

ANGIOGENESIS IN THE NASAL MUCOSA

by

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ABSTRACT

Nasal polyposis is a common disease affecting 2-4 % of the general population. The aetiology and pathogenesis are far from clear. Recent publications have suggested up-regulation of several pro-angiogenic factors including VEGF. The aim of this study was to assess and quantify the degree of angiogenesis in nasal polyposis and to determine if angiogenesis was the driving force behind polyposis. We started by developing a novel triple stain to assess remodelling in the nasal mucosa. For the first time we were able to categorically refute the common belief of angiogenesis driven polyposis. We then carried out genomic studies and identified upregulation of genes controlling the cell cycle and apoptosis, suggesting cell turnover is an important part of the pathogenesis of nasal polyps. Our gene expression data was confirmed by TUNEL staining, indicating an increased level of apoptosis in nasal polyp tissue, counterbalancing the increased cell proliferation. Inflammatory genes are also upregulated, however the data collected so far cannot distinguish between different types of inflammatory response. We carried out proteomic studies using the luminex system but this did not clarify the situation despite using matched samples that were used in the gene array. They highlight the protein differences occurring in the polyps themselves. We have shown chemoattractants for eosinophils & macrophages (which are found in polyps), and significantly in iNOS, which is novel.

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CHAPTER 1

GENERAL INTRODUCTION

1 General Introduction

1.1 Angiogenesis

1.1.1 What is angiogenesis?

1.1.1.1 Definition

Angio genesis (Latin) is the growth of new blood vessels from an existing vasculature, and is an important natural process occurring both in health and in disease. This occurs mainly in the capillaries and may affect venules.

1.1.1.2 History

The term “angiogenesis” was first used in 1787 by the Scottish surgeon Dr. John Hunter to describe blood vessels growing in reindeer antler after the annual shedding (Palmer, 1837). This process is multifactorial and related to day light length changes and sexual hormone level variations. In 1935, Boston pathologist Dr Arthur Tremain Hertig (Hertig, 1935) was the first to describe the actual process of angiogenesis in the placenta of pregnant monkeys. This again is multifactorial and regulated by cyclical hormonal variations.

In 1971 Dr Judah Folkman (Folkman et al., 1971) showed a soluble factor separable from human and animal tumours that was mitogenic for endothelial cells and responsible for formation of new capillaries. He hypothesized that tumour growth in all mammals studied (including man) was dependent upon angiogenesis and thus antiangiogenic therapy could be developed as a treatment modality in solid tumours.

Cartilage, which has no blood supply but receives its nutrition by diffusion, was the first tissue found to inhibit angiogenesis and Drs. Henry Brem and Judah Folkman hypothesized that with further purification, morselised cartilage may prove useful as a means of maintaining tumour dormancy by “anti-angiogenesis” (Brem and Folkman, 1975). The factor was unknown.

In vitro studies of tissue culture allowed the identification and extraction of these growth factors. The first angiogenic factor (subsequently identified as basic fibroblast growth factor, bFGF) was purified at Harvard Medical School in 1984 because of its affinity to heparin, which allowed its extraction. Basic fibroblast growth factor stimulated capillary endothelial cell proliferation (Shing et al., 1984), although it proved to be a non-specific mitogen so unsuitable for targeted therapy.

Vascular endothelial growth factor (VEGF) which is one of the most important angiogenic factors was discovered simultaneously by Dr. Napoleone Ferrara (Ferrara and Henzel, 1989) and by Dr Jean Plouet in 1989 (Plouet et al., 1989). It turned out to be identical to a molecule called vascular permeability factor (VPF) discovered 6 years previously by Dr. Harold Dvorak (Senger et al., 1983).

The first anti-angiogenic compound, angiostatin, was discovered by O’Reily and Folkman in 1994 (O’Reilly et al., 1994) from serum and urine of tumour bearing mice.

1.1.1.3 Clinical relevance

Angiogenesis is ubiquitous in health and in many disease processes. Physiological angiogenesis occurs in a variety of conditions, most notably; wound healing to restore blood

flow to tissues after injury or assault, menstruation to rebuild the uterus lining, to mature the egg during ovulation, during pregnancy to build the placenta, lactation, formation of adipose tissue, and during endurance training in skeletal muscle. Pathological angiogenesis is equally common occurring in both neoplastic and non- neoplastic pathologies. Neoplastic conditions include both benign and malignant tumours anywhere in the body and the non-neoplastic conditions include diabetic blindness, age related macular degeneration, atherosclerosis, rheumatoid arthritis and psoriasis (Folkman, 1995). Diseases in which there is insufficient angiogenesis include, ischaemic heart disease, peripheral vascular disease, stroke, delayed wound healing, and certain muscular dystrophies. The body controls angiogenesis through a series of angiogenesis stimulating growth factors balanced with angiogenesis inhibitors. A better understanding of the complex processes and controls of angiogenesis will undoubtedly help develop better management strategies in many of these conditions.

1.1.2 Types of angiogenesis

Angiogenesis is a mechanism by which a pre-existing microvascular network is expanded in response to a changing metabolic and mechanical environment (Hudlicka et al., 1992). There are a number of discreet processes, through which new blood vessels are formed; sprouting angiogenesis, splitting angiogenesis – intussusceptive and longitudinal splitting, elongation and vasculogenesis.

1.1.2.1 Sprouting Angiogenesis

During this process, endothelial cells form sprouts that break through the basement membrane and begin tube formation that grows as endothelial cells proliferate, forming a new capillary. This new capillary then fuses with another capillary and blood starts to flow through it enabling it to mature into a functional capillary with reformation of the basement membrane and recruitment of perivascular cells (Ausprunck and Folkman, 1977, Hudlicka, 1991, Carmeliet, 2000). This is most often seen in tumour angiogenesis, but the blood supply of a tumour is rarely as patent and well organized as the normal vasculature. Vessels are often leaky, badly organised and have blind ending tubes (Papetti and Herman, 2002), and may be lined with non-endothelial cells (Folberg and Maniotis, 2004).

1.1.2.2 Splitting Angiogenesis

Capillaries can split *in vivo* in two different ways (Figure 1.1), by intussusception and luminal division.

1.1.2.2.1 Intussusceptive Angiogenesis

This was first described in rat lung and occurs when interstitial cells press into the capillary, forming a column which then propagates down the capillary, resulting in the formation of two separate capillaries (Burri and Tarek, 1990). Intussusceptive angiogenesis is thought to be an important process during development and possibly some tumours (Djonov et al., 2003).

1.1.2.2.2 Luminal Division

In contrast to this, luminal division (also known as longitudinal splitting) involves endothelial

initiation (as opposed to interstitial initiation), whereby endothelial cells send filopodial processes into the capillary lumen, which join and form a septation that propagates down the capillary (Zhou et al., 1998, Egginton et al., 2001). This has been seen in adult rat and mouse skeletal muscle in response to increased blood flow and is a different process to intussusceptive angiogenesis (Williams et al., 2006b, Zhou et al., 1998, Egginton et al., 2001). The clinical relevance of luminal division is unclear, as it has not been investigated, but as it is associated with much less mitotic activity than the traditional (sprouting) form of angiogenesis it may be more efficient in terms of energy cost and rapidity of response (Egginton, 2011).

1.1.2.3 Elongation

During the menstrual cycle, endometrial angiogenesis occurs by a combination of capillary splitting (whether luminal division or intussusception is unknown) and capillary elongation (Rogers and Gargett, 1998). This capillary elongation is a specialised process that increases capillary supply mainly due to the unique spiral shape of capillaries found in the endometrium. Capillary elongation is difficult to detect using the standard two dimensional measurements of angiogenesis commonly used, thus may be more prevalent than currently realised.

1.1.2.4 Vasculogenesis

This is another method of new blood vessel formation first described in chick embryos (Gonzalez-Crussi, 1971, Watterson, 1949). It is not the same as angiogenesis, which requires pre-existing endothelial lined vessels. Vasculogenesis is the *de novo* formation of blood

vessels from mesodermal endothelial precursors which coalesce to form a blood island, and then form endothelial tubes resulting in immature and isolated vascular beds, that subsequently form complex networks through angiogenesis (Risau, 1997). Circulating endothelial precursor cells (EPCs) may also lead to new blood vessel formation, which some authors have stated as being a type of vasculogenesis (Asahara et al., 1997, Ribatti et al., 2001). EPCs are clinically relevant as they may be incorporated into tumour vasculature (Peters et al., 2005). This appears to be a different process to classic embryonic vasculogenesis.

