursement. With a current average waiting time of over 18 months for the procedure, the availability of a cheap, non-invasive filter test to aid in prioritisation of patients would be extremely useful. The AUC for this cut-off level of Ang-2 was 0.695, which is below par for use in clinical practice, however there is great scope for improving the AUC, through incorporation with other significant serum angiogenic factors, including TIMP1 or Endostatin, and further

assessments of these factors in this cohort are already planned in our unit. Another filter test for prioritising SBA which has been incorporated into a trial in our unit is the use of a faecal immunochemical test (FIT) prior to CE (319). FIT detects the presence of human globin in stool and is predominantly used in bowel cancer screening services internationally. However, it has been shown that 40-60% of patients with a positive FIT result do not have colonic lesions at endoscopy to explain their blood loss, and it has been proposed that a proportion of these patients may have a small bowel bleeding source (320, 321). In this study of 54 patients a positive FIT test prior to CE was found to have a positive predictive value for small bowel pathology of 50% and a negative predictive value of 89%, with SBA accounting for 23% of their positive findings. In addition, they found that a combination of anaemia and a positive FIT increased the positive predictive value to 67%. Similarly to measurement of serum levels of Ang-2, FIT is a cheap, non-invasive test which is widely available and patient friendly, and it would be interesting to measure FIT levels alongside serum Ang-2, Endostatin or TIMP1 levels to see whether a combination of these filter tests may increase the subsequent diagnostic yield of CE, particularly helping to prioritise patients with SBA for an earlier diagnosis and more targeted treatment.

Another major challenge in the care of patients with SBA, is the lack of any prognostic markers for disease course or activity. The severity of SBA has not definitively been associated with the number of lesions in the small bowel, and anecdotally we have seen patients with few lesions suffer a more severe and even fatal clinical course than those with more multiple lesions. The use of antithrombotics and anticoagulants is known to increase the risk of all GI bleeding and the cessation of these medications is often undertaken after a bleeding episode if possible, after consultation with the patient's

various other treating physicians and attempting to apply a risk benefit assessment for the medications. However, without an objective prognostic tool this is often difficult to do. As a result, we often end up following patients up intensely with Hb monitoring and advise them to be aware of episodes of overt bleeding. As neither of these factors are helpful in predicting a future bleeding episode, it means recurrent bleeding can only be detected after it has occurred, risking the development of severe anaemia and exacerbation of co-morbidities. Hence, the availability of some form of prognostic marker with the ability to herald a future bleed, which could be measured at intervals alongside current disease assessment tools would be of great advantage in identifying patients at an earlier stage and hopefully preventing progression to severe bleeding episodes. Serum measurements of Ang-1, Ang-2, Endostatin and TIMP1 at various stages of disease presentation would be an interesting assessment and may prove clinically useful in this regard. Park et al performed a study looking at the use of serum levels of Ang-1 and Ang-2 as a prognostic marker for future disease course in patients with resected early stage lung cancer (322). They initially observed a similar pattern to our SBA cohort, with a reduced serum Ang-1 level and elevated Ang-2 level in affected patients and controls, and they concluded that a higher level of Ang-2 was associated with a more progressive stage of disease, and that a higher level of Ang-1 was associated with a lower rate of disease recurrence during follow-up. Furthermore, a study by Ganter et al identified higher serum levels of Ang-2 at presentation in patients following major trauma as a poor prognostic factor in terms of disease severity, recovery time and overall clinical outcome (323). However, serial Ang-2 levels were not monitored during recovery to determine whether fluctuations may be associated with changes in the clinical course of disease. A study by Helfrich et al assessed the use of assessment of

serum levels of Ang-2 at various intervals to determine its use as a predictive or prognostic marker of disease severity in metastatic malignant melanoma and found that serum Ang-2 levels taken at various time intervals during the progression of disease from stage III to stage IV showed an increase of up to 400% (324). They determined that serum Ang-2 levels could be useful prognostic markers in terms of predicting disease severity both at the outset of diagnosis and also when used at intervals to assess disease progression. In addition to Ang-2 levels, serum Endostatin levels as a prognostic marker have also been evaluated in various disease types. Suzuki et al reported that higher levels of Endostatin in patients with non-small cell lung cancer was associated with a poorer prognosis in terms of stage of disease and overall survival (325). Similarly, Mo et al have determined that an elevated serum level of Endostatin in patients with nasopharyngeal carcinoma was an independent unfavourable prognostic factor for disease progression and overall survival (326). In addition, serum TIMP-1 levels have been reported to be useful as diagnostic and prognostic markers in other diseases. Poruk et al reported the successful use of TIMP-1 in differentiating pancreatic adenocarcinoma from chronic pancreatitis, and found that patients with resectable disease had significantly lower serum levels of TIMP-1 than patients with advanced disease (327). A similar study by Wang et al in patients with gastric cancer found that although serum TIMP-1 levels were not useful in diagnosis, a significantly elevated level was associated with more progressive disease and a reduced survival, making it a useful prognostic marker (328). These studies provide the basis for future work in SBA, where the assessment of specific serum levels of Ang-1, Ang-2, Endostatin and TIMP-1 may be used to highlight at risk patients who warrant closer monitoring. Furthermore, the assessment of serum levels of each of these factors at various intervals, particularly

around bleeding episodes, may prove them to be useful prognostic factors which could be used as an adjunct to the current clinical and Hb tools used in disease monitoring. Finally, although this thesis was focused on identifying angiogenic factors specifically associated with SBA and its underlying pathophysiology, there are a number of related vascular mucosal conditions which affect the gastrointestinal tract and result in refractory gastrointestinal bleeding which may share a common underlying aetiology in terms of pathophysiology. In particular, GAVE and portal hypertensive gastropathy (PHG) affect the stomach, with similar mucosal vascular appearances to GIAD, although the surface area is usually more extensive. Both conditions result in intermittent gastrointestinal bleeding at unpredictable intervals, which can be severe. Although GAVE can be treated endoscopically using APC or band ligation, both approaches usually require multiple endoscopic sessions and recurrence rates post-treatment remain high, with refractory patients occasionally requiring a distal gastrectomy in severe cases (329). PHG is most commonly associated with liver cirrhosis, although non-cirrhotic portal hypertension is also less commonly detected. Gastrointestinal bleeding from PHG is usually chronic and obscure but can lead to severe anaemia in a cohort of patients who often have multiple other co-morbidities (330). Treatment of bleeding from PHG is primarily through the prophylactic use of beta blockers which are often poorly tolerated. No specific medical treatment for either GAVE or PHG is available due to limited knowledge behind the pathophysiology for their development, as for SBA. Our hypothesis was that the alterations in serum abnormalities that we detected in patients with SBA may be due to an alteration in the local gastrointestinal vasculature, and may as a result be shared by other local conditions, such as GAVE and PHG. Our group have already carried out an assessment of serum angiogenic factors in patients with GAVE

and PHG, alongside SBA patients and healthy controls and have recently had the results accepted for publication in the World Journal of Gastrointestinal Pharmacology and Therapeutics. We measured serum levels of Ang-1, Ang-2, and VEGF in all groups and found that mean levels of Ang-1 were significantly lower in patients with SBA, GAVE and PHG, compared to controls, while mean Ang-2 levels were significantly higher, in a similar pattern to what we observed in this thesis in our SBA cohort. Additionally, there was no significant difference in serum VEGF levels between patients in any group and controls. Based on these findings, it would be reasonable to presume that GAVE and PHG may share a common angiogenic pathway with SBA formation, at least at a systemic level, and that potential diagnostic and prognostic markers earmarked for future assessment in patients with SBA, may be useful in patients with GAVE and PHG also. Further work assessing these angiogenic factors at a tissue level in patients with GAVE and PHG will hopefully yield further information as to whether the observed serum abnormalities are also present at a tissue level, as in SBA. If found to share a common angiogenic pathway this may mean that angiogenic factors identified as potential therapeutic targets in patients with SBA may also be useful in patients with GAVE and PHG, thereby increasing the number of patients available for further research and medication development, and increasing the potential therapeutic impact to a much larger patient group.

References:

1. Regula J, Wronska E, Pachlewski J. Vascular lesions of the gastrointestinal tract. Best practice and research, clinical gastroenterology. Vol 22, no.2 pg 313-328, 2008.
2. Boley SJ, Sammartano R, Adams A, et al. On the nature and etiology of vascular ectasias of the colon. Degenerative lesions of aging. Gastroenterology. 1977;72:650–60
3. Baum S, Athanasoulis CA, Waltman AC, Galdabini J, Schapiro RH, Warshaw AL, Ottinger LW. Angiodysplasia of the right colon: a cause of gastrointestinal bleeding. AJR Am J Roentgenol. 1977 Nov;129(5):789-94.
4. D E Roskell, S C Biddolph, and B F Warren. Apparent deficiency of mucosal vascular collagen type IV associated with angiodysplasia of the colon. J Clin Pathol. 1998 January; 51(1): 18–20.
5. Heyde EC (1958). "Gastrointestinal bleeding in aortic stenosis". N. Engl. J. Med. 259 (4): 196.
6. Junquera F, Saperas E, de Torres I, Vidal MT, Malagelada JR. Increased expression of angiogenic factors in human colonic angiodysplasia. Am J Gastroenterol. 1999;94:1070-6.
7. Rockey DC, Koch J, Cello JP, Sanders LL, McQuaid K. Relative frequency of upper gastrointestinal and colonic lesions in patients with positive fecal-occult blood tests. N Eng J Med 1998;339:153-9.

8. Schmit A, Gay F, Adler M, Cremer M, Van Gossum A. Diagnostic efficacy of push enteroscopy and long term follow up of patients with small bowel angiodysplasia. *Dig Dis Sci* 1996;41:2348-2352.
9. Sami SS , Al-Araji SA , Ragunath K . Review article: gastrointestinal angiodysplasia - pathogenesis, diagnosis and management. *Aliment Pharmacol Ther* 2014;39:15–34
10. X Dray, Camus M, Coelho J, Ozenne V, Pocard M, Marteau P. Treatment of gastrointestinal angiodysplasia and unmet needs. *Dig Liver dis* 2011 Jul;43(7):515-22. Epub 2011 Jan 15.
11. Bollinger E, Raines D, Saitta P. Distribution of bleeding gastrointestinal angioectasias in a Western population. *World J Gastroenterol*. 2012 Nov 21;18(43):6235-9.
12. Holleran G, Hall B, Zgaga L, Breslin N, McNamara D. The natural history of small bowel angiodysplasia. *Scand J Gastroenterol*. 2016;51(4):393-9.
13. Nardone G, Compare D, Scarpignato C, Rocco A. Long acting release-octreotide as "rescue" therapy to control angiodysplasia bleeding: A retrospective study of 98 cases. *Dig Liver Dis*. 2014 Aug;46(8):688-94.
14. Lecleire S, Iwanicki-Caron I, Di-Fiore A, et al. Yield and impact of emergency capsule enteroscopy in severe obscure-overt gastrointestinal bleeding. *Endoscopy* 2012; 44: 337–42.
15. Ueno S, Nakase H, Kasahara K, Uza N, Kitamura H, Inoue S et al. Clinical features of Japanese patients with colonic angiodysplasia. *J Gastroenterol Hepatol*. 2008 Aug;23(8 Pt 2):e363-6.