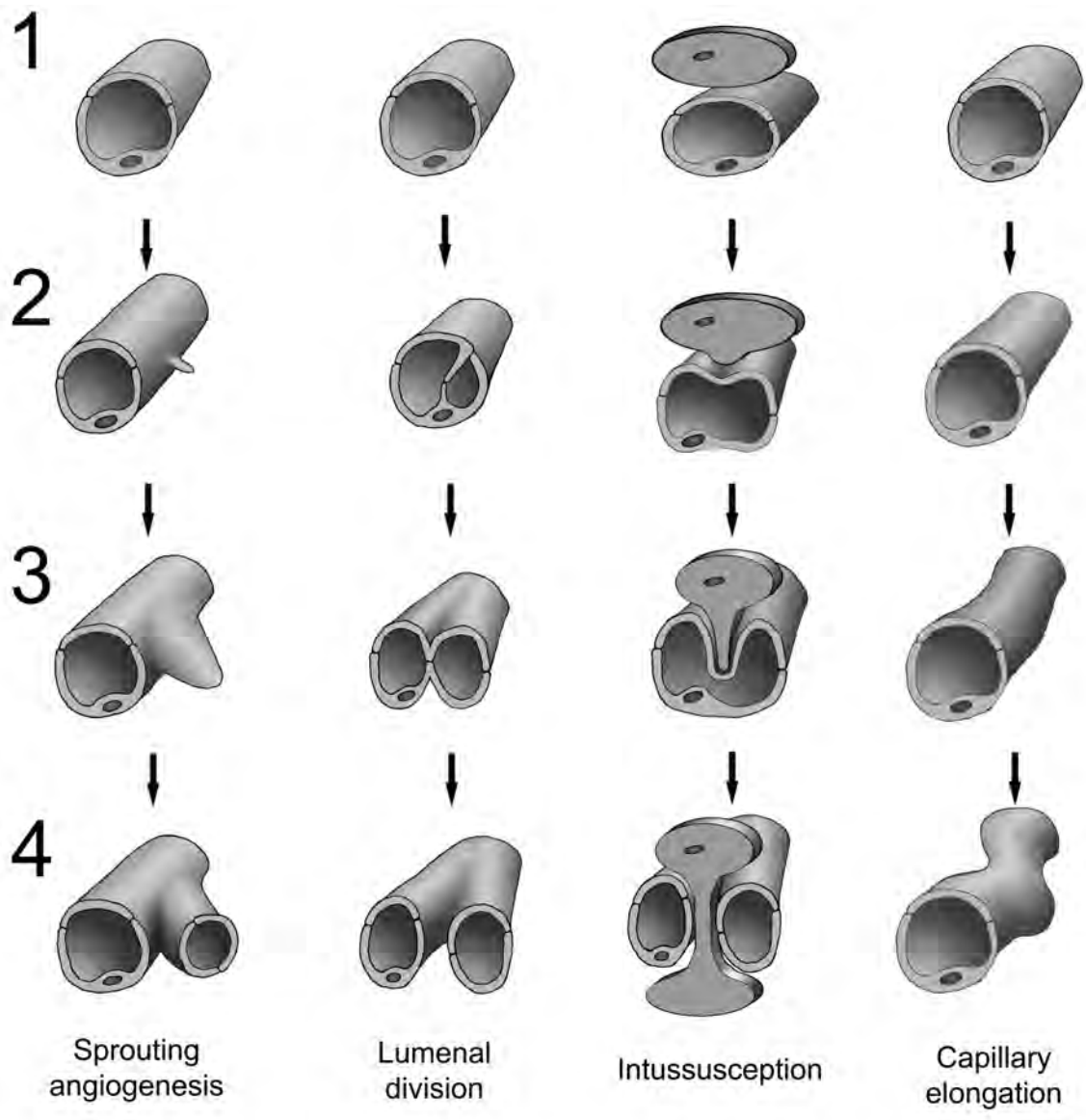


Figure 1.1 Four morphological forms of angiogenesis; sprouting angiogenesis, splitting (both luminal division and intussusception) angiogenesis and capillary elongation.

Despite there being morphological differences between the different types of angiogenesis there are many processes that are required in all of them, such as alteration of cell contacts,

disruption of basement membrane, endothelial cell proliferation, and capillary maturation. All these processes require very precise spatial and temporal control, which in turn requires a complex balance of pro and anti angiogenic factors. Numerous pro and anti-angiogenic factors and inflammatory mediators have been described (Folkman, 1996, Friedlander et al., 1996), mainly from *in vitro* studies, but what is becoming apparent is that angiogenesis *in vivo* involves a cascade of feed-forward and feed-back pathways. Vascular endothelial growth factor (VEGF) and the angiopoietins are the most specific for endothelial cells and may therefore have the most important role in angiogenesis (Gale and Yancopoulos, 1999).

1.1.3 Molecular Basis of Angiogenesis

1.1.3.1 The vascular endothelial growth factor system

Vascular endothelial growth factor (VEGF) is the most important factor in the promotion of angiogenesis (Clauss et al., 1990). A single allele knockout is lethal *in utero* (Carmeliet et al., 1996) and it is essential for maintaining vascular integrity (Tang et al., 2004). VEGF was first described in 1989 (Ferrara and Henzel, 1989) and has since been found in a range of different tissues (Ferrara, 2004). Members of the vascular growth factor family (VEGF [VEGF-A], placental derived growth factor, VEGF-B, VEGF-C, and VEGF-D) are currently known as the major inducers of angiogenesis and lymphangiogenesis (Saaristo et al., 2000a). VEGF is also known to be more potent than histamine in increasing capillary permeability to plasma proteins (Dvorak et al., 1995), and VEGF signalling often plays a critical rate limiting step in physiological angiogenesis (Ferrara, 2004, Ferrara and Davis-Smyth, 1997).

VEGF-A, commonly referred to simply as VEGF, is the most abundant type and has four isoforms, having 121, 165, 189 and 206 amino acids respectively, after signal sequence cleavage (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆) (Houck et al., 1991, Ferrara, 2004). VEGF-B also has a wide tissue distribution and is particularly abundant in the heart and skeletal muscle (Olofsson et al., 1996). VEGF-C and VEGF-D are closely related and form a subfamily of the VEGF-related proteins (Achen et al., 1998, Robinson and Stringer, 2001). They are more specific for lymphatic channels and are involved in lymphangiogenesis (Kukk et al., 1996, Jeltsch et al., 1997, Joukov et al., 1996, Ristimaki et al., 1998). The VEGF family mediate their effects through binding with varying affinity to vascular endothelial growth factor receptors (VEGFR) that form the family of receptor tyrosine kinases (Robinson and Stringer, 2001, Ferrara et al., 2003). Three VEGF receptor types (R1-R3) have been identified. VEGFR-1 and VEGFR-2 are both high affinity receptors for VEGF-A whereas VEGFR-3 binds to VEGF-C and VEGF-D and appears to be localised to lymph vessels (Karkkainen et al., 2002). VEGF-B seems to selectively bind only to VEGFR-1 (Olofsson et al., 1998). VEGFR-2, also called *Flk-1* (for foetal liver kinase, or *KDR* for kinase insert domain receptor), mediates most of the direct effects of VEGFs on the endothelial cell, and binds the remaining VEGFs (Waltenberger et al., 1994). VEGFR-1 is also known as *Flt-1* (for *fms* -like tyrosine kinase-1) and may mediate secondary effects such as tissue factor upregulation as apposed to direct proliferative effects on endothelial cells (Clauss et al., 1990, Park et al., 1994).

1.1.3.2 Basic fibroblast growth factor

Basic fibroblast growth factor (bFGF), also known as fibroblast growth factor-2 (FGF-2) was

originally described as an angiogenic fraction from a tumour line and was found to be a heparin binding protein in 1984 (Shing et al., 1984, Folkman et al., 1971) capable of inducing angiogenesis *in vivo* and cell proliferation *in vitro* (Presta et al., 2005). They are in fact a family of heparin binding growth factors and like VEGF, exist as four isoforms (18, 21.5, 22 and 24 kDa) (Bikfalvi et al., 1998). bFGF is much less specific in its expression and action on endothelium than VEGF and can stimulate angiogenesis in a variety of *in vivo* models (Yayon and Klagsbrun, 1990, Presta et al., 2005). VEGF knockout mice die *in utero* from vascular defects (Carmeliet et al., 1996, Ferrara et al., 1996), whereas bFGF knockout mice are viable although with impaired wound healing (Ortega et al., 1998), suggesting that VEGF is more important in angiogenesis.

1.1.3.3 Platelet derived growth factor

Platelet derived growth factor (PDGF) is a dimeric glycoprotein that has five isoforms (A, B, C, D, and AB) that activate cellular response. It plays an important role in regulating cell growth and division, particularly in blood vessel formation (Matsui et al., 1989). The receptor for PDGF, PDGFR is a cell surface receptor tyrosine kinase. Two types of PDGFRs have been identified, alpha (PDGFRA) and beta (PDGFRB) (Heidaran et al., 1991) that dimerise upon binding resulting in tyrosine phosphorylation, leading to enzymatic activity. Expression of both receptors and each of the five isoforms is under independent control. In addition, different external stimuli such as inflammation, embryonic development or differentiation modulate cellular receptor expression and some cell types display only one of the PDGFR isoforms while other cells express both isoforms simultaneously or separately (Heldin and Westermark, 1999). PDGF has been shown to recruit pericytes and promote vessel maturation

(Carmeliet, 2000). It can help wound healing (Robson et al., 1992) and was the first licensed clinical pro-angiogenic factor for treating diabetic foot ulcers (Steed, 1995). Thus the PDGF/PDGFR system has many complex external controlling mechanisms affording it high flexibility in regulating cell growth and division, particularly in angiogenesis.