16. Igawa A, Oka S, Tanaka S, et al. Major predictors and management of small-bowel angioectasia. *BMC Gastroenterol* 2015; 15: 108.
17. Richter JM, Christensen MR, Colditz GA, et al. Angiodysplasia. Natural history and efficacy of therapeutic interventions. *Dig Dis Sci* 1989; 34: 1542–1546.
18. May A, Friesing-Sosnik T, Manner H, et al. Long-term outcome after argon plasma coagulation of small-bowel lesions using double balloon enteroscopy in patients with mid gastrointestinal bleeding. *Endoscopy* 2011; 43:759–765.
19. Grooteman KV, van Geenen EJ, Drenth JP. High variation in treatment strategies for gastrointestinal angiodysplasias. *Eur J Gastroenterol Hepatol*. 2016 Sep;28(9):1082-6.
20. Swanson E, Mahgoub A, MacDonald R, et al. Medical and endoscopic therapies for angiodysplasia and gastric antral vascular ectasia: a systematic review. *Clin Gastroenterol Hepatol* 2014; 12: 571–582.
21. Vargo JJ. Clinical applications of the argon plasma coagulator. *Gastrointest Endosc* 2004;59:81-88
22. Olmos JA, Marcolongo M, Pogorelsky V, Herrera L, Tobal F, Davolos JR. Long-term outcome of argon plasma ablation therapy for bleeding in 100 consecutive patients with colonic angiodysplasia. *Dis Colon Rectum* 2006; 49: 1507–16.
23. Olmos JA, Marcolongo M, Pogorelsky V, Varela E, Davolos JR. Argon plasma coagulation for prevention of recurrent bleeding from GI angiodysplasias. *Gastrointest Endosc* 2004; 60: 881–6.
24. May A, Friesing-Sosnik T, Manner H, Pohl J, Ell C. Long-term outcome after argon plasma coagulation of small-bowel lesions using double-balloon enteroscopy in patients with mid-gastrointestinal bleeding. *Endoscopy*. 2011 Sep;43(9):759-65.

25. Rahmi G, Samaha E, Vahedi K, Delvaux M, Gay G, Lamouliatte H, et al. Long-term follow-up of patients undergoing capsule and double-balloon enteroscopy for identification and treatment of small-bowel vascular lesions: a prospective, multicenter study. *Endoscopy*. 2014 Jul;46(7):591-7. doi: 10.1055/s-0034-1365514.
26. Landi B, Cellier C, Gaudric M, Demont H, Guimbaud R, Cuillerier E, Couturier D, Barbier JP, Marteau P. Long-term outcome of patients with gastrointestinal bleeding of obscure origin explored by push enteroscopy. *Endoscopy*. 2002 May;34(5):355-9.
27. Samaha E, Rahmi G, Landi B, Lorenceau-Savale C, Malamut G, Canard JM, Bloch F, Jian R, Chatellier G, Cellier C. Long-term outcome of patients treated with double balloon enteroscopy for small bowel vascular lesions. *Am J Gastroenterol*. 2012 Feb;107(2):240-6.
28. Bauditz J, Lochs H, Voderholzer W. Macroscopic appearance of intestinal angiodysplasias under antiangiogenic treatment with thalidomide. *Endoscopy* 2006;38:1036-1039
29. Bauditz J, Lochs H. Angiogenesis and vascular malformations: antiangiogenic drugs for treatment of gastrointestinal bleeding. *World J Gastroenterol* 2007;13:5979-5984
30. Moschler O, May AD, Muller MK, et al. Complications in double balloon enteroscopy: results of the German DBE registry (in German). *Z Gastroenterol* 2008;46:266-270
31. Suzuki N, Arebi N, Saunders BP. A novel method of treating colonic angiodysplasia. *Gastrointest Endosc* 2006; 64: 424–7.

32. Kwan V, Bourke MJ, Williams SJ, et al. Argon plasma coagulation in the management of symptomatic gastrointestinal vascular lesions: experience in 100 consecutive patients with long-term follow-up. *Am J Gastroenterol* 2006; 101:58–63.
33. F. Junquera, E. Brullet, R. Campo, X. Calvet, V. Puig-Divi, M. Vergara. Usefulness of endoscopic band ligation for bleeding small bowel vascular lesions. *Gastrointest Endosc*, 58 (2003), pp. 274–279
34. Rutgeerts P, van Gompel F, Geboes K et al. Long-term results of treatments of vascular malformations of the gastrointestinal tract by neodymium YAG laser photocoagulation. *GUT* 1985;26:586-593
35. Harrison DF. Use of estrogen in treatment of familial hemorrhagic telangiectasia. *Laryngoscope* 1982;92:314-20.
36. Szilagyi A, Ghali MP. Pharmacological therapy of vascular malformations of the gastrointestinal tract. *Can J Gastroenterol*. 2006 Mar;20(3):171-8. Review
37. Junquera F, Santos J, Saperas E, Armengol JR, Malagelada JR. Estrogen and progestagen treatment in digestive hemorrhage caused by vascular malformations. *Gastroenterol Hepatol*. 1995 Feb;18(2):61-5
38. Barkin JS, Ross BS. Medical therapy for chronic gastrointestinal bleeding of obscure origin. *Am J Gastroenterol* 1998;93:1250-4.
39. Van Cutsem E, Rutgeert P, Vantrappen G. Long-term effect of hormonal therapy for bleeding gastrointestinal vascular malformations. *Eur J Gastroenterol Hepatol* 1993;5:439–43.

40. Carey EJ, Leighton JA, Heigh RI, et al. A single-center experience of 260 consecutive patients undergoing capsule endoscopy for obscure gastrointestinal bleeding. *Am J Gastroenterol* 2007;102:89-95.
41. Bauditz J, Schachschal G, Wedel S, et al. Thalidomide for treatment of severe intestinal bleeding. *Gut* 2004;53:609–12.
42. Shurafa M, Kamboj G. Thalidomide for the treatment of bleeding angiodysplasias. *Am J Gastroenterol* 2003;98:221–2.
43. De Koning DB, Drenth JP, Friederich P, et al. Thalidomide for the treatment of recurrent gastrointestinal blood loss due to intestinal angiodysplasias. *Ned Tijdschr Geneeskd* 2006;150:1994–7.
44. Kamalaporn P, Saravanan R, Cirocco M, May G, Kortan P, Kandel G, Marcon N. Thalidomide for the treatment of chronic gastrointestinal bleeding from angiodysplasias: a case series. *Eur J Gastroenterol Hepatol*. 2009 Dec;21(12):1347-50.
45. Fernández Salazar LI, Velayos Jiménez B, Fernández Galante I, Aller de la Fuente R, González Hernández JM. Efficacy of thalidomide in digestive bleeding caused by angiodysplasias. *Rev Esp Enferm Dig*. 2009 May;101(5):371
46. Ge ZZ, Chen HM, Gao YJ, Liu WZ, Xu CH, Tan HH, Chen HY, Wei W, Fang JY, Xiao SD. Efficacy of thalidomide for refractory gastrointestinal bleeding from vascular malformation. *Gastroenterology*. 2011 Nov;141(5):1629-37.e1-4
47. Bauditz J, Lochs H, Voderholzer W. Macroscopic appearance of intestinal angiodysplasias under antiangiogenic treatment with thalidomide. *Endoscopy* 2006;38:1036–9.

48. Izquierdo Navarro MC , Hernando Verdugo M , Cardaba Garcia E , et al.
Therapeutic failure with thalidomide in patients with recurrent intestinal
bleeding due to angiodysplasias. *Farm Hosp* 2016;40:230–2
49. Kurosaki M, Saegert W, Abe T, Ludecke DK. Expression of vascular endothelial
growth factor in growth hormone-secreting pituitary adenomas: special
reference to the octreotide treatment. *Neurol Res* 2008; 30: 518–22.
50. Ludwig D, Terai S, Bruning A, Stange EF. Long-term haemodynamic effects of
octreotide on postprandial splanchnic hyperemia in humans: a placebocontrolled
echo-doppler study. *Aliment Pharmacol Ther* 1999; 13: 1119–29.
51. Junquera F, Saperas E, Videla S, et al. Long-term efficacy of octreotide in the
prevention of recurrent bleeding from gastrointestinal angiodysplasia. *Am J
Gastroenterol* 2007; 102: 254–60.
52. Nardone G, Rocco A, Balzano T, Budillon G. The efficacy of octreotide therapy in
chronic bleeding due to vascular abnormalities of the gastrointestinal tract.
Aliment Pharmacol Ther. 1999 Nov;13(11):1429-36
53. Scaglione G, Pietrini L, Russo F, Franco MR, Sorrentini I. Long-acting octreotide as
rescue therapy in chronic bleeding from gastrointestinal angiodysplasia. *Aliment
Pharmacol Ther* 2007; 26: 935–42.
54. Molina IJ, Perez GB, Hernandez AM, Mateos Rodriguez JM, Duenas SC, Fernandez
BM. Octreotide long acting release for severe obscure gastrointestinal
haemorrhage in elderly patients with serious comorbidities. *Med Clin (Barc)*
2009; 133: 667–70.
55. Bon C, Aparicio T, Vincent M, Mavros M, Bejou B, Raynaud JJ, Zampeli E, Airinei
G, Sautereau D, Benamouzig R, Michopoulos S. Long-acting somatostatin

- analogues decrease blood transfusion requirements in patients with refractory gastrointestinal bleeding associated with angiodysplasia. *Aliment Pharmacol Ther.* 2012 Sep;36(6):587-93.
56. Holleran G, Hall B, Breslin N, McNamara D. Long-acting somatostatin analogues provide significant beneficial effect in patients with refractory small bowel angiodysplasia: Results from a proof of concept open label mono-centre trial. *United European Gastroenterol J.* 2016 Feb;4(1):70-6.
57. Grooteman KV, van Geenen EJ, Drenth JP. Multicentre, open-label, randomised, parallel-group, superiority study to compare the efficacy of octreotide therapy 40 mg monthly versus standard of care in patients with refractory anaemia due to gastrointestinal bleeding from small bowel angiodysplasias: a protocol of the OCEAN trial. *BMJ Open.* 2016 Sep 12;6(9):e011442.
58. Benamouzig R, Benallaoua M, Saurin JC, Boubaya M, Cellier C, Laugier R, Vincent M, Boustière C, Gincul R, Samaha E, Grandval P, Aparicio T, Airinei G, Bejou B, Bon C, Raynaud JJ, Levy V, Sautereau D. Efficacy and safety of pasireotide-LAR for the treatment of refractory bleeding due to gastrointestinal angiodysplasias: results of the ANGIOPAS multicenter phase II noncomparative prospective double-blinded randomized study. *Therap Adv Gastroenterol.* 2018 Feb 16;11:1756283X18756260. doi: 10.1177/1756283X18756260. eCollection 2018.
59. Hawkey GM, Cole AT, McIntyre AS, Long RG, Hawkey CJ. Drug treatments in upper gastrointestinal bleeding: value of endoscopic findings as surrogate end points. *Gut* 2001;49:372-9.