1.1.3.4 Tumour necrosis factor

Tumour necrosis factors (or the TNF family) refer to a group of cytokines, each with 157 amino acids that can cause apoptosis as well as a wide range of pro-inflammatory actions. TNF- α (TNF- α) is the most well known, and is a multifunctional inflammatory cytokine released by leukocytes and endothelial cells, with effects on lipid metabolism, coagulation, insulin resistance, and the function of endothelial cells lining blood vessels (Yoshida et al., 1997, Huang et al., 2000). TNF acts via the TNF receptor (TNF-R) and blocking the action of TNF has been shown to be beneficial in reducing inflammation. It has an important role in angiogenesis and can have pro- or anti- angiogenic effects, depending on the system studied (Patterson et al., 1996). It also affects angiogenesis through leukocyte behaviour (Norrby, 1997, Polverini, 1997).

1.1.3.5 Nitric oxide

Nitric oxide (NO) is a gaseous signalling molecule that has several potential effects on angiogenesis. It is synthesised from arginine by one of three nitric oxide synthases (NOS) – endothelial (eNOS/NOS3), neuronal (nNOS/NOS1) or inducible (iNOS/NOS2). In skeletal muscle, eNOS is found in endothelial cells, whilst nNOS is found at a high level in the sarcolemma of muscle fibres and iNOS can be induced in the endothelium by inflammatory

factors (Stamler and Meissner, 2001). Whilst eNOS has been thought of as being most important in angiogenesis (Smith et al., 2002, Lee et al., 1999, Amano et al., 2003), both nNOS and iNOS are capable of exposing the endothelium to higher concentrations of nitric oxide (Kavdia and Popel, 2004). Nitric oxide has a very short half life in the blood and is constitutively produced as a tonic vasodilating agent, which may in part explain its angiogenic activity (Egginton, 2009).

NO can upregulate the mitogen-activated protein (MAP) kinase pathway and lead to secretion of VEGF *in vitro* (Ziche and Morbidelli, 2002), and addition of exogenous eNOS can stimulate angiogenesis (Smith et al., 2002), however nitric oxide may also have an anti-angiogenic role (Bussolati et al., 2001, Lau and Ma, 1996, Pipili-Synetos et al., 1993).

NO is also found in exhaled air and most originates from the paranasal sinuses (Lundberg et al., 1995). This will be discussed further in section 1.2.1.5

1.1.3.6 Matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent endopeptidases that can collectively degrade almost all extra cellular matrix (ECM) components and are implicitly involved in ECM remodelling. Proteolysis of the extracellular matrix is essential for angiogenesis to physically enable capillaries to grow into an existing tissue (Nguyen et al., 2001). There are over 20 MMPs (Rundhaug, 2005), of which 1, 2, 3, and 9 are produced by endothelial cells (Haas and Madri, 1999). The activity of MMPs is tightly regulated, as with all the components of the angiogenic response, by a four-fold mechanism. Transcriptional control of MMPs is tightly regulated by a host of factors which

up- or down-regulate MMP production (Chase and Newby, 2003). After transcription, MMPs must be converted from a latent to an active form, and this active form is inhibited by the activity of the tissue inhibitors of metalloproteinases (TIMPs) (Silletti et al., 2001), although the TIMPs may be necessary for activation of MMPs. Once MMPs are activated, the products they generate can inhibit them. MMPs are thought to be of critical importance in angiogenesis, and inhibition of MMPs results in abolition of angiogenesis in rat skeletal muscle (Haas et al., 2000) when examining the sprouting form of angiogenesis where interstitial migration of endothelial cells is essential. It appears not to be important for longitudinal splitting, where the interstitium is unaffected (Williams et al., 2006c) (Williams et al 2006).

1.2 The Nose

1.2.1 Functions of the nose

1.2.1.1 Anatomy:

The nose is made up of bone and cartilage. The septum is a part bony and cartilaginous structure that divides the nose into two similar halves. The nose contains shelf-like structures called turbinates that help trap particles entering the nasal passages. Material deposited in the nose is transported by ciliary action to the back of the throat in 10-15 minutes. A space called the meatus lies inferior to the turbinates and is a drainage pathway for mucociliary flow to the postnasal space. The cribriform plate of the ethmoid bone internally forms the roof of the nose.

1.2.1.2 Physiology:

The nasal cavity is divided into two segments: the respiratory and the olfactory segment. The respiratory segment is lined with ciliated pseudostratified columnar epithelium, whereas the olfactory segment is lined with olfactory epithelium, which contains receptors for the sense of the smell.

The nose has two primary functions: olfaction, and filtration & humidification of inhaled air. The turbinates on the lateral wall of nasal cavity interrupt the airflow into the nasal passage, forcing it through narrow passages that are covered with moist nasal mucosa. The airflow across this mucosal surface is humidified and warmed by the turbulent flow and greater surface area created by the turbinates.

1.2.1.3 Blood flow in the nose

Blood supply comes from branches of the carotid artery, including the facial and the maxillary arteries. The arteries within the nose are the anterior ethmoidal artery, the Sphenopalatine and Greater palatine arteries that are branches of the maxillary artery, as well as facial arteries that supply to the anterior part of the nasal cavity.

1.2.1.4 Nasal cycle

The nasal cycle is the alternating congestion and decongestion of the nasal airway, every two to six hours, controlled by the autonomic nervous system. Changes in hormone levels, temperature, and humidity all affect the nasal cycle(Chaaban and Corey, 2011).

1.2.1.5 Nitric oxide

Most of the NO that can be detected in exhaled air is from the paranasal sinuses (Lundberg et al., 1995). It is involved in ciliary motility; being low in Kartagener's Syndrome (Lundberg et al., 1994) and also correlates with ciliary beat frequency (Lindberg et al., 1997). Infusion of NO donors increases ciliary beat frequency and blood flow (Runer and Lindberg, 1998) whereas NO inhibition causes vascular constriction and nasal decongestion (Rinder and Lundberg, 1996).

1.3 Vascular responses in nasal disease

1.3.1 Asthma

The nose and lungs are anatomically and physiologically closely related organs. The nose plays an important role within the airways, as a filter and humidifier, together with other important functions. Asthmatic patients are said to have an increased swelling of the nasal mucosa, which results in a reduced ability to filter air and nasal diseases are associated with several lower airways diseases such as asthma. Other upper airway conditions, such as nasal polyposis and chronic sinusitis are thought to be factors involved in influencing lower airway function hence asthma exacerbation.

1.3.2 Allergic rhinitis

Rhinitis is simply inflammation of the nasal passages. Allergic rhinitis is inflammation of the nasal passages through an allergic mechanism. Allergic rhinitis can be seasonal or perennial. The symptoms of allergic rhinitis are: sneezing; rhinorrhea or runny nose, usually clear and colorless in allergy; itching of the nose; and obstructed nasal passages.

20 - 30% of the population forms a type of antibody (IgE) to the offending allergens. The IgE antibody sits on mast cells and basophils which contain granules of mediators especially histamine. When the allergen becomes bound by the IgE antibodies on the surface of these cells, it causes them to release their histamine and other mediators. Histamine released during this reaction causes dilatation of blood vessels which causes them to leak fluid. Histamine also creates itching. Moreover as the allergic reaction continues, other cells such as eosinophils are

called from the blood that release other toxic granules that further inflame the surrounding tissue in a more long-lasting fashion (Braido et al., 2011).

Allergic patients have a twofold increase in nasal nitric oxide (NO) concentration (Arnal et al., 1997)

1.4 Nasal polyps

1.4.1 Definition:

Nasal polyps are oedematous, semi translucent, benign masses that develop from mucosal linings of the paranasal cavity, usually originating from the mucosa in the osteomeatal complex,(Larsen and Tos, 2004) so strictly speaking should be referred to as sinonasal polyps. They protrude into the nasal cavity as grape-like structures causing nasal blockage and obstructing airflow to olfactory mucosa. Polyps consist of loose connective tissue, oedema, inflammatory cells (predominantly eosinophils), capillaries and some glands, mainly covered with respiratory pseudostratified epithelium with cilia and goblet cells. The relationship between sinonasal polyposis and chronic rhinosinusitis is much debated, but should now be regarded as part of the same disease spectrum.(Fokkens et al., 2007)

1.4.2 Tissue remodelling and angiogenesis in nasal polyps

Ito et al. first described expression of VEGF messenger RNA and its receptor VEGFR-1, in nasal polyps using northern blotting and *in situ* hybridisation (Ito et al., 1995b). They revealed VEGF mRNA expressing cells that were scattered in the oedematous stroma of nasal polyps. Yang et al. used immunohistochemistry to compare nasal polyps with inferior turbinate

mucosa and identified expression of both VEGF protein and its receptor VEGFR-2, to be in greater abundance in the vascular endothelium and glands of nasal polyps (Yang et al., 1998a). In 2000 Coste et al., demonstrated *in vitro* expression of VEGF mRNA and *in vitro* secretion of VEGF protein from primary human cultures of nasal epithelial cells (Coste et al., 2000a). The authors concluded that VEGF, inducing oedema and angiogenesis, was involved in the pathogenesis of nasal polyps. Lee et al.,(Lee et al., 2000) demonstrated respiratory syncytial virus (RSV) to be a potent stimulator of VEGF and suggested VEGF had an important role in the pathogenesis of RSV-induced disorders. Guo et al.,(Guo et al., 2001a) used immunohistochemistry to compare nasal polyps with middle turbinate mucosa. They demonstrated VEGF positivity in vascular endothelium and in glandular cells that was significantly higher in their nasal polyp group and concluded that VEGF played a key role in the formation of nasal polyps. Li et al.,(Li et al., 2001) evaluated the expression of VEGF and basic fibroblastic growth factor (bFGF or FGF-2) in nasal polyps from patients with different degrees of polyposis, using immunohistochemistry. The detection rates were significantly higher in the group with more severe nasal polyposis and the authors concluded that the overexpression of VEGF and bFGF in nasal polyps might contribute to their growth and development. Jiang et al.,(Jiang et al., 2001) demonstrated that VEGF expression was upregulated in nasal polyps, supporting the potential role of VEGF in nasal polyposis. Jiang et al.,(Jiang et al., 2002, Jiang et al., 2003) used cultured human nasal epithelial cells from polyps and showed them to be actively producing vast amounts of VEGF in response to hypoxia. They suggested that this was the major cause for nasal polyp formation in the middle meatus of the nose, a site at which most of the paranasal sinuses drain into the nasal cavity and where minimal mucosal oedema can result in complete occlusion, with resultant hypoxia in

the sinus cavities. In 2002 Wittenkindt et al.,(Wittekindt et al., 2002) studied the immunohistochemical expression of VEGF and VEGF receptors 1 and 2 in nasal polyps. They demonstrated strong immunostaining for VEGF in nasal polyps as well as VEGFR-1 in the vascular endothelium in polyps. Mononuclear round cells and the endothelium of capillaries revealed immunoreactivity to VEGFR-2. In 2004, using immunohistochemistry, Wang et al.,(Wang et al., 2004b) showed increased expression of VEGF and its receptors in nasal polypoid tissue as compared to normal nasal mucosa. They conclude that VEGF and its receptors participate in the regulation and formation of nasal polyps.

To summarise, VEGF is a potent stimulator of angiogenesis and permeability. There is a body of evidence to support the presence of VEGF in the nasal mucosa in patients with nasal polyposis. VEGF protein and its receptors have been shown in nasal polyps, around inflammatory cells, in the mucosal glands and in the vascular endothelium of the nasal mucosa. VEGF-C and VEGFR-3 are usually found in lymphatics, however, Saaristo et al.,(Saaristo et al., 2000a, Saaristo et al., 2000b) demonstrated both VEGF-C and its receptor VEGFR-3 in the vascular endothelium of the nasal mucosa. The remaining splice variants of VEGF have not specifically been looked for in the nasal mucosa.

The VEGF-C/ VEGFR-3 signalling pathway is critical for lymphangiogenesis (Jeltsch et al., 1997). Northern blot analysis in mouse and human embryos were used to isolate mRNA from nasal mucosa and immunohistochemistry with *in situ* hybridisation used to measure VEGF-C protein and VEGFR-3 expression (Saaristo et al., 2000a, Saaristo et al., 2000b). Both VEGF-C and VEGFR-3 expression were demonstrated in the nasal mucosa. *In situ* hybridisation of mouse embryos showed strong expression of VEGF-C mRNA in the developing nasal conchae

and VEGFR-3 mRNA in the submucosal plexus, suggesting this ligand-receptor may have an important role in the modelling of this unique vascular plexus. (Saaristo et al., 2000a, Saaristo et al., 2000b)

1.5 Pathogenesis of nasal polyposis

The reason why polyps develop in some patients and not in others is unknown. It is a heterogeneous condition associated with chronic sinus inflammation. Symptomatic polyposis occurs in up to 4% of the general population (Hedman et al., 1999) but there is a higher prevalence in patients with specific conditions such as asthma, aspirin sensitivity, and cystic fibrosis. Autopsy studies have demonstrated prevalence rates of 32-42% in unselected cadavers (Larsen and Tos, 1996, Larsen and Tos, 2004) suggesting that a large proportion of patients with polyposis remain asymptomatic. The pathogenesis of nasal polyposis is far from clear, with suggestions of them being adenoma formations (Billroth, 1885), arising from inflammation (Bachert et al., 2000a, Jenkins, 1932), oedema (Krajina, 1963), epithelial rupture (Tos, 1990), or pseudocysts (Bachert et al., 2000b). A number of studies have proposed a role for angiogenesis (Caye-Thomasen et al., 2004, Ito et al., 1995b, Coste et al., 2000a) however none of these theories explain all of the histopathological findings and research is now moving toward molecular biology, gene array and proteomic array technologies (Figueiredo et al., 2007, Wang et al., 2006).

1.5.1 Allergy:

Chronic rhinosinusitis (CRS) can be subdivided according to the presence or absence of sinonasal polyps.(Fokkens et al., 2007) Mucosal inflammation in CRS without polyposis is usually neutrophil predominant, but when sinonasal polyposis is present is eosinophil predominant. This eosinophilic infiltrate, the fact that patients commonly complain of watery

rhinorrhoea with nasal obstruction, and have a degree of mucosal swelling has lead many groups to believe that allergy plays a significant role in polyp formation. However, there is no difference in the prevalence of allergy in patients with sinonasal polyposis compared to the general population, and there is no difference in prevalence of nasal polyps in patients with allergy.(Caplin et al., 1971b, Drakelee et al., 1984, Bunnag et al., 1983) Polyps are found in 0.5 to 1.5% of patients with positive skin prick tests for common allergens.(Settipane and Chafee, 1977, Caplin et al., 1971b) Moreover, specific treatment by allergen avoidance and/or antihistamines have no effect on polyp size(Haye et al., 1998) and conditions associated with a high prevalence of polyposis, such as the Samter triad group, are not based on IgE mediated allergy. The exception is allergic fungal rhinosinusitis. This condition is found in up to 8% of CRS patients requiring surgery(Bent and Kuhn, 1994) and polyposis occurs in almost all patients with this condition. Specific IgE to the fungal allergen can be demonstrated in sinus mucin in 71% of this patient group.(Collins et al., 2004)

1.5.2 Samter's triad:

Up to 96% of patients with asthma and aspirin (or non steroidal inflammatory drug (NSAID)) intolerance develop polyposis(Caplin et al., 1971b) demonstrating a very strong relationship. This relationship was described by Samter in 1967 (Samter and Beers, 1967) and represents the most aggressive form of the disease, however, not all asthmatics with NSAID sensitivity develop nasal polyps. It is not an IgE mediated allergy, but rather a pharmacological intolerance due to dysregulation of prostaglandin and leukotriene metabolism. Asthmatics

without an intolerance to aspirin have a prevalence of polyposis of up to 13%, which is greater than that of non-asthmatics.(Drakelee et al., 1984, Settipane, 1996)

1.5.3 Cystic fibrosis, primary ciliary dyskinesia, Young's syndrome:

37% of adults with cystic fibrosis (CF) have nasal polyps visible at nasal endoscopy.(Hadfield et al., 2000) Similarly deficient mucociliary clearance and recurrent bacterial infections result in sinonasal polyposis in 40% of patients with primary ciliary dyskinesia (PCD)(Pedersen and Mygind, 1982). PCD is classically manifested in Kartagener's syndrome, which is inherited in an autosomal recessive manner.(Afzelius, 1986) Young's syndrome encompasses a combination of bronchiectasis, rhinosinusitis +/- nasal polyposis and reduced fertility.(Handelsman et al., 1984) In all three conditions nasal polyps have a lymphocyte/neutrophil infiltrate as opposed to an eosinophilic infiltrate.

1.5.4 Heredity:

There seems to be increasing evidence for a genetic role. In a study of 224 patients with sinonasal polyposis 52% had a positive family history.(Rugina et al., 2002) Greisner et al.(Greisner and Settipane, 1996) studied 50 patients with polyposis and found 14% had a positive family history with between 1-3 immediate family members having sinonasal polyposis. This compared to a matched control group in which none had a family member with polyposis. As well as shared environmental factors, the known heredity of polyp associated conditions, such as asthma and allergy, make it difficult to establish whether the

findings are more than this.

1.5.5 Location:

The majority of sinonasal polyps arise from the osteomeatal complex. The reason for this is unknown but theories put forward include 'touching mucous membranes' resulting in the release of proinflammatory cytokines, special air currents including the Bernoulli principle, in which there is internal pressure reduction with increased air stream velocity, or it may be neurogenic as nerve endings at the osteomeatal complex are thin(Cauna et al., 1972) and more susceptible to cytotoxic damage.

1.5.6 Neurogenic:

Sensory, autonomic secretory and vasomotor nerves cannot be identified within the stroma of polyps.(Cauna et al., 1972) It is postulated that this denervation induces an abnormal vascular permeability leading to polyp formation.(Cauna et al., 1972)

1.5.7 Histology and inflammation:

At microscopy there is marked oedema of connective tissue with prominent lymphatic dilatation.(Michaels, 1987) Different types of epithelium have been found, most typically respiratory pseudostratified epithelium with ciliary cells and goblet cells, but low cubic or cylindrical, stratified squamous non-keratinized, and transitional epithelium can also be seen. The basement membrane is often thickened and the stroma is markedly oedematous with a myxomatous appearance and contains variable numbers of fibroblasts. An associated

inflammatory infiltrate is predominantly composed of eosinophils in 80% of European polyps.(Stoop et al., 1993) There are also increased numbers of mast cells and T lymphocytes with their humeral products, the cytokines.(Van Zele et al., 2006)

1.5.8 Biofilms:

CRS possesses all the hallmarks of biofilm mediated disease and there is good evidence of biofilm formation in CRS,(Harvey and Lund, 2007) however, little is known about its potential role in the pathogenesis of sinonasal diseases.