60. Von Holstein CC, Eriksson SB, Kallen R. Tranexamic acid as an aid to reducing blood transfusion requirements in gastric and duodenal bleeding. *BMJ* 1987;294:7-10.
61. Ker K, Edwards P, Perel P, Shakur H, Roberts I. Effect of tranexamic acid on surgical bleeding: systematic review and cumulative meta-analysis. *BMJ* 2012;344:e3054.
62. Grooteman KV, van Geenen EJM, Drenth JPH. Tranexamic acid in treatment-resistant chronic transfusion-dependent gastrointestinal angiodysplasia bleeding. *BMJ Case Rep.* 2017 Nov 1;2017.
63. Manno D1, Ker K, Roberts I. How effective is tranexamic acid for acute gastrointestinal bleeding? *BMJ.* 2014 Feb 17;348:g1421. doi: 10.1136/bmj.g1421.
64. Marlu R, Barthelon J, Durand A, Mathieu N, Barro C, Granger V, Tatu A, Pernod G, Polack B, Bonaz B. Long-term therapy with bevacizumab in a patient with Glanzmann's thrombasthenia and recurrent digestive bleeding due to gastrointestinal angiodysplastic lesions. *Am J Gastroenterol.* 2015 Feb;110(2):352-3.
65. Fiorito JJ, Brandt LJ, Kozicky O, Grosman IM, Sprayragen S. The diagnostic yield of superior mesenteric angiography: correlation with the pattern of gastrointestinal bleeding. *Am J Gastroenterol.* 1989 Aug;84(8):878-81.
66. Pai M, Frampton AE, Virk JS, Nehru N, Kyriakides C, Limongelli P, Jackson JE, Jiao LR. Preoperative superselective mesenteric angiography and methylene blue injection for localization of obscure gastrointestinal bleeding. *JAMA Surg.* 2013 Jul;148(7):665-8.

67. Cappell MS, Gupta A. A changing epidemiology of gastrointestinal angiodysplasia with increasing recognition of clinically milder cases: angiodysplasia tend to produce mild chronic gastrointestinal bleeding in a study of 47 consecutive patients admitted from 1980-1989. *Am J Gastroenterology* 1992;87:201-6.
68. Kheterpal S. Angiodysplasia; a review. *Journal of the Royal Soc Med.* 1991;84:615-18.
69. Kim DB, Chung WC, Lee SJ, Sung HJ, Woo S, Kim HS, Jeong YO, Lee H, Kim YJ. Analysis of risk factor and clinical characteristics of angiodysplasia presenting as upper gastrointestinal bleeding. *Korean J Intern Med.* 2016 Jul;31(4):669-77.
70. Werkentin TE, Moore JC, Anand SS, Lonn EM, Morgan DG. Gastrointestinal bleeding; angiodysplasia, cardiovascular disease and acquired von Willebrand syndrome. *Transfusion Med Rev* 2003;4:272-86.
71. Gordon FH, Watkinson A, Hodgson H. Vascular malformations of the gastrointestinal tract. *Best Practice and Research, clinical Gastroenterology* 2001;15:41-58
72. Holleran G, Hall B, Hussey M, McNamara D. Small bowel angiodysplasia and novel disease associations: a cohort study. *Scand J Gastroenterol.* 2013 Apr;48(4):433-8.
73. Jackson CS, Gerson LB. Management of gastrointestinal angiodysplastic lesions (GIADs): a systematic review and meta-analysis. *Am J Gastroenterol.* 2014 Apr;109(4):474-83; quiz 484.
74. Foutch PG, Rex DK, Lieberman DA. Prevalence and natural history of colonic angiodysplasia among healthy asymptomatic people. *Am J Gastroenterol.* 1995 Apr;90(4):564-7

75. Nishimura N, Mizuno M, Shimodate Y, Doi A, Mouri H, Matsueda K, Yamamoto H. Risk factors for active bleeding from colonic angiodysplasia confirmed by colonoscopic observation. *Int J Colorectal Dis*. 2016 Dec;31(12):1869-1873. Epub 2016 Sep 5.
76. Grooteman KV, Matheeuwsen M, van Geenen EJM, Drenth JPH. Decreased health-related quality of life in angiodysplasia patients: A cross-sectional cohort. *PLoS One*. 2017 May 26;12(5):e0177522.
77. Bouïs D, Kusumanto Y, Meijer C, Mulder NH, Hospers GA. A review on pro- and anti-angiogenic factors as targets of clinical intervention. *Pharmacol Res*. 2006 Feb;53(2):89-103.
78. Durand MJ, Ait-Aissa K, Gutterman DD. Regenerative Angiogenesis: Quality Over Quantity. *Circ Res*. 2017 Apr 28;120(9):1379-1380.
79. Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003 Jun;9(6):653-60. Review.
80. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;9:669-676.
81. Bopp S. Anti-VEGF for neovascular ARMD: visual improvement as the goal of therapy? *Br J Ophthalmol*. 2007 Oct;91(10):1259-60.
82. Rosen LS, Jacobs IA, Burkes RL. Bevacizumab in Colorectal Cancer: Current Role in Treatment and the Potential of Biosimilars. *Target Oncol*. 2017 Oct;12(5):599-610. doi: 10.1007/s11523-017-0518-1.
83. Tamura R, Tanaka T, Miyake K, Yoshida K, Sasaki H. Bevacizumab for malignant gliomas: current indications, mechanisms of action and resistance, and markers

- of response. *Brain Tumor Pathol.* 2017 Apr;34(2):62-77. doi: 10.1007/s10014-017-0284-x. Epub 2017 Apr 6.
84. Escudier B, Pluzanska A, Koralewski P et al. Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind Phase III trial. *Lancet* 2007;370(9605): 2103–2111
 85. Ojeda-Fernandez L, Barrios L, Rodriguez-Barbero A, Recio-Poveda L, Bernabeu C, Botella LM. Reduced plasma levels of Ang-2 and sEng as novel biomarkers in hereditary hemorrhagic telangiectasia (HHT). *Clin Chim Acta.* 2010 Apr 2;411(7-8):494-9.
 86. Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, ten Dijke P. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J.* 2004 Oct 13;23(20):4018-28. Epub 2004 Sep 23.
 87. Fagiani E, Christofori G. Angiopoietins in angiogenesis. *Cancer Lett.* 2013 Jan 1;328(1):18-26
 88. Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, Kriz W, Thurston G, Augustin HGet al.. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood.* 2004 Jun 1;103(11):4150-6.
 89. Randi AM1, Laffan MA, Starke RD. Von Willebrand factor, angiodyplasia and angiogenesis. *Mediterr J Hematol Infect Dis.* 2013 Sep 2;5(1):e2013060.
 90. Peter S. Unfolding the plot of obscure gastrointestinal bleeding:excellent or elemental. *Clin Gastroenterol Hepatol* 2012 Dec;10(12):1386-8.

91. Foutch PG. Angiodysplasia of the gastrointestinal tract. *Am J Gastroenterol* 1993;88:807-18.
92. Junquera F, Feu F, Papo M, Videla S, Armengol JR, Bordas JM, Saperas E, Piqué JM, Malagelada JR. A multicenter, randomized, clinical trial of hormonal therapy in the prevention of rebleeding from gastrointestinal angiodysplasia. *Gastroenterology*. 2001 Nov;121(5):1073-9.
93. Saperas E, Dot J, Videla S, et al. Capsule endoscopy versus computed tomographic or standard angiography for the diagnosis of obscure gastrointestinal bleeding. *Am J Gastroenterol* 2007;102:731-737.
94. Lee SS, Oh TS, Kim HJ et al (2011) Obscure gastrointestinal bleeding: diagnostic performance of multidetector CT enterography. *Radiology* 259:739–748
95. Milano A, Balatsinou C, Fillipone A, Caldarella MP, Laterza F, Lapenna D et al. A prospective evaluation of iron deficiency anaemia in the GI endoscopy setting: role of standard endoscopy, video-capsule endoscopy, and CT-enterocolysis. *Gastrointest Endosc*. 2011 May;73(5):1002-8
96. Triester SL, KLeighton JA, Leontiadis JL, Gurudu SR, Fleischer DE, Hara AK, et al. A meta-analysis of the yield of capsule endoscopy compared to other diagnostic modalities in patients with obscure gastrointestinal bleeding. *Am J Gastroenterol* 2005;100:2407-18.
97. Holleran G, Hall B, Hussey M, McNamara D. Small bowel angiodysplasia and novel disease associations: a cohort study *Scand J Gastroenterol*. 2013 Apr;48(4):433-8.
98. Nissen AR, Goodnough LT, Dubois LW. Anaemia: not just an innocent bystander?. *Arch Intern Med* Jun 2003;163(12):1400-4.

99. Szilagyi A, Ghali MP. Pharmacological therapy of vascular malformations of the gastrointestinal tract. Review. *Can J Gastroenterol*. 2006 Mar;20(3):171-8.
100. Cappell MS, Gupta A. A changing epidemiology of gastrointestinal angiodysplasia with increasing recognition of clinically milder cases: angiodysplasia tend to produce mild chronic gastrointestinal bleeding in a study of 47 consecutive patients admitted from 1980-1989. *Am J Gastroenterology* 1992;87:201-6.
101. Junquera F, Saperas E, de Torres I, Vidal MT, Malagelada JR. Increased expression of angiogenic factors in human colonic angiodysplasia. *Am J Gastroenterol*. 1999;94:1070-6.
102. Ojeda-Fernandez L, Barrios L, Rodriguez-Barbero A, Recio-Poveda L, Bernabeu C, Botella LM. Reduced plasma levels of Ang-2 and sEng as novel biomarkers in hereditary hemorrhagic telangiectasia (HHT). *Clin Chim Acta*. 2010 Apr 2;411(7-8):494-9.
103. Lebrin F, Srun S, Raymond K, Martin S, van den Brink S, Freitas C, Bréant C, Mathivet T, Larrivée B, Thomas JL, Arthur HM, Westermann CJ, Disch F, Mager JJ, Snijder RJ, Eichmann A, Mummery CL. Thalidomide stimulates vessel maturation and reduces epistaxis in individuals with hereditary hemorrhagic telangiectasia. *Nat Med*. 2010 Apr; 16(4):420-8.
104. Kümpers P, Lukasz A, David S, Horn R, Hafer C, Faulhaber-Walter R, et al. Excess circulating angiopoietin-2 is a strong predictor of mortality in critically ill medical patients. *Crit Care*. 2008; 12(6): R147
105. Tran HT, Liu Y, Zurita AJ, Lin Y, Baker-Neblett KL, Martin AM, Figlin RA, Hutson TE, Sternberg CN, Amado RG, Pandite LN, Heymach JV. Prognostic or

predictive plasma cytokines and angiogenic factors for patients treated with pazopanib for metastatic renal-cell cancer: a retrospective analysis of phase 2 and phase 3 trials. *Lancet Oncol.* 2012 Aug;13(8):827-37.

106. Ge ZZ, Chen HM, Gao YJ et al. Efficacy of thalidomide for refractory gastrointestinal bleeding from vascular malformation. *Gastroenterology* 2011; 141: 1629–37
107. Tan H, Chen H, Xu C, Ge Z, Gao Y, Fang J, Liu W, Xiao S. Role of vascular endothelial growth factor in angiodysplasia: an interventional study with thalidomide. *Journal of gastroenterology and hepatology.* 2012 Jun;27(6):1094-101.
108. Thomas M, Augustin HG. The role of the Angiopoietins in vascular morphogenesis. *Angiogenesis.* 2009 Jun 1;12(2):125.
109. Fagiani E, Christofori G. Angiopoietins in angiogenesis. *Cancer Lett.* 2013 Jan 1;328(1):18-26
110. Davis S, Aldrich TH, Jones PF et al (1996) Isolation of angiopoietin- 1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 87:1161–1169.
111. Maisonpierre PC, Suri C, Jones PF et al (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277:55–60.
112. Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, Kriz W, Thurston G, Augustin HG. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood.* 2004 Jun 1;103(11):4150-6.

113. Kim I, Kim JH, Moon SO et al (2000) Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 30-kinase/Akt signal transduction pathway. *Oncogene* 19:4549–4552
114. Seegar TC, Eller B, Tzvetkova-Robev D, Kolev MV, Henderson SC, Nikolov DB, Barton WA. Tie1-Tie2 interactions mediate functional differences between angiopoietin ligands. *Mol Cell*. 2010 Mar 12;37(5):643-55.
115. Sato TN, Tozawa Y, Deutsch U et al (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376:70–74.
116. Wong AL, Haroon ZA, Werner S et al (1997) Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. *Circ Res* 81:567–574
117. Peters KG, Coogan A, Berry D et al (1998) Expression of Tie2/ Tek in breast tumour vasculature provides a new marker for evaluation of tumour angiogenesis. *Br J Cancer* 77:51–56
118. Suri C, Jones PF, Patan S et al (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87:1171–1180.
119. Ward NL, Van Slyke P, Sturk C et al (2004) Angiopoietin 1 expression levels in the myocardium direct coronary vessel development. *Dev Dyn* 229:500–509.
120. Hammes HP, Lin J, Wagner P et al (2004) Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. *Diabetes* 53:1104–1110.
121. Reiss Y, Droste J, Heil M, Tribulova S et al (2007) Angiopoietin-2 impairs revascularization after limb ischemia. *Circ Res* 101:88–96.

122. Davis J, Yeo T, Piera K, Woodberry T, Celermajer D, Stephens D, Anstey N. Angiopoietin-2 is increased in sepsis and inversely associated with nitric oxide-dependent microvascular reactivity. *Crit Care*. 2010; 14(3): R89.
123. Wilson PM, LaBonte MJ, Lenz HJ. Assessing the in vivo efficacy of biologic antiangiogenic therapies. *Cancer Chemother Pharmacol*. 2013 Jan;71(1):1-12.
124. Ferrara N, Houch KA, Jakeman LB, et al. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 1992;13:18 –32.
125. Leung DW, Cachianes G, Kuang WJ, et al. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989;246:1306 –9.
126. Witzigmann-Voos S, Breier G, Risau W, et al. Up-regulation of vascular endothelial growth factor and its receptors in von Hippel-Lindau disease-associated and sporadic hemangioblastomas. *Cancer Res* 1995;55:1358–64.
127. Maeda K, Chung YS, Ogawa Y, et al. Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer* 1996;77:858–63.
128. Harada K, Friedman M, Lopez JJ, et al. Vascular endothelial growth factor in chronic myocardial ischemia. *Am J Physiol* 1996;270:H1791–802
129. Tudor RM, Flook BE, Voelkel NF. Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lung exposed to acute or chronic hypoxia. *J Clin Invest* 1995;95:1798–807.
130. Folkman J. Clinical applications of research on angiogenesis. *N Eng J Med* 1995;333:1757– 63.

131. Tan H, Chen H, Xu C, Ge Z, Gao Y, Fang J, Liu W, Xiao S. Role of vascular endothelial growth factor in angiodysplasia: an interventional study with thalidomide. *Journal of gastroenterology and hepatology*. 2012 Jun;27(6):1094-101.
132. Cirulli A, Liso A, D'Ovidio F, Mestice A, Pasculli G, Gallitelli M, Rizzi R, Specchia G, Sabbà C. Vascular endothelial growth factor serum levels are elevated in patients with hereditary hemorrhagic telangiectasia. *Acta Haematol*. 2003;110(1):29-32.
133. Duray PH, Marcal JM, LiVolsi VA, Fisher R, Scholhamer C, Brand MH. Small intestinal angiodysplasia in the elderly. *J Clin Gastroenterol* 1984 Aug;6(4):311-19.
134. Letamendia A, Lastres P, Botella LM, et al. Role of endoglin in cellular responses to transforming growth factor- α . A comparative study with betaglycan. *J Biol Chem* 1998;273:33011-9.
135. Bobik A. Transforming growth factor- α s and vascular disorders. *Arterioscler Thromb Vasc Biol* 2006;26:1712-20.
136. Lebrin F, Deckers M, Bertolino P, ten Dijke P. TGF- α receptor function in the endothelium. *Cardiovasc Res* 2005;65:599-608.
137. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrel J, McCormick MK. Endoglin, a TGF- β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nature genetics*. 1994 Dec;8(4):345.

138. Dallas NA, Samuel S, Xia L, Fan F, Gray MJ, Lim SJ, Ellis LM. Endoglin (CD105): a marker of tumor vasculature and potential target for therapy. *Clinical Cancer Research*. 2008 Apr 1;14(7):1931-7.
139. Ding S, Li C, Lin S, et al. Comparative evaluation of microvessel density determined by CD34 or CD105 in benign and malignant gastric lesions. *Hum Pathol* 2006;37:861^6.
140. Saad RS, El-GoharyY, Memari E, LiuYL, Silverman JF. Endoglin (CD105) and vascular endothelial growth factor as prognostic markers in esophageal adenocarcinoma. *Hum Pathol* 2005;36:955^61.
141. Yang LY, LuWQ, Huang GW, WangW. Correlation between CD105 expression and postoperative recurrence and metastasis of hepatocellular carcinoma. *BMC Cancer* 2006;6:110.
142. Arthur HM, Ure J, Smith AJ, et al. Endoglin, an ancillary TGF β receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol* 2000;217:42–53.
143. Venkatesha S, Toporsian M, Lam C, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 2006;12:642–9.
144. Li C, Guo B, Wilson PB, et al. Plasma levels of soluble CD105 correlate with metastasis in patients with breast cancer. *Int J Cancer* 2000;89:122–6.
145. Ojeda-Fernandez L, Barrios L, Rodriguez-Barbero A, Recio-Poveda L, Bernabeu C, Botella LM. Reduced plasma levels of Ang-2 and sEng as novel biomarkers in hereditary hemorrhagic telangiectasia (HHT). *Clinica Chimica Acta*. 2010 Apr 2;411(7-8):494-9.

146. Shankaran H, Alexandridis P, Neelamegham S: Aspects of hydrodynamic shear regulating shear-induced platelet activation and self-association of von Willebrand factor in suspension. *Blood* 101:2637-2645, 2003
147. Veyradier A, Jenkins CSP, Fressinaud E, et al: Acquired von Willebrand syndrome: from pathophysiology to management. *Thromb Haemost* 84:175-182, 2000
148. Fressinaud E, Meyer D: International survey of patients with von Willebrand disease and angiodysplasia. *Thromb Haemost* 70:546 (letter), 1993
149. Loscalzo J. From clinical observation to mechanism—Heyde's syndrome. *New England Journal of Medicine*. 2012 Nov 15;367(20):1954-6.
150. Panzer S, Badr Eslam R, Schneller A, et al. Loss of high-molecular-weight von Willebrand factor multimers mainly affects platelet aggregation in patients with aortic stenosis. *Thromb Haemost* 2010;103:408-414
151. Crawley JT, de Groot R, Xiang Y, Luken BM, Lane DA. Unraveling the scissile bond: how ADAMTS 13 recognizes and cleaves von Willebrand factor. *Blood* 2011;118:3212-3221
152. Junquera F, Saperas E, Anglés A, Abadía C, Monasterio J, Malagelada JR. Increased plasma fibrinolytic activity in bleeding gastrointestinal angiodysplasia. *Eur J Gastroenterol Hepatol*. 2005 Feb;17(2):199-205.
153. Prochorec-Sobieszek M, Windyga J, Maryniak RK, Misiak A, Szczepanik A. Angiodysplasia as a cause of recurrent bleeding from the small bowel in patients with von Willebrand disease. Report of 4 patients. *Pol J Pathol*. 2004;55(4):173-6.
154. Hashizume H, Falcón BL, Kuroda T, Baluk P, Coxon A, Yu D, Bready JV, Oliner JD, McDonald DM. Complementary actions of inhibitors of angiopoietin-2

- and VEGF on tumor angiogenesis and growth. *Cancer Res.* 2010 Mar 15;70(6):2213-23. doi: 10.1158/0008-5472.CAN-09-1977.
155. Felcht M, Luck R, Schering A, Seidel P, Srivastava K, Hu J, et al. Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signalling. *J Clin Invest.* 2012 June 1; 122(6): 1991–2005
 156. Arakelyan L, Vainstein V, Agur Z. A computer algorithm describing the process of vessel formation and maturation, and its use for predicting the effects of anti-angiogenic and anti-maturation therapy on vascular tumor growth. *Angiogenesis.* 2002 Sep 1;5(3):203-14.
 157. Pasha SF, Hara AK, Leighton JA. Diagnostic evaluation and management of obscure gastrointestinal bleeding: a changing paradigm. *Gastroenterol Hepatol (NY).* 2009 Dec;5(12):839---50.
 158. Holleran G, Hall B, Hussey M, McNamara D. Small bowel angiodysplasia and novel disease associations: a cohort study. *Scand J Gastroenterol.* 2013 Apr;48(4):433-8. doi: 10.3109/00365521.2012.763178.
 159. JM Richter, MR Christensen, GA Colditz, N Nishioka. Angiodys-plasia. Natural history and efficacy of therapeutic interventions. *Dig Dis Sci*, 34 (1989), pp. 1542–1546
 160. Holleran G, Hall B, Zgaga L, Breslin N, McNamara D. The natural history of small bowel angiodysplasia. *Scand J Gastroenterol.* 2016;51(4):393-9. doi: 10.3109/00365521.2015.1102317. Epub 2015 Nov 5.
 161. Jackson CS1, Gerson LB2. Management of gastrointestinal angiodysplastic lesions (GIADs): a systematic review and meta-analysis. *Am J Gastroenterol.* 2014 Apr;109(4):474-83; quiz 484. doi: 10.1038/ajg.2014.19. Epub 2014 Mar 18.