1.5.9 Superantigens:

Superantigens, predominantly derived from *Staphylococcus aureus*, are potent activators of T-cells, induce the synthesis of IgE in B-cells and have many direct affects on pro-inflammatory cells, such as eosinophils. IgE antibody to *S.aureus* enterotoxins have been described in polyp tissue, linked to both local IgE production and an aggravation of eosinophilic inflammation.(Bachert et al., 2003)

1.5.10 Nitric oxide:

Epithelial cells in CRS show a stronger expression for inducible nitric oxide synthase (iNOS) than controls, iNOS being upregulated in nasal epithelium.(Wang et al., 2004a) In a prospective randomised trial in patients with CRS who failed medical therapy with nasal corticosteroids, the rise in nasal NO seen on both medical and surgical treatments correlated with symptom score, saccharin clearance time, endoscopic changes and polyp size suggesting that nasal NO provides a non-invasive measure of the response for CRS to therapy.(Ragab et

al., 2006)

1.5.11 Summary:

In summary, sinonasal polyposis is a multifactorial disease process with local and general patient factors determining disease progression. The precise cause and mechanism of polyposis in the majority of patients remains unknown.

1.6 Treatment options

Treatment depends upon patient symptomatology, which includes nasal blockage, nasal discharge, facial pain or pressure and hypo- or anosmia. Loss of sense of smell with associated taste disturbance caused by polyp obstruction of the upper part of the nasal cavity is a common feature in many patients. Treatment can either be medical and/ or surgical. Medical treatment consists of intranasal and/ or systemic corticosteroids.

1.6.1 Intranasal corticosteroids:

Topical intranasal corticosteroid sprays have a documented positive effect on bilateral sinonasal polyposis and on their associated symptoms.(Tos et al., 1998) Nasal drops are more effective than nasal spray in symptom control and are more efficacious in improving the sense of smell.(Tos et al., 1998, Aukema et al., 2005) However, this is balanced with the slightly increased systemic absorption and potential side effects. Intranasal corticosteroids have been extensively used for 30 years without any serious adverse event.(Mygind and Lund, 2006)

1.6.2 Systemic corticosteroids:

Systemic corticosteroids should be used in patients with severe symptoms as a means of creating a medical polypectomy (Lildholdt et al., 1988) to create space for intranasal corticosteroid sprays or used when topical nasal sprays are ineffective. They are also increasingly being used in the perioperative period to reduce intraoperative bleeding and improve longer term surgical outcomes in patients with CRS with polyposis undergoing endoscopic sinus surgery.(Wright and Agrawal, 2007) A short oral course is indeed as effective as a simple polypectomy with a snare(Lildholdt et al., 1988) and the therapeutic effect outlasts the medication for a variable period. The beneficial effect on olfaction is also most pronounced with oral corticosteroids than nasal drops or topical nasal spray.

1.6.3 Novel medical treatments:

1.6.3.1 Capsaicin:

Capsaicin is the active substance in hot chilli peppers. It is a neurotoxin leading to long term damage of axons when repeatedly applied to the nasal mucosa. The neurogenic hypothesis in nasal polyp formation has lead to trials in capsaicin treatment for nasal polyposis that have shown potential benefit for this novel treatment.(Filiaci et al., 1996, Baudoin et al., 2000) The commonest side effect, if the nose is not topically anaesthetised, is a severe burning sensation.

1.6.3.2 Antileukotrienes:

Leukotrienes are upregulated in asthma and nasal polyposis especially in association with

aspirin sensitivity. Current evidence does not yet support the routine use of leukotriene antagonists for sinonasal polyposis.

1.6.3.3 Aspirin desensitization:

In CRS patients with sinonasal polyposis and aspirin intolerance, systemic aspirin desensitisation or topical lysine-aspirin treatment may protect against polyp recurrence.(Stevenson et al., 1996, Mardiney and Borish, 2001) Aspirin desensitisation by graded oral doses can be effective, but must only be carried out by a specialist under controlled supervised conditions.

1.6.4 Surgery

Due to the inflammatory nature of mucous membranes in sinonasal polyposis, surgery cannot be expected to cure the disease. In most patients sinonasal surgery is reserved for those who do not satisfactorily respond to medical treatment. The aim is to reduce the amount of inflammatory tissue, open up the nasal airway and improve ventilation of the paranasal sinuses. Although a simple intranasal polypectomy was performed repeatedly before, most patients should be referred to a rhinologist for endoscopic sinus surgery, preceded by a computed tomography (CT) scan. Endoscopic sinus surgery is well established and there are many techniques (Fokkens et al., 2007). However, the risks of minor and major complications exist and this has to be balanced with the expected result of operative verses conservative treatment. If surgery is chosen then the extent of surgery is up to the individual surgeon and

will also be determined by the extent of sinus disease as well as patient symptoms. Surgery gives the greatest improvement in relieving nasal obstruction and the least improvement in sense of smell. Surgery can range from simple intranasal polypectomy to nasalisation with removal of the middle turbinate and large anrostomies in all paranasal sinuses, including a median drainage procedure for the frontal sinuses. There is as yet no convincing evidence that more radical surgery is more beneficial.

1.7 Hypothesis and Aims

Nasal polyposis is a common disease, whose aetiology is currently unknown. If further understanding of the driving force of nasal polyposis can be made, this opens the possibility of new treatment modalities never considered before for this clinically important disease process. Despite the prevailing opinion at the time of starting this research, we concluded that the evidence for polyposis being an angiogenesis-dependent pathology was poor. We therefore tested the null hypothesis, that the formation of nasal polyps is independent of angiogenesis (chapter 6). Subsequently, we attempted to derive evidence for the underlying stimuli with particular reference to vasoactive elements such as NO (chapters 7, 8).

CHAPTER 2
GENERAL METHODS

2 General Methods

2.1 Human tissue

All human tissue was obtained from patients undergoing routine surgery at University Hospitals Birmingham NHS Trust (numbers 0692 and 06/Q2702/15), following informed consent (appendix II and IV) under either local or general anaesthesia. To date, no adverse effects from any biopsy has been detected or reported.

2.2 Muscle Samples

Local ethical committee approval was granted for the work and all patients gave informed consent for the biopsy to be taken.

18 muscle biopsy samples were analysed, from 17 different subjects.

4 samples demonstrated fibre hypertrophy in male quadriceps muscle and 2 samples demonstrated fibre atrophy in male quadriceps muscle. These were obtained by needle biopsy in a Middlesex hospital to determine the cause of each patients muscle weakness. The remaining 12 samples were obtained by open biopsy at either the Queen Elizabeth or Selly Oak hospitals in Birmingham. All were carried out by the same surgeon (Mr Adrian Drake-Lee) who took biopsies from the same part of each muscle, thus reducing variability. 6 samples were of the Medial Gastrocnemius muscle from limb ischaemia patients taken during a femoral-distal artery bypass. These were compared to age matched controls of the same muscle from varicose veins operation patients. 4 final samples of normal muscle were taken from varicose veins patients to compare fibre type distributions in the following normal muscles: Sartorius, Medial Adductor Longus and Medial Gastrocnemius.

All 12 open biopsies were obtained under the local anaesthetic Prilocaine. Potential muscle samples were sought on the basis of the types of operations at the various hospitals. However, this was found to be very time consuming due to the serendipitous nature of the operating lists. After removal the samples were immediately mounted in an embedding medium (Tissue-tek, O.C.T. compound) with fibres orientated vertically, using fine tipped forceps, on a labelled cork disk. A fine pin was also pushed into the cork to help support the vertical fibres and to enable more even freezing, the pin acting as a thermal conduction shaft through the tissue. The mounted muscle block was then snap frozen in isopentane cooled in liquid nitrogen. Thus a flask of liquid nitrogen and other apparatus were taken into theatre for every sample taken, reducing the loss of labile enzymes that occurs if the tissue is not frozen soon after removal (Loughlin, 1993). The samples were transported from the hospital to be stored at -70°C until analysis. Serial transverse sections, 12 μm thick, were cut from the muscle blocks at -20°C using a Bright 5020 cryostat machine and mounted on slides for staining.

2.3 Muscle fibre types

The fibre types were identified by using differential sensitivity of the myofibrillar ATPase to inactivation after preincubation in acidic and alkali solutions. The enzyme ATPase is responsible for catalysing the breakdown of ATP to ADP and inorganic phosphate. Fast myosins, as in type II fibres, are inactivated at acid pH whereas slow myosins, as in type I fibres, are inactivated at alkaline pH. Having preincubated the section and inactivated one set of fibres the myosin ATPase activity can be visualised in the other set. The histochemical reaction depends upon a complex series of reactions for the production of its end product (see Loughlin, 1993).

Serial sections were also stained for fibres using succinate dehydrogenase (SDH), a mitochondrial enzyme, thereby ensuring unambiguous identification of fibres.

2.4 Myosin ATPase Stain - Method (adapted from Brooke and Kaiser, 1970)

1. fgb Air dry unfixed sections for 30 minutes.
2. Pre-incubate sections at pH 4.35, 4.60 and 10.2 at room temperature for 8 minutes.
3. Rinse in distilled water once.
4. Rinse in glycine/ calcium buffer once.
5. Incubate sections at pH 9.6 for 25 minutes at room temperature.
6. Rinse in distilled water 3 times.
7. Rinse in 3 changes of 2% CaCl_2 .
8. incubate in 1% CoCl_2 for 3 minutes.
9. Rinse in distilled water 5 times.
10. Incubate in 1% Ammonium sulphide for 2 minutes.
11. Rinse in distilled water.
12. Mount in Aquamount.

Solutions - for coplin jar (30ml, 6 - 8 slides)

2% CaCl_2 solution.

1% CoCl_2 solution.

1% Ammonium sulphide solution (in fume cupboard).

Glycine/ CaCl_2 solution.

3.0g glycine plus 2.94g Cl_2 /l

200ml

Acid Pre-incubation solutions

Sodium acetate, 3.282g/ 200ml 60ml

Adjust to pH 4.35 and 4.60 with Acetic acid (1:1 dil. with water).

Alkali

Boric acid, 3.09g/ l 30ml

Adjust to pH 10.2 with 1molar KOH

Incubation solution

Take 50ml Glycine/ Ca Cl₂ solution and adjust pH to 9.60 with 0.1M KOH

ATP (disodium salt). To be measured out just before staining. 45mg

Add this to 50ml of the glycine/ Cl₂ solution and readjust the pH to 9.6

2.5 Lectin staining

Working Solution -For 1ml (covering 10 slides)

10ul stock +990ul P.B.S. (stock solution =2mg UEA-1 / ml)

Vectastain ABC Kit

Refer to kit's instructions

DAB substrate Kit

Refer to kit's instructions.

Lectins are plant proteins that bind to specific sugar residues. The lectin used here binds to galactose residues, which are found at a high concentration on the proteoglycans in the glycocalyx which lines blood vessels. The sarcolemma of muscle fibres is also faintly stained, allowing quantification of fibres.

2.5.1.1 Method

Air dry sections (30 min).

Fix sections in pre-cooled acetone (4°C or less) for 5 sec.

Apply rhodamine-conjugated *Griffonia simplicifolia* lectin – 1 (Vector Labs) or other lectin eg *Ulex europaeus* agglutinin 1 (UEA-1) at a concentration of 20µg/ml (1:100 - 5µl lectin in 500µl PBS) for 30 min at RT. Keep sections out of direct light.

Rinse in PBS: 3 x 5 min washes.

Rinse in distilled water (dH₂O).

2.6 Counting capillaries

Place two to four sampling squares randomly on the slide and count the number of capillaries, fibres and larger vessels inside these squares. The top and left sides of the square are designated ‘inclusion lines’ with the bottom and right sides designated ‘exclusion lines’. Any capillary or fibre touching the exclusion lines is not counted, and capillaries or fibres exclusively touching the inclusion lines are counted along with those contained within the square (Gundersen, 1977, Egginton and Turek, 1990).

Fibre boundaries can be visualised by the lectin slightly staining the sarcolemma of muscle fibres, which may be digitally enhanced on the computer to allow easy visualisation of fibres.

Capillaries are defined as being stained objects of 12µm in diameter or less.

A graticule is used to measure the sample area, and capillary density (CD, mm⁻²) and fibre size (a(f), µm²) can be calculated from this. The capillary to fibre ratio (C:F) can then also be calculated.

2.7 Immunohistochemical Staining

Samples taken for confocal analysis were immediately fixed in 4% buffered formalin and left for 24 hours. Samples were then washed in 3 changes of phosphate buffered saline (PBS) for a further 24 hours before being stained whole with dilutions of either fluorescein-conjugated *Ulex europeaus* lectin (Vector Labs), or a fluorescein-conjugated lectin mixture (1:1:1 *Ulex europeaus*, *Griffonia simplicifolia*, *Euonymus europeaus*, Vector Labs) to account for different affinities associated with blood groups (Capaldi et al., 1985). Samples were then washed and stored in PBS until being viewed under a confocal Leica DM IRE2 microscope. A total of 60 samples were stained with the single lectin protocol, and 50 different samples stained with the triple lectin protocol.

Samples taken for immunostaining were snap frozen in liquid nitrogen-cooled isopentane immediately in theatre, and 8 µm sections were subsequently cut on a cryostat. These were allowed to air dry before being fixed in 4% buffered formalin (for 1, 5, 10, 20 or 60 minutes) or ice-cold acetone (for 10, 30, 60 or 300 seconds). Antigen retrieval was performed in citrate buffer (10 mM citric acid adjusted to pH 6.0 with NaOH, 0.05% Tween 20) heated to 95°C for 5 min then allowed to cool to room temperature. Slides were then blocked for 30 min at room temperature in wash buffer (either 5% foetal bovine serum (FBS) or 1.5% w/v BSA, 0.6% v/v Triton X-100 in PBS, or 5% FBS in PBS), then incubated for 2 hours with primary antibodies (Ki-67, Dako/PCNA, Santa Cruz) diluted in wash buffer to label proliferating nuclei. Sections were then washed in PBS and incubated for 1 hour with secondary antibody (TRITC polyclonal rabbit anti-mouse, Dako) diluted in wash buffer. Sections were rinsed in PBS and then incubated for 30 min with lectin to stain blood vessels. Sections were washed in PBS,

rinsed with dH₂O and mounted using Vectashield containing DAPI (4',6-diamidino-2-phenylindole) to label all nuclei, and enable calculation of a proliferation index (Ki-67 count / DAPI count). All counts were made on either the same section, or on a serial section from the same sample.

2.8 PCNA/Capillary Dual Staining

PCNA (proliferating cell nuclear antigen), also called cyclin, is a DNA polymerase co-factor expressed by cells undergoing mitosis, and so can act as a measure of cell proliferation. Co-localisation of PCNA with capillaries can give a measure of cell proliferation associated with the capillaries, and that associated with interstitial cells. This can be performed using fluorescent filters that excite and observe at different wavelengths, allowing two different fluorophores to be observed separately on the same section.

Solutions

4% buffered formalin

100ml 37% formaldehyde

900ml distilled water (dH₂O)

Washing buffer

1.5% bovine serum albumin, 0.6% Triton X-100

500µl 6% Triton X-100 stock

666µl 7.5% BSA stock

3.83ml PBS

2.8.1.1 Method

Air dry cryostat sections (30 min).

Fix in 4% buffered formalin for 5 min.

Wash in PBS for 5 min.

Block with washing buffer at RT for 30 min.

Incubate with primary antibody (rabbit anti-PCNA antibody, Santa Cruz) 1:100 for 1hr at RT (5µl antibody in 500µl washing buffer).

Rinse in PBS: 3 x 7 min washes.

Incubate with secondary antibody (CY2 conjugated donkey anti-rabbit, Jackson) 1:50, and 1:100 rhodamine conjugated Ulex europeaus lectin (vector) for 1 hour at RT (10µl antibody, 5µl lectin in 500µl washing buffer). Keep sections covered and out of direct light.

Rinse in PBS: 3 x 7 min washes.

Rinse in distilled water.

Mount with glycerol if required.

2.9 Triple stain – Ki67/ Lectin/ DAPI

Solutions:

Citrate buffer

2.94g Tri-sodium citrate (dihydrate)

Dissolve in 1L dH₂O, adjust to pH 6.0 with HCl, then add 0.5ml Tween 20

or

1.92g Citric acid (anhydrous)

Dissolve in 1L dH₂O, adjust to pH 6.0 with NaOH, then add 0.5ml Tween 20

Wash buffer

500µl Normal rabbit serum

500µl of 6% v/v Triton X-100 stock

4ml PBS

2.9.1.1 Method

Air dry slides for 30 min at RT.

Use wax pen now if slides are poly-lysine coated, otherwise wait until after heat treatment.

Use at least two concentric ellipses round the sections.

Fix slides in 4% formalin solution (1 in 10 dilution of 37% formalin in PBS) for 20 min

Wash 3 x 2 min in PBS.

Heat citrate buffer to 90-95°C on heater (about 10 min at heat 10, then drop to 6.5) in a glass beaker (NOT slide jar, they break!), then add slides for 20 min (drop heat to about 3). After 20 min, remove beaker from heat and allow to stand for about 30-40 min until cooled to under 35°C.

Alternatively, put slides in plastic coplin jar, add buffer and microwave for 5 min at power 20, followed by 2 min at power 10.

Remove slides from citrate buffer, rinse in PBS (add PBS to coplin jar, remove immediately), then wash for 5 min.

If using non-poly-lysine coated slides, wipe slides clean round the edges and reverse side, tilt slides and blot the bottom of the drop with a torn edge of a tissue (facilitating capillary action up the tissue) to allow slides to dry (wait about 30 min). Once sections are dry, apply wax pen or crayon round the sections.

Block for 30 min by applying wash buffer. Tip excess solution away, then blot the slides as previously described to minimise the amount of buffer on the slide.

Incubate with primary antibody (Clone Ki67, Dako), diluted 1:100 in wash buffer (5µL antibody in 500µL wash buffer) for 2 hours at RT, (overnight in the fridge should work just as well, if not better).

Wash 3 x 5 min in PBS, then wipe and blot slides.