162. Romagnuolo J1, Brock AS, Ranney N. Is Endoscopic Therapy Effective for Angioectasia in Obscure Gastrointestinal Bleeding?: A Systematic Review of the Literature. *J Clin Gastroenterol*. 2014 Dec 16.
163. May A, Friesing-Sosnik T, Manner H, Pohl J, Ell C. Long-term outcome after argon plasma coagulation of small-bowel lesions using double-balloon enteroscopy in patients with mid-gastrointestinal bleeding. *Endoscopy*. 2011 Sep;43(9):759-65. doi: 10.1055/s-0030-1256388.
164. Landi B, Cellier C, Gaudric M, Demont H, Guimbaud R, Cuillerier E, Couturier D, Barbier JP, Marteau P. Long-term outcome of patients with gastrointestinal bleeding of obscure origin explored by push enteroscopy. *Endoscopy*. 2002 May;34(5):355-9.
165. Shurafa M, Kamboj G. Thalidomide for the treatment of bleeding angiodysplasias. *Am J Gastroenterol* 2003;98:221–2.
166. Bon C, Aparicio T, Vincent M, Mavros M, Bejou B, Raynaud JJ, Zampeli E, Airinei G, Sautereau D, Benamouzig R, Michopoulos S. Long-acting somatostatin analogues decrease blood transfusion requirements in patients with refractory gastrointestinal bleeding associated with angiodysplasia. *Aliment Pharmacol Ther*. 2012 Sep;36(6):587-93.
167. Holleran G, Hall B, Breslin N, McNamara D. Long-acting somatostatin analogues provide significant beneficial effect in patients with refractory small bowel angiodysplasia: Results from a proof of concept open label mono-centre trial. *United European Gastroenterol J*. 2016 Feb;4(1):70-6. doi: 10.1177/2050640614559121

168. Liao Z, Gao R, Xu C, Li ZS. Indications and detection, completion and retention rates of small bowel capsule endoscopy: a systematic review. *Gastrointest Endosc.* 2010;71:280-6.
169. Koulaouzidas A, Yung DE, Lam J, Smirnidis A, Douglas S, Plevris JN. The use of small bowel capsule endoscopy in iron deficiency anaemia alone; be aware of the young anaemic patient. *Scand J Gastroenterol.* 2012 Aug;online:1-7
170. James MW, Chen CH, Goddard WP, Scott BB, Goddard AF. Risk factors for gastrointestinal malignancy in patients with iron deficiency anaemia. *Eur J Gastroenterol Hepatol.* 2005(Nov);17(11):1197-203.
171. Ribeiro I, Pinho R, Rodrigues A, Silva J, Ponte A, Rodrigues J, Carvalho J. What is the long-term outcome of a negative capsule endoscopy in patients with obscure gastrointestinal bleeding? *Rev Esp Enferm Dig.* 2015 Dec;107(12):753-8.
172. Niikura R, Yamada A, Nagata N, Kobayashi Y, Okamoto M, Mitsuno Y, Ogura K, Yoshida S, Shimbo T, Hirata Y, Koike K. New predictive model of rebleeding during follow-up of patients with obscure gastrointestinal bleeding: A multicenter cohort study. *J Gastroenterol Hepatol.* 2016 Apr;31(4):752-60. doi: 10.1111/jgh.13201.
173. Okamoto J, Tominaga K, Sugimori S, Kato K, Minamino H, Ominami M, Fukunaga S, Nagami Y, Kamata N, Yamagami H, Tanigawa T, Shiba M, Watanabe T, Fujiwara Y, Arakawa T. Comparison of Risk Factors Between Small Intestinal Ulcerative and Vascular Lesions in Occult Versus Overt Obscure Gastrointestinal Bleeding. *Dig Dis Sci.* 2016 Feb;61(2):533-41. doi: 10.1007/s10620-015-3904-5. Epub 2015 Oct 6

174. Igawa A, Oka S, Tanaka S, Kuniyama S, Nakano M, Aoyama T, Chayama K. Major predictors and management of small-bowel angiodysplasia. *BMC Gastroenterol*. 2015 Aug 25;15:108. doi: 10.1186/s12876-015-0337-8.
175. Ribeiro I, Pinho R, Rodrigues A, Marqués J, Fernandes C, Carvalho J. Obscure gastrointestinal bleeding: Which factors are associated with positive capsule endoscopy findings? *Rev Esp Enferm Dig*. 2015 Jun;107(6):334-9.
176. Min YW, Kim JS, Jeon SW, Jeon YT, Im JP, Cheung DY, Choi MG, Kim JO, Lee KJ, Ye BD, Shim KN, Moon JS, Kim JH, Hong SP, Chang DK. Long-term outcome of capsule endoscopy in obscure gastrointestinal bleeding: a nationwide analysis. *Endoscopy*. 2014 Jan;46(1):59-65. doi: 10.1055/s-0033-1358803. Epub 2013 Nov 19
177. Holleran G, Hall B, O'Regan M, Smith S, McNamara D. Expression of Angiogenic Factors in Patients With Sporadic Small Bowel Angiodysplasia. *J Clin Gastroenterol*. 2015 Nov-Dec;49(10):831-6. doi: 10.1097/MCG.0000000000000260.
178. Davis J, Yeo T, Piera K, et al. Angiopoietin-2 is increased in sepsis and inversely associated with nitric oxide-dependent microvascular reactivity. *Crit Care*. 2010;14:R89.
179. Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. *BMJ* 2009;339:b2535.
180. Wells GA, Shea B, O'Connell D et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality if nonrandomized studies in meta-analyses. http://www.ohrica/programs/clinical_epidemiology/oxford.htm 2009 [cited 2016 Oct

12];Available from: URL:

http://www.ohri.ca/programs/clinical_epidemiology/oxford.htm

181. Chen H, Cohen P, Chen S. How Big is a Big Odds Ratio? Interpreting the Magnitudes of Odds Ratios in Epidemiological Studies. *Communications in Statistics - Simulation and Computation*. 2010;39(4).
182. Lanas A, García-Rodríguez LA, Polo-Tomás M et al. The changing face of hospitalisation due to gastrointestinal bleeding and perforation. *Aliment Pharmacol Ther*. 2011 Mar;33(5):585-91.
183. Igawa A, Oka S, Tanaka S et al. Major predictors and management of small-bowel angioectasia. *BMC Gastroenterol*. 2015 Aug 25;15:108.
184. Holleran G, Hall B, Hussey M, McNamara D. Small bowel angiodysplasia and novel disease associations: a cohort study. *Scand J Gastroenterol*. 2013 Apr;48(4):433-8.
185. Macdonald J, Porter V, Scott NW, McNamara D. Small bowel lymphangiectasia and angiodysplasia: a positive association; novel clinical marker or shared pathophysiology? *J Clin Gastroenterol*. 2010 Oct;44(9):610-4.
186. Chak A, Koehler MK, Sundaram SN, Cooper GS, Canto MI, Sivak MV Jr. Diagnostic and therapeutic impact of push enteroscopy: analysis of factors associated with positive findings. *Gastrointest Endosc*. 1998 Jan;47(1):18-22.
187. Clouse RE, Costigan DJ, Mills BA, Zuckerman GR. Angiodysplasia as a cause of upper gastrointestinal bleeding. *Arch Intern Med*. 1985 Mar;145(3):458-61.
188. Cochrane J, Jackson C, Schlepp G, Strong R. Gastrointestinal angiodysplasia is associated with significant gastrointestinal bleeding in patients

- with continuous left ventricular assist devices. *Endosc Int Open*. 2016 Mar;4(3):E371-7.
189. Blackshear JL, Wysokinska EM, Safford RE et al. Shear stress-associated acquired von Willebrand syndrome in patients with mitral regurgitation. *J Thromb Haemost*. 2014 Dec;12(12):1966-74.
 190. French JB, Pamboukian SV, George JF et al. Gastrointestinal bleeding in patients with ventricular assist devices is highest immediately after implantation. *ASAIO J*. 2013 Sep-Oct;59(5):480-5.
 191. Sotoudehmanesh R, Ali Asgari A, Ansari R, Nouraie M. Endoscopic findings in end-stage renal disease. *Endoscopy*. 2003 Jun;35(6):502-5.
 192. Duchini A, Sessoms SL. Gastrointestinal hemorrhage in patients with systemic sclerosis and CREST syndrome. *Am J Gastroenterol*. 1998 Sep;93(9):1453-6.
 193. Bhutani MS, Gupta SC, Markert RJ, Barde CJ, Donese R, Gopalswamy N. A prospective controlled evaluation of endoscopic detection of angiodysplasia and its association with aortic valve disease. *Gastrointest Endosc*. 1995 Nov;42(5):398-402.
 194. Marcuard SP, Weinstock JV. Gastrointestinal angiodysplasia in renal failure. *J Clin Gastroenterol*. 1988 Oct;10(5):482-4.
 195. Rockey DC, Cello JP. Evaluation of the gastrointestinal tract in patients with iron deficiency anaemia. *N Eng J Med* 1993;329:1691-5.
 196. Goddard AF, James MW, McIntyre AS, Scott BB. Guidelines for the management of iron deficiency anaemia. *GUT* 2011;60:1309-16.

197. Yamada A1, Watabe H, Kobayashi Y, Yamaji Y, Yoshida H, Koike K. Timing of capsule endoscopy influences the diagnosis and outcome in obscure-overt gastrointestinal bleeding. *Hepatogastroenterology*. 2012 May;59(115):676-9. doi: 10.5754/hge12180.
198. Cúrdia Gonçalves T, Magalhães J, Boal Carvalho P, Moreira MJ, Rosa B, Cotter J. Is it possible to predict the presence of intestinal angioectasias? *Diagn Ther Endosc*. 2014;2014:461602. doi: 10.1155/2014/461602. Epub 2014 Mar 17.
199. Sakai E1, Endo H, Taniguchi L, Hata Y, Ezuka A, Nagase H, Yamada E, Ohkubo H, Higurashi T, Sekino Y, Koide T, Iida H, Hosono K, Nonaka T, Takahashi H, Inamori M, Maeda S, Nakajima A. Factors predicting the presence of small bowel lesions in patients with obscure gastrointestinal bleeding. *Dig Endosc*. 2013 Jul;25(4):412-20. doi: 10.1111/den.12002. Epub 2012 Dec 17.
200. DeBenedet AT1, Saini SD, Takami M, Fisher LR. Do clinical characteristics predict the presence of small bowel angioectasias on capsule endoscopy? *Dig Dis Sci*. 2011 Jun;56(6):1776-81. doi: 10.1007/s10620-010-1506-9. Epub 2010 Dec 10.
201. Lee BJ, Chun HJ, Koo JS, Keum B, Park SH, Kim du R, Kwon YD, Kim YS, Jean YT, Lee HS, Um SH, Lee SW, Choi JH, Kim CD, Ryu HS. Analysis of the factors that affect the diagnostic yield of capsule endoscopy in patients with obscure gastrointestinal bleeding]. *Korean J Gastroenterol*. 2007 Feb;49(2):79-84
202. Fiedler U, Scharpfenecker M, Koidl S, et al. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood*. 2004;103:4150–4156.

203. Felcht M, Luck R, Schering A, et al. Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signalling. *J Clin Invest*. 2012;122:1991–2005.
204. Chen J, Zhi Y, Chang X, Zhang S, Dai D. Expression of ADAMTS1 and its correlation with angiogenesis in primary gastric cancer and lymph node metastasis. *Dig Dis Sci*. 2013 Feb;58(2):405-13. doi: 10.1007/s10620-012-2379-x.
205. Xu Z, Yu Y, Duh EJ. Vascular endothelial growth factor upregulates expression of ADAMTS1 in endothelial cells through protein kinase C signaling. *Investigative ophthalmology & visual science*. 2006 Sep 1;47(9):4059-66.
206. Lee M, Rodansky ES, Smith JK, Rodgers GM. ADAMTS13 promotes angiogenesis and modulates VEGF-induced angiogenesis. *Microvascular research*. 2012 Sep 1;84(2):109-15.
207. Starke RD, Ferraro F, Paschalaki KE, Dryden NH, McKinnon TA, Sutton RE, Payne EM, Haskard DO, Hughes AD, Cutler DF, Laffan MA. Endothelial von Willebrand factor regulates angiogenesis. *Blood*. 2011 Jan 20;117(3):1071-80.
208. Scheers NM, Pereira DIA, Faria N, Powell JJ. Ferric citrate and ferric EDTA but not ferrous sulfate drive amphiregulin-mediated activation of the MAP kinase ERK in gut epithelial cancer cells. *Oncotarget*. 2018 Mar 30;9(24):17066-17077.
209. Kariagina A, Xie J, Leipprandt JR, Haslam SZ. Amphiregulin mediates estrogen, progesterone, and EGFR signaling in the normal rat mammary gland and in hormone-dependent rat mammary cancers. *Horm Cancer*. 2010;1:229–44
210. Abe K, Shoji M, Chen J, Bierhaus A, Danave I, Micko C, Casper K, Dillehay DL, Nawroth PP, Rickles FR. Regulation of vascular endothelial growth factor

production and angiogenesis by the cytoplasmic tail of tissue factor. *Proc Natl Acad Sci U S A*. 1999 Jul 20; 96(15):8663-8.

211. Staton CA, Chetwood AS, Cameron IC, Cross SS, Brown NJ, Reed MW. The angiogenic switch occurs at the adenoma stage of the adenoma carcinoma sequence in colorectal cancer. *Gut*. 2007;56:1426–1432.
212. Ellis LM. Epidermal growth factor receptor in tumour angiogenesis. *Hematol Oncol Clin North Am* 2004; 18: 1007–21.
213. Corlan AS, Cîmpean AM, Jitariu AA, Melnic E, Raica M. Endocrine Gland-Derived Vascular Endothelial Growth Factor/Prokineticin-1 in Cancer Development and Tumor Angiogenesis. *Int J Endocrinol*. 2017;2017:3232905.
214. Goi T., Fujioka M., Satoh Y., et al. Angiogenesis and tumor proliferation/metastasis of human colorectal cancer cell line sw620 transfected with endocrine glands-derived-vascular endothelial growth factor, as a new angiogenic factor. *Cancer Research*. 2004;64(6):1906–1910.
215. Goi T., Nakazawa T., Hirono Y., Yamaguchi A. The anti-tumor effect is enhanced by simultaneously targeting VEGF and PROK1 in colorectal cancer. *Oncotarget*. 2015;6(8):6053–6061
216. Walia A, Yang JF, Huang YH, Rosenblatt MI, Chang JH, Azar DT. Endostatin's emerging roles in angiogenesis, lymphangiogenesis, disease, and clinical applications. *Biochim Biophys Acta*. 2015 Dec;1850(12):2422-38.
217. Bouallegue A, Bou Daou G, Srivastava AK. Endothelin-1-induced signaling pathways in vascular smooth muscle cells. *Current vascular pharmacology*. 2007 Jan 1;5(1):45-52.

218. Lankhorst S, Danser AH, van den Meiracker AH. Endothelin-1 and antiangiogenesis. *Am J Physiol Regul Integr Comp Physiol*. 2016 Feb 1;310(3):R230-4.
219. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev*. 2005 Apr;16(2):159-78. Epub 2005 Feb 2.
220. Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES, Shapiro RL, Galloway AC, Rifkin DB, Mignatti P. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol*. 1998 Jun 29;141(7):1659-73.
221. Rogers MS, Rohan RM, Birsner AE, D'Amato RJ. Genetic loci that control the angiogenic response to basic fibroblast growth factor. *Faseb J*. 2004;18:1050-9.
222. Jazwa A, Tomczyk M, Taha HM, Hytonen E, Stoszko M, Zentilin L, Giacca M, Yla-Herttuala S, Emanuelli C, Jozkowicz A, Dulak J. Arteriogenic therapy based on simultaneous delivery of VEGF-A and FGF4 genes improves the recovery from acute limb ischemia. *Vasc Cell*. 2013 Jul 1;5:13.
223. Narita K, Fujii T, Ishiwata T, Yamamoto T, Kawamoto Y, Kawahara K, Nakazawa N, Naito Z. Keratinocyte growth factor induces vascular endothelial growth factor-A expression in colorectal cancer cells. *Int J Oncol*. 2009 Feb;34(2):355-60.
224. Rajavashisth TB, Andalibi A, Territo MC, Berliner JA, Navab M, Fogelman AM, Lusis AJ. Induction of endothelial cell expression of granulocyte and

macrophage colony-stimulating factors by modified low-density lipoproteins.

Nature. 1990 Mar;344(6263):254.

225. Zhao J, Chen L, Shu B, Tang J, Zhang L, Xie J, Qi S, Xu Y.
Granulocyte/macrophage colony-stimulating factor influences angiogenesis by regulating the coordinated expression of VEGF and the Ang/Tie system. PloS one. 2014 Mar 21;9(3):e92691.
226. Rocourt DV, Mehta VB, Besner GE. Heparin-binding EGF-like growth factor decreases inflammatory cytokine expression after intestinal ischemia/reperfusion injury. J Surg Res. 2007;139:269–273.
227. Ming YC, Chao HC, Chu SM, Luo CC. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) protected intestinal ischemia-reperfusion injury through JNK and p38/MAPK-dependent pathway for anti-apoptosis. Pediatr Neonatol. 2018 Aug 17: S1875-9572(18)30244-4.
228. Si Y, Zhang H, Ning T, Bai M, Wang Y, Yang H, Wang X, Li J, Ying G, Ba Y. miR-26a/b Inhibit Tumor Growth and Angiogenesis by Targeting the HGF-VEGF Axis in Gastric Carcinoma. Cell Physiol Biochem. 2017;42(4):1670-1683.
229. Bach LA. IGF-binding proteins. J Mol Endocrinol. 2018 Jul;61(1):T11-T28.
230. Azar WJ, Zivkovic S, Werther GA, Russo VC. IGFBP-2 nuclear translocation is mediated by a functional NLS sequence and is essential for its pro-tumorigenic actions in cancer cells. Oncogene. 2014 Jan;33(5):578.
231. Gopinathan G, Milagre C, Pearce OM, Reynolds LE, Hodivala-Dilke K, Leinster DA, Zhong H, Hollingsworth RE, Thompson R, Whiteford JR, Balkwill F. Interleukin-6 Stimulates Defective Angiogenesis. Cancer Res. 2015 Aug 1; 75(15):3098-107.

232. Martin D, Galisteo R, Gutkind JS. CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex. *J Biol Chem*. 2009 Mar 6; 284(10):6038-42.
233. Katz LH, Li Y, Chen JS, Muñoz NM, Majumdar A, Chen J, Mishra L. Targeting TGF- β signaling in cancer. *Expert Opin Ther Targets*. 2013 Jul; 17(7):743-60
234. Muppala S, Xiao R, Krukovets I, Verbovetsky D, Yendamuri R, Habib N, Raman P, Plow E, Stenina-Adognravi O. Thrombospondin-4 mediates TGF- β -induced angiogenesis. *Oncogene*. 2017 Sep 7;36(36):5189-5198.
235. Sobrinho Santos EM, Guimarães TA, Santos HO, Cangussu LMB, de Jesus SF, Fraga CAC, Cardoso CM, Santos SHS, de Paula AMB, Gomez RS, Guimarães ALS, Farias LC. Leptin acts on neoplastic behavior and expression levels of genes related to hypoxia, angiogenesis, and invasiveness in oral squamous cell carcinoma. *Tumour Biol*. 2017 May;39(5):1010428317699130.
236. McMurtry V, Simeone AM, Nieves-Alicea R. Leptin utilizes Jun N-terminal kinases to stimulate the invasion of MCF-7 breast cancer cells. *Clin Exp Metastasis* 2009; 26(3): 197–204.
237. Ehling J, Bartneck M, Wei X. CCL2-dependent infiltrating macrophages promote angiogenesis in progressive liver fibrosis *Gut* 2014;63:1960-1971.
238. Liao YY, Tsai HC, Chou PY, Wang SW, Chen HT, Lin YM, Chiang IP, Chang TM, Hsu SK, Chou MC, Tang CH, Fong YC. CCL3 promotes angiogenesis by dysregulation of miR-374b/ VEGF-A axis in human osteosarcoma cells. *Oncotarget*. 2016 Jan 26;7(4):4310-25.

239. Raza SL, Cornelius LA. Matrix metalloproteinases: pro- and anti-angiogenic activities. *J Invest Dermatol Symp Proc*. 2000 Dec;5(1):47-54.
240. Sugreev V, Kousik K, Nilanjan G, Sayantan J, Snehasikta S. Matrix metalloproteinases and gastrointestinal cancers: Impacts of dietary antioxidants. *World J Biol Chem*. 2014 Aug 26; 5(3): 355–376.
241. Chengqiang W, Chun G, Lang L, Yiheng P, Zhongli T, Jing W. Expression and secretion of neuregulin-1 in cardiac microvascular endothelial cells treated with angiogenic factors. *Exp Ther Med*. 2018 Apr; 15(4): 3577–3581.
242. Rusnati M, Camozzi M, Moroni E, et al. Selective recognition of fibroblast growth factor-2 by the long pentraxin PTX3 inhibits angiogenesis. *Blood* 2004;104:92–99.
243. Rodriguez-Grande B, Varghese L, Molina-Holgado F, et al. Pentraxin 3 mediates neurogenesis and angiogenesis after cerebral ischaemia. *J Neuroinflamm* 2015;12:15
244. Steineger J, Ueland T, Aukrust P, Michelsen A, Osnes T, Heimdal K, Dheyaudeen S. Pentraxin 3 level is elevated in hereditary hemorrhagic telangiectasia and reflects the severity of disease-associated epistaxis. *Laryngoscope*. 2019 Jan;129(1):E44-E49.
245. Usuki K, Saras J, Waltenberger J, Miyazono K, Pierce G, Thomason A, Heldin C: Platelet-derived endothelial cell growth factor has thymidine phosphorylase activity. *Biochem Biophys Res Commun* 1992, 184:1311-1316
246. Griffiths L, Stratford IJ. Platelet-derived endothelial cell growth factor thymidine phosphorylase in tumour growth and response to therapy. *Br J Cancer*. 1997; 76(6): 689–693.