Incubate with secondary antibody (Polyclonal rabbit anti-mouse TRITC, Dako), diluted 1:100 in wash buffer for 1 hr at RT.

Rinse 3 times in PBS, then wipe and blot slides.

Add lectin (normally *Ulex europeaus* lectin-1, fluorescein) diluted 1:250 in PBS (2µL lectin in 500µL PBS).

Wash 3 x 5 min in PBS.

Rinse in dH₂O and allow slides to dry (wiping and blotting the sections as previously described).

Once slides are dry, coverslip with diluted DAPI solution (in fridge, made from 1ml Vectashield with DAPI with 2ml Vectashield).

Slides are now ready for viewing. If saving for future analysis, cover from light and leave in

the fridge (overnight, or one or two days), or seal with clear nail varnish, then cover and refrigerate (3-10 days).

2.10 Succinate Dehydrogenase Staining

Succinate dehydrogenase is a mitochondrial enzyme that catalyses the conversion of succinate to fumarate, reducing NAD^+ to NADH in the process. When used for staining, sodium metaborate is used as the electron acceptor, and the reduced form acts on nitro-blue tetrazolium to form a blue precipitate in areas with a high oxidative capacity. In skeletal muscle, this can therefore be used to differentiate between muscle fibre types

Solutions

Stock buffer

0.29g potassium dihydrogen orthophosphate (KH_2PO_4)

2.19g di-sodium hydrogen orthophosphate (Na_2HPO_4)

4.50g sodium succinate

Up to 1L dH_2O

Store at 4°C

Sodium metaborate solution

6.89g sodium metaborate (NaBO_2)

Up to 1L dH_2O

Buffered sucrose formol

30g sucrose

50ml 37% formaldehyde

5g calcium nitrate [Ca(NO₃)₂]

Adjust to pH 7.2 with sodium metaborate solution

Store at 4°C

Working buffer

50ml stock buffer solution

25mg NBT (nitro blue tetrazolium)

Store at 4°C. Mix well and bring to RT before use

2.10.1.1 Method

Air dry fresh cryostat sections at RT for 30 min

Incubate slides in working buffer for 30 min at about 37°C

Rinse in distilled water

Post-fix in sucrose-formol for 2 min

Rinse in 3 changes of distilled water

Mount with aquamount and cover slip

2.11 Power calculations

Based on the control values,

Expect a 50% change from control, no change seen

30% (conservative) change in Vv requires n=1

15% change in Vv requires a sample size of 3 for 80% power (sigma = 0.05)

Expect a 50% change from control, no change seen

30% change in Sv requires n=1 for 80% power

15% change requires n=3

Expect a 30% change from control, no change seen

30% change in cap density requires n=2

15% change requires n=6

As we mentioned, capillary density is dependent on interstitial volume

Expect a 2-400% change from control, compared to 850% change seen in the interstitium of polyps

100% increase in proliferation requires n=2

50% increase in proliferation requires n=7

All these calculations assume a normal distribution from the mean, which may not necessarily be the case with increases in Vv, Sv, or capillary density (these often increase in a single-tailed manner as is common in closed biological systems), but these errors should be minute compared to the error in estimating calculations.

2.12 Sensiscript Reverse Transcription

Solutions

Master mix:

2.0µL	10x Buffer RT (Qiagen)
2.0µL	dNTP mix (Qiagen)
1.0µL	Primer (oligo dT 50µM or random hexamers)
1.0µL	RNase inhibitor (10 units/µl)

1.0 μ L Sensiscript reverse transcriptase (Qiagen)

ddH₂O up to 20 μ L with RNA template

2.12.1.1 Methods

Thaw solutions on ice.

Prepare a fresh master mix on ice. Mix by briefly vortexing solution, then centrifuge briefly to collect residual liquid.

Distribute master mix into reaction tubes on ice.

Add template RNA to the master mix. Mix thoroughly.

Incubate for 60 min at 37°C

2.13 Gene expression in nasal polyps

2.13.1 High Capacity cDNA extraction

Solutions

2x Master mix:

2.0 μ L 10x RT Buffer (Applied Biosystems)

0.8 μ L 25x dNTP mix (100mM, Applied Biosystems)

2.0 μ L 10x RT random hexamers or oligo dT

1.0 μ L Multiscribe reverse transcriptase (Applied Biosystems)

1.0 μ L RNase inhibitor (10 units/ μ l) (Qiagen)

3.2 μ L nuclease free H₂O (Sigma)

2.13.1.1 Method

Thaw solutions on ice

Prepare a fresh master mix on ice. Mix by briefly vortexing solution, then centrifuge briefly to collect residual liquid.

Distribute master mix into reaction tubes on ice.

Add template RNA to the master mix. Mix thoroughly.

Incubate for 60 min at 37°C.

2.14 Northern Blot

Everything must be Thoroughly cleaned of RNAses, and gloves must be worn at all times.

Use RNase-ZAP or some other commercially available RNase cleaner to clean the work area and all equipment that may come into contact, directly or indirectly, with the RNA.

Clean everything with 95 % ethanol. This will also help any residual RNase-ZAP evaporate.

Solutions

10x TBE Buffer pH8.3

54g	Tris base
27.5g	Boric acid
4.65g	Sodium EDTA (NaEDTA.2H ₂ O)
ddH ₂ O	up to 500ml

6x Loading buffer

10μM	Tris/HCl (pH7.5)
------	------------------

50 μ M	EDTA
10%	Tricoll 400
0.25%	Xylene cyanol
0.25%	Bromophenol Blue
150 μ g/ml	Ethidium Bromide

2.14.1.1 Method

Assemble the tray, combs and clamp according to the manufacturers' instructions.

Dissolve 1.5g of agarose in 100ml TBE buffer (for 300-500bp, 1g for longer, 2g for smaller), and pour gel into the tray to a depth of 5mm. Remove any bubbles with a Pasteur pipette.

Allow the gel to set for 1hr before placing the gel in the gel holder and removing the gel combs. Cover with 2mm of TBE buffer.

Prepare the samples with 2 μ l of loading buffer with 10 μ l of sample, and load the samples carefully into the wells, with a 100bp DNA ladder (100bp molecular ruler, BioRad).

Attach the cover and run the gel at 75V until the dye front nearly reaches the bottom of the gel.

Remove the gel into a tray filled with ddH₂O, and photograph gel with excitation at 254nm (Gel Logic, Kodak) and Photofinish software.

2.15 Western Blotting Protocol

Solutions

0.5M TrisCl/SDS (pH 6.8):

Dissolve 6.05g Trizma base (Sigma) in 40ml ddH₂O. Adjust pH to 6.8 with 1M HCl, then add

ddH₂O to 1000ml. Filter through a 0.45µm filter, then add 0.4g SDS. Store at 4°C.

1.5M TrisCl/SDS (pH 8.8):

Dissolve 18.17g Trizma base (Sigma) in 40 ml ddH₂O. Adjust pH to 8.8 with 1M HCl, then add ddH₂O to 1000ml. Filter through a 0.45µm filter, then add 0.4g SDS. Store at 4°C.

4x SDS sample buffer

0.5ml 0.5M TrisCl/SDS pH 6.8
0.4ml 100% glycerol
0.8ml 10%(w/v) SDS (in ddH₂O)
0.2ml bromophenol blue
1.9ml ddH₂O
0.2ml β-mercaptoethanol

10x SDS electrophoresis buffer

30g tris base
144g glycine
10g SDS
ddH₂O up to 1000ml

Resolving Gel

7.5% / 10% / 12% acrylamide

6.25ml/ 8.25ml/12.5ml 30% acrylamide/bis (Biorad)

12.1ml/ 10.1ml/ 6.0ml ddH₂O

6.25ml	1.5M TrisCl/SDS pH 8.8
0.25ml	10% SDS
12.5µl	TEMED (N,N,N',N'-Tetramethyl-1-,2 diaminomethane)
125µl	APS (Ammonium persulphate)

Stacking Gel

0.55ml	30% acrylamide/bis
1.04ml	0.5M TrisCL/SDS pH 6.8
50µl	10% SDS
2.5ml	ddH ₂ O
4µl	TEMED
21µl	APS

Blotting Transfer Buffer (pH 8.3)

3.03g	Tris base	(25mM)
14.4g	Glycine	(192mM)
150ml	Methanol	(15%)
ddH ₂ O to 1000ml		

Do not adjust the pH. If pH is incorrect, prepare a new solution. Store at 4°C.

Tris-buffered saline Tween (TTBS) pH 7.6

2.42g	Tris base	(20mM)
8g	Sodium Chloride	(137mM)

Dissolve in 40ml ddH₂O. Adjust pH to 6.8 with 1M HCl, then add ddH₂O to 1000ml.

Add 1ml Tween-20 (Sigma). Store at 4°C.

Stripping buffer

3.8ml β -mercaptoethanol	(100mM)
Tris base adjusted to pH 6.7 with 1M HCl	(62.5mM)
10g SDS	(2%)
ddH ₂ O to 500ml	

2.15.1.1 Method**Gel Preparation**

Assemble glass plates according to the manufacturers instructions (MiniProtean3, Biorad).

Place the comb in the plates, and mark a level approximately 1cm below the bottom of the comb. Fill to this mark with the resolving gel solution. When preparing the gels, mix all the reagents except for the APS and TEMED, then allow the solution to de-gas for a few minutes before adding the final reagents and gently swirling to mix.