247. Sengupta S, Sellers LA, Matheson HB, Fan TP D. Thymidine phosphorylase induces angiogenesis in vivo and in vitro: an evaluation of possible mechanisms. *Br J Pharmacol*. 2003 May; 139(2): 219–231.
248. Lin S, Lai H, Qin Y, Chen J, Lin Y. Thymidine phosphorylase and hypoxia-inducible factor 1- α expression in clinical stage II/III rectal cancer: association with response to neoadjuvant chemoradiation therapy and prognosis. *Int J Clin Exp Pathol*. 2015; 8(9): 10680–10688.
249. Raica M, Cimpean AM. Platelet-Derived Growth Factor (PDGF)/PDGF Receptors (PDGFR) Axis as Target for Antitumor and Antiangiogenic Therapy. *Pharmaceuticals (Basel)*. 2010 Mar 11;3(3):572-599.
250. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev*. 2008;22:1276–1312
251. Thomas M, Augustin HG. The role of the Angiopoietins in vascular morphogenesis. *Angiogenesis*. 2009;12(2):125-37.
252. Liu T, Ma W, Xu H, Huang M, Zhang D, He Z, Zhang L, Brem S, O'Rourke DM, Gong Y, Mou Y, Zhang Z, Fan Y. PDGF-mediated mesenchymal transformation renders endothelial resistance to anti-VEGF treatment in glioblastoma. *Nat Commun*. 2018 Aug 27;9(1):3439.
253. Kano MR, Morishita Y, Iwata C, Iwasaka S, Watabe T, Ouchi Y, Miyazono K, Miyazawa K. VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFRbeta signaling. *J Cell Sci*. 2005 Aug 15; 118(Pt 16):3759-68.

254. Aidoudi S, Bikfalvi A. Thromb Interaction of PF4 (CXCL4) with the vasculature: a role in atherosclerosis and angiogenesis. *Haemost.* 2010 Nov;104(5):941-8.
255. Petersen F, Brandt E, Lindahl U, Spillmann D. Characterization of a neutrophil cell surface glycosaminoglycan that mediates binding of platelet factor 4. *Journal of Biological Chemistry.* 1999 Apr 30;274(18):12376-82.
256. Ribatti D. The discovery of the placental growth factor and its role in angiogenesis: a historical review. *Angiogenesis.* 2008;11(3):215-21.
257. Zhang M, Volpert O, Shi YH, Bouck N. Maspin is an angiogenesis inhibitor. *Nat Med.* 2000 Feb;6(2):196-9.
258. McMahon GA, Petitclerc E, Stefansson S, Smith E, Wong MK, Westrick RJ, Ginsburg D, Brooks PC, Lawrence DA. Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. *J Biol Chem.* 2001 Sep 7; 276(36):33964-8.
259. Wu J, Strawn TL, Luo M, Wang L, Li R, Ren M, Xia J, Zhang Z, Ma W, Luo T, Lawrence DA, Fay WP. Plasminogen activator inhibitor-1 inhibits angiogenic signaling by uncoupling vascular endothelial growth factor receptor-2- α V β 3 integrin cross talk. *Arterioscler Thromb Vasc Biol.* 2015 Jan;35(1):111-20.
260. Ren JG, Jie C, Talbot C. How PEDF prevents angiogenesis: a hypothesized pathway. *Med Hypotheses.* 2005;64(1):74-8.
261. Hidalgo, M., Eckhardt, S.G. Development of matrix metalloproteinase inhibitors in cancer therapy. *J. Natl. Cancer* 2001;93:178–193.
262. Fernández CA, Moses MA. Modulation of angiogenesis by tissue inhibitor of metalloproteinase-4. *Biochemical and biophysical research communications.* 2006 Jun 23;345(1):523-9.

263. Fernández CA, Louis G, Moses MA. Characterization of the anti-angiogenic effects of TIMP-4 (2004): 530-530.
264. Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proceedings of the National Academy of Sciences*. 1990 Sep 1;87(17):6624-8.
265. Taraboletti G, Roberts D, Liotta LA, Giavazzi R. Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor. *The Journal of cell biology*. 1990 Aug 1;111(2):765-72.
266. MacLauchlan SC, Calabro NE, Huang Y, Krishna M, Bancroft T, Sharma T, Yu J, Sessa WC, Giordano F, Kyriakides TR. HIF-1 α represses the expression of the angiogenesis inhibitor thrombospondin-2. *Matrix Biology*. 2018 Jan 1;65:45-58.
267. Sun R, Wu J, Chen Y, Lu M, Zhang S, Lu D, Li Y. Down regulation of Thrombospondin2 predicts poor prognosis in patients with gastric cancer. *Mol Cancer*. 2014 Sep 28;13:225.
268. Rabbani SA, Mazar AP. The role of the plasminogen activation system in angiogenesis and metastasis. *Surgical oncology clinics of North America*. 2001 Apr 1;10(2):393-415.
269. Ploplis VA, Carmeliet P, Vazirzadeh S, Van Vlaenderen I, Moons L, Plow EF, et al. Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. *Circulation* 1995; 92:2585-93;

270. Ellis V, Wun TC, Behrendt N, Rønne E, Danø K. Inhibition of receptor-bound urokinase by plasminogen-activator inhibitors. *J Biol Chem* 1990; 265:9904-8.
271. Watanabe K, Hasegawa Y, Yamashita H, Shimizu K, Ding Y, Abe M, Ohta H, Imagawa K, Hojo K, Maki H, et al: Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. *J Clin Invest.* 114:898–907. 2004.
272. Shen J, Yang X, Xiao WH, Hackett SF, Sato Y and Campochiaro PA: Vasohibin is up-regulated by VEGF in the retina and suppresses VEGF receptor 2 and retinal neovascularization. *FASEB J.* 20:723–725. 2006
273. Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev*, 2004, 56(4):549–580.
274. Witzenbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu JS, Isner JM. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *The American journal of pathology.* 1998 Aug 1;153(2):381-94.
275. Meurer SK, Alsamman M, Scholten D, Weiskirchen R. Endoglin in liver fibrogenesis: Bridging basic science and clinical practice. *World J Biol Chem.* 2014 May 26;5(2):180-203.
276. Khong TL, Thairu NG, Larsen H, Dawson PM, Kiriakidis S, Paleolog EM. Identification of the angiogenic gene signature induced by EGF and hypoxia in colorectal cancer. *BMC Cancer.* 2013; 13: 518.

277. Fukumoto S, Morifuji M, Katakura Y, Nakamura S. Endostatin inhibits lymph node metastasis by a down-regulation of the vascular endothelial growth factor C expression in tumor cells. *Clin Exp Metastasis*. 2005;22(1):31–8.
278. Li K, Shi M, Qin S. Current Status and Study Progress of Recombinant Human Endostatin in Cancer Treatment. *Oncology and Therapy*. 2018 Jun 1:1-23.
279. Kim YM, Hwang S, Kim YM, Pyun BJ, Kim TY, Lee ST, Gho YS, Kwon YG. Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. *J Biol Chem*. 2002; 277:27872–27879.
280. Wickstrom SA, Alitalo K, Keski-Oja J. Endostatin associates with integrin $\alpha 5 \beta 1$ and caveolin-1, and activates Src via a tyrosyl phosphatase-dependent pathway in human endothelial cells. *Cancer Res*. 2002; 62:5580–5589.
281. Walia A, Yang JF, Huang YH, Rosenblatt MI, Chang JH, Azar DT. Endostatin's emerging roles in angiogenesis, lymphangiogenesis, disease, and clinical applications. *Biochimica et Biophysica Acta (BBA)-General Subjects*. 2015 Dec 1;1850(12):2422-38.
282. Deininger MH, Fimmen BA, Thal DR, Schluesener HJ, Meyermann R. Aberrant neuronal and paracellular deposition of endostatin in brains of patients with Alzheimer's disease. *J Neurosci*. 2002; 22:10621–10626.
283. Sodha NR, Clements RT, Boodhwani M, Xu SH, Laham RJ, Bianchi C, Sellke FW. Endostatin and angiostatin are increased in diabetic patients with coronary artery disease and associated with impaired coronary collateral formation. *Am J Physiol Heart Circ Physiol*. 2009; 296:H428–H434.

284. Lerman LO, Chade AR. Angiogenesis in the kidney: a new therapeutic target? *Curr Opin Nephrol Hypertens*. 2009; 18:160–165.
285. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. Endostatin: An Endogenous Inhibitor of Angiogenesis and Tumor Growth. *Cell*. 1997;88:277–285.
286. Folkman J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol*. 2002; 29:15–18
287. Lee TY, Tjin Tham Sjin RM, Movahedi S, Ahmed B, Pravda EA, Lo KM, Gillies SD, Folkman J, Javaherian K. Linking antibody Fc domain to endostatin significantly improves endostatin half-life and efficacy. *Clin Cancer Res*. 2008; 14:1487–1493.
288. Chen J, Hamm LL, Kleinpeter MA, Husserl F, Khan IE, Chen CS, Liu Y, Mills KT, He C, Rifai N, Simon EE, He J. Elevated plasma levels of endostatin are associated with chronic kidney disease. *Am J Nephrol*. 2012; 35:335–340.
289. Wang X, Xu X, Tang L, Song S, Fan C. Interactions between endostatin and vascular endothelial growth factor (VEGF) and inhibition of choroidal neovascularization. *International journal of molecular sciences*. 2007 Jan 31;8(1):61-9.
290. Hu Y, Hu MM, Shi GL, Han Y, Li BL. Imbalance between vascular endothelial growth factor and endostatin correlates with the prognosis of operable non-small cell lung cancer. *European Journal of Surgical Oncology (EJSO)*. 2014 Sep 1;40(9):1136-42.