Immediately overlay the solution with about 200 μ l of a 1:1 mixture of butanol/ddH₂O to avoid evaporation and obtain a good border between the two gels.

Leave the gel to polymerise for about 1hr.

Drain the water/butanol mixture from the plates, and pour the stacking gel solution on top of the solid resolving gel. Insert the comb, taking care to avoid air bubbles, and ensure that the gel level reaches the top of the comb teeth.

Leave the gel to polymerise for about 45 min before carefully removing the gel and assembling in the electrophoresis cell as per the manufacturer's instructions. Fill the cell with 1x SDS electrophoresis buffer, and fill the outside of the cell to a level where both the

electrodes are completely submerged.

Sample preparation

Dilute 50µg of sample (in a volume of 12µl) 4:1 with sample buffer and heat at 95°C for 5 min, then immediately place on ice for 5 min and briefly spin down.

Load the samples into the wells created by the comb, then run the gel at a constant current of 40mA for about 1hr, taking care to check the progress of the electrophoresis.

Protein blotting

Dismantle the gels from the gel holder and cut away the resolving gel with a clean scalpel blade and soaking the resolving gel in transfer buffer for 5 min.

Cut a piece of Hybond-ECL polyvinyl difluoride (PVDF, Amersham) membrane to the size of the gel, wet fully with methanol, and soak for a few minutes in the transfer buffer.

Place the gel on top of a piece of filter paper soaked in the transfer buffer, place the gel on the membrane and cover with another soaked filter paper. Remove air bubbles by rolling a glass pipette over the paper. Sandwich the filter papers between two fibre pads saturated in the transfer buffer, and place these in the blotting cassettes, loading the cassette so that the membrane is on the red (positive/anode) side of the blotting holder relative to the gel.

Fill the apparatus with 1x blotting transfer buffer (pre-cooled in the fridge), and the proteins transferred to the membrane at 30V overnight or 80V for 3 hours, keeping the apparatus at 4°C or less by immersing in ice-water.

Once the transfer is complete, remove the membrane from the cassette and rinse in TTBS.

Stain with Ponceau S as per the manufacturer's method.

2.16 Protein Measurement

Construct a set of dilutions using 1.37mg ml⁻¹ bovine serum albumin (Biorad), using a 25µl sample volume with 0, 5, 10, 15, 20 and 25µl of the standard diluted in ddH₂O.

Dilute the samples to roughly 1 mg ml⁻¹ based on the amount of sample placed in the volume of extraction buffer, and ignoring the loss of material in the pellet. Assay 25µl of these diluted samples.

Using a detergent compatible protein assay kit (Biorad, based on the Lowry assay (Lowry et al. 1951)), add 1ml of reagent B (Folin phenol reagent) to each sample, followed by 125µl reagent A (alkaline copper tartrate), to which 10µl of reagent S (a surfactant) had been added per 1ml reagent A.

Transfer the samples to cuvettes and leave to incubate for 15 min before assaying the absorbance at 650nm. Take two absorbance readings for each sample, running through the samples in order, then in reverse order.

2.16.1 Detergent Protein Extraction

Solutions

Detergent solution:

50µl 20% NP-40 stock (Sigma)

50µl 10% sodium deoxycholate (NaDOC, Sigma) stock

5µl 20% sodium dodecyl sulphate (SDS, Sigma) stock

2.16.1.1 Method

Cool mortar and pestle in dry ice with Eppendorf tubes.

Cool PBS and proteinase inhibitor cocktail (PIC, Sigma) on ice.

Crush 25mg muscle in the mortar, transfer to an Eppendorf and homogenise in 137.5µl 1:1 PIC in PBS using a hand homogeniser.

Add 15.75µl detergent solution to give final concentrations of 1% NP-40, 0.5% NaDOC, 0.1% SDS.

Re-homogenise, then leave on ice for 20 min.

Centrifuge at 12,000g at 4°C for 20 min to pellet.

Remove supernatant, assay for protein levels, freeze in liquid nitrogen and store at -80°C.

2.16.2 Cryosectioning

Take excised biopsies and place on a cork disc. Coat the muscle with OCT Tissue Tek compound (Leica Instruments) for structural support and cryoprotection.

Orientate the muscle perpendicular to the cork by leaning the muscle against a pin, then carefully drop the disc into liquid nitrogen-cooled isopentane. The isopentane should be at its freezing point, which can be ascertained through an increase in the viscosity and clouding whilst stirring.

Place discs in the liquid nitrogen for short-term storage before sectioning. Long-term storage is at -80°C.

Cut 10µm sections on a cryostat at -20 ± 3°C, depending on the rigidity of the sections obtained, which varies from patient to patient. Pick up slides on poly-L-lysine coated slides for better adhesion on subsequent immunohistochemistry. Allow sections to air-dry for 30-60min before staining or re-freezing and storage at -20°C.

2.16.3 Endpoint cDNA method:

End-point primer amplification

Solutions

Reaction mix:

12.5µL Taqman Universal PCR Master Mix (Applied Biosystems)

2.5µL TGF-β forward primer (Binding Site)

2.5µL TGF-β backwards primer (Binding Site)

1µL cDNA sample

6.5µL ddH₂O

Methods

Thaw solutions on ice

Make up the reaction mix, then transfer to thermal cycler.

Run thermal cycler using the following program:

Step 1: 95°C 10 min AmpliTaq Gold Amplification (Applied Biosystems)

Step 2: 95°C 15 sec Denature

Step 3: 60°C 1 min Extend

Step 4: Repeat steps 2 and 3 for 40 cycles

2.16.4 TRIZOL method

2.16.4.1 Equipment treatment

To reduce the effect of environmental RNAases on the samples, all plasticware that came into contact with the samples or solutions use either RNAase free disposable plasticware, or soak in 0.5 M NaOH for 1 hour, then rinse with reverse-osmosis filtered water and autoclave. Bake glass homogenisers at 150°C for 4 hours. Clean disposable gloves and forceps with RNAZap (Ambion).

2.16.4.2 Sample treatment

Pre-cool glass homogenisers and TRIZOL reagent (Life Sciences) on ice. Pre-weigh tissue samples and homogenise in 1ml of ice-cold TRIZOL, transfer to a labelled eppendorf tube, then incubate for 10 min at RT. Add 0.2ml of chloroform to each tube, then shake the tubes vigorously by hand for 15 sec, and incubate at RT for 3 min. Centrifuge tubes at 12,000g for 15 min at 4°C. This gives a colourless aqueous phase containing RNA, an interphase and a lower red, phenol-chloroform phase.

2.16.4.3 RNA extraction

Remove the aqueous phase carefully and transfer to a fresh labelled eppendorf, keeping phenolic phase at -20°C for subsequent DNA and protein extraction. Precipitate RNA by

addition of 0.5ml isopropyl alcohol and incubate for 10 min at RT. Centrifuge samples at 12,000g for 10 min at 4°C to pellet the RNA. Discard the supernatant, and wash the pellet with 1 ml of 75% ethanol in RNAase free water. Vortex the pellet off the bottom of the eppendorf, then centrifuge at 7,500g for 5 min at 4°C. Store sample at -80°C until used. Before use, discard the supernatant, and allow the RNA pellet to air-dry for 15 min before resuspending in 25µl of RNAase free water.

2.16.4.4 DNA extraction

Remove the remaining traces of aqueous phase from the phenolic phase and discard, then add 0.3ml of 100% ethanol to precipitate the DNA. Invert samples several times to mix, and incubate for 3 min at RT. Sediment DNA by centrifugation at 2,000g for 5 min at 4°C. Split the phenolic supernatant equally into three labelled eppendorfs for protein extraction.

2.16.4.5 Protein extraction

Add 1 ml of acetone to each eppendorf containing approximately 250 µl phenolic supernatant. Incubate samples for 10 min at RT, then sediment protein by centrifuging at 12,000g for 10 min at 4°C. Remove the supernatant and wash each protein pellet three times in 800 µl of 0.3M guanidine hydrochloride in 95% ethanol. During each wash, store the protein pellet in the wash solution for 20 min at RT and centrifuge at 7,500g for 5 min at 4°C. After the final wash, vortex the protein pellets in 700µl ethanol. Store in ethanol at -20°C until use. To use, centrifuge pellet at 7,500g for 5 min at 4°C, remove supernatant and air dry the pellet for 15-20 min. Dissolve in 9.5M Urea/2% CHAPS and measure concentration.

2.16.4.6 Gene array

Gene arrays consist of DNA fragments spotted onto a substrate. When a cDNA sample is added to the array, complimentary sample strands will specifically bind to the immobilised ligand. Non-binding strands will be washed away, and non-specific fragments on the array will remain unbound. If the cDNA sample is labelled with a reporter, such as Cy3 in this case, the presence of the reporter will give the position and intensity of bound cDNA fragments. As the efficiency of binding will vary between sequences (both due to A-T/C-G binding differences and shape), and differences in spotting efficiency between arrays, a reference sample containing cDNA for every DNA sequence on the array is added. This reference uses a Cy5 reporter dye, and allows differences to be controlled for by measuring the Cy3 signal against the Cy5 signal. The reference sample was constructed from RNA extracted from the buffy coat of healthy