291. Hashizume H, Falco' n BL, Kuroda T, et al. Complementary actions of inhibitors of angiopoietin-2 and VEGF on tumor angiogenesis and growth. *Cancer Res.* 2010;70:2213–2223.
292. Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. In *Seminars in cancer biology* 2000 Dec 1 (Vol. 10, No. 6, pp. 415-433). Academic Press.
293. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol.* 1997 Oct;74(2):111-22.
294. Lambert E, Boudot C, Kadri Z, Soula-Rothhut M, Marie-Line SO, Mayeux P, Hornebeck W, Bernard HA, Petitfrere E. Tissue inhibitor of metalloproteinases-1 signalling pathway leading to erythroid cell survival. *Biochemical Journal.* 2003 Jun 15;372(3):767-74.
295. Chirco R, Liu XW, Jung KK, Kim HR. Novel functions of TIMPs in cell signaling. *Cancer Metastasis Rev.* 2006;25(1):99–113.
296. Van Geest RJ, Klaassen I, Lesnik-Oberstein SY, Tan HS, Mura M, Goldschmeding R, et al. Vitreous TIMP-1 levels associate with neovascularization and TGF-beta2 levels but not with fibrosis in the clinical course of proliferative diabetic retinopathy. *Journal of cell communication and signaling.* 2013;7(1):1–9.
297. Ikenaka Y, Yoshiji H, Kuriyama S, Yoshii J, Noguchi R, Tsujinoue H, et al. Tissue inhibitor of metalloproteinases-1 (TIMP-1) inhibits tumor growth and angiogenesis in the TIMP-1 transgenic mouse model. *Int J Cancer.* 2003;105(3):340–6.

298. Waas ET, Wobbes T, Ruers T, Lomme RM, Hendriks T. Circulating gelatinases and tissue inhibitor of metalloproteinase-1 in colorectal cancer metastatic liver disease. *Eur J Surg Oncol.* 2006 Sep;32(7):756-63.
299. Honkavuori M, Talvensaaari-Mattila A, Puistola U, Turpeenniemi-Hujanen T, Santala M. High serum TIMP-1 is associated with adverse prognosis in endometrial carcinoma. *Anticancer Res.* 2008 Sep-Oct;28(5A):2715-9.
300. Monach PA, Warner RL, Tomasson G, Specks U, Stone JH, Ding L, Fervenza FC, Fessler BJ, Hoffman GS, Iklé D, Kallenberg CG, Krischer J, Langford CA, Mueller M, Seo P, St Clair EW, Spiera R, Tchao N, Ytterberg SR, Johnson KJ, Merkel PA. Serum proteins reflecting inflammation, injury and repair as biomarkers of disease activity in ANCA-associated vasculitis. *Ann Rheum Dis.* 2013 Aug;72(8):1342-50.
301. Määttä M, Talvensaaari-Mattila A, Turpeenniemi-Hujanen T, Santala M. Matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) and their tissue inhibitors (TIMP-1 and TIMP-2) in differential diagnosis between low malignant potential (LMP) and malignant ovarian tumours. *Anticancer Res.* 2007 Jul-Aug;27(4C):2753-8.
302. Creemers EE, Davis JN, Parkhurst AM, Leenders P, Dowdy KB, Hapke E, Hauet AM, Escobar PG, Cleutjens JP, Smits JF, Daemen MJ. Deficiency of TIMP-1 exacerbates LV remodeling after myocardial infarction in mice. *American Journal of Physiology-Heart and Circulatory Physiology.* 2003 Jan 1;284(1):H364-71.
303. Westerhoff M, Tretiakova M, Hovan L, Miller J, Noffsinger A, Hart J. CD61, CD31, and CD34 improve diagnostic accuracy in gastric antral vascular ectasia and portal hypertensive gastropathy: An immunohistochemical and digital

- morphometric study. *Am J Surg Pathol*. 2010 Apr;34(4):494-501. doi: 10.1097/PAS.0b013e3181d38f0a.
304. Junquera F, Saperas E, de Torres I, Vidal MT, Malagelada JR. Increased expression of angiogenic factors in human colonic angiodysplasia. *Am J Gastroenterol*. 1999 Apr;94(4):1070-6.
 305. Tan H, Chen H, Xu C, Ge Z, Gao Y, Fang J, Liu W, Xiao S. Role of vascular endothelial growth factor in angiodysplasia: an interventional study with thalidomide. *J Gastroenterol Hepatol*. 2012 Jun;27(6):1094-101.
 306. Prapansilp P, Medana I, Mai NT, Day NP, Phu NH, Yeo TW, Hien TT, White NJ, Anstey NM, Turner GD. A clinicopathological correlation of the expression of the angiopoietin-Tie-2 receptor pathway in the brain of adults with *Plasmodium falciparum* malaria. *Malar J*. 2013 Feb 5;12:50. doi: 10.1186/1475-2875-12-50.
 307. Rossi A, Ferreira L, Cuevas-Nunez M, et al. Angiopoietin-2 is expressed in oral Kaposi's sarcoma. *J Oral Pathol Med*. 2017;46:1011–1014.
 308. Hong S, Jung HI, Ahn TS, Kim HJ, Lee KT, Baek MJ, Bae SB. Expressions and Clinical Significances of Angiopoietin-1, Angiopoietin-2, and Tie-2 Receptor in Patients With Colorectal Cancer. *Ann Coloproctol*. 2017 Feb;33(1):9-15. doi: 10.3393/ac.2017.33.1.9. Epub 2017 Feb 28.
 309. Li C, Sun CJ, Fan JC, Geng N, Li CH, Liao J, Mi K, Zhu GQ, Ma H, Song YF, Tang YL, Chen Y. Angiopoietin-2 expression is correlated with angiogenesis and overall survival in oral squamous cell carcinoma. *Med Oncol*. 2013;30(2):571.
 310. Staton CA, Valluru M, Hoh L, Reed MW, Brown NJ. Angiopoietin-1, angiopoietin-2 and Tie-2 receptor expression in human dermal wound repair and scarring. *Br J Dermatol*. 2010 Nov;163(5):920-7.

311. Ye K, Li J, Li X, Chang S, Zhang Z. Ang1/Tie2 induces cell proliferation and migration in human papillary thyroid carcinoma via the PI3K/AKT pathway. *Oncol Lett.* 2018 Jan;15(1):1313-1318.
312. Kather JN, Friedrich J, Woik N, Sticht C, Gretz N, Hammes HP, Kroll J. Angiopoietin-1 is regulated by miR-204 and contributes to corneal neovascularization in KLEIP-deficient mice. *Invest Ophthalmol Vis Sci.* 2014 Jun 10;55(7):4295-303.
313. Lee YJ, Ke CY, Tien N, Lin PK. Hyperhomocysteinemia Causes Chorioretinal Angiogenesis with Placental Growth Factor Upregulation. *Sci Rep.* 2018 Oct 25;8(1):15755.
314. Lee YJ, Chiu CC, Ke CY, Tien N, Lin PK. Homocysteine Facilitates Prominent Polygonal Angiogenetic Networks of a Choroidal Capillary Sprouting Model. *Invest Ophthalmol Vis Sci.* 2017 Aug 1;58(10):4332–4343.
315. Tan H, Chen H, Xu C, Ge Z, Gao Y, Fang J, Liu W, Xiao S. Role of vascular endothelial growth factor in angiodysplasia: an interventional study with thalidomide. *J Gastroenterol Hepatol.* 2012 Jun;27(6):1094-101. doi: 10.1111/j.1440-1746.2011.06967.x.
316. Murphy RM, Lamb GD. Important considerations for protein analyses using antibody based techniques: down-sizing Western blotting up-sizes outcomes. *J Physiol.* 2013 Dec 1;591(23):5823-31.
317. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet.* 2012 Mar 13;13(4):227-32. doi: 10.1038/nrg3185.

318. Kumar D, Bansal G, Narang A, Basak T, Abbas T, Dash D. Integrating transcriptome and proteome profiling: Strategies and applications. *Proteomics*. 2016 Oct;16(19):2533-2544. doi: 10.1002/pmic.2
319. Judge C, Tighe D, Barry L, O'Neill J, Wong J, Shahin A, Moran N, Stack R, Hussey M, Breslin N, O'Connor A, Ryan B, Buckley M, McNamara D. Predicting pathology on small bowel capsule endoscopy: a good FIT. *Endosc Int Open*. 2019 Nov;7(11):E1379-E1385.
320. Navarro M, Nicolas A, Ferrandez A et al. Colorectal cancer population screening programs worldwide in 2016: An update. *World J Gastroenterol*. 2017;23:3632–3642
321. Park J J, Cheon J H. Small bowel evaluation in asymptomatic fecal immunochemical test-positive patients with a negative colonoscopy: is it necessary? *Dig Dis Sci*. 2011;56:2773–2775.
322. Park JH, Choi H, Kim YB, Kim YS, Sheen SS, Choi JH, Lee HL, Lee KS, Chung WY, Lee S, Park KJ. Serum angiopoietin-1 as a prognostic marker in resected early stage lung cancer. *Lung cancer*. 2009 Dec 1;66(3):359-64.
323. Ganter MT, Cohen MJ, Brohi K, Chesebro BB, Staudenmayer KL, Rahn P, Christiaans SC, Bir ND, Pittet JF. Angiopoietin-2, marker and mediator of endothelial activation with prognostic significance early after trauma?. *Annals of surgery*. 2008 Feb 1;247(2):320-6.
324. Helfrich I, Edler L, Sucker A, Thomas M, Christian S, Schadendorf D, Augustin HG. Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. *Clinical Cancer Research*. 2009 Feb 15;15(4):1384-92.

325. Suzuki M, Iizasa T, Ko E, Baba M, Saitoh Y, Shibuya K, Sekine Y, Yoshida S, Hiroshima K, Fujisawa T. Serum endostatin correlates with progression and prognosis of non-small cell lung cancer. *Lung Cancer*. 2002 Jan 1;35(1):29-34.
326. Mo HY, Luo DH, Qiu HZ, Liu H, Chen QY, Tang LQ, Zhong ZL, Huang PY, Zhao ZJ, Zhang CQ, Zhang Y. Elevated serum endostatin levels are associated with poor survival in patients with advanced-stage nasopharyngeal carcinoma. *Clinical Oncology*. 2013 May 1;25(5):308-17.
327. Poruk KE, Firpo MA, Scaife CL, Adler DG, Emerson LL, Boucher KM, Mulvihill SJ. Serum osteopontin and TIMP-1 as diagnostic and prognostic biomarkers for pancreatic adenocarcinoma. *Pancreas*. 2013 Mar;42(2):193.
328. Wang CS, Wu TL, Tsao KC, Sun CF. Serum TIMP-1 in gastric cancer patients: a potential prognostic biomarker. *Annals of Clinical & Laboratory Science*. 2006 Dec 21;36(1):23-30.
329. Nguyen, H., C. Le, and H. Nguyen, Gastric antral vascular ectasia (watermelon stomach)—an enigmatic and often-overlooked cause of gastrointestinal bleeding in the elderly. *The Permanente Journal*, 2009. 13(4): p. 46
330. Gjeorgjievski, M. and M.S. Cappell, Portal hypertensive gastropathy: A systematic review of the pathophysiology, clinical presentation, natural history and therapy. *World journal of hepatology*, 2016. 8(4): p. 231
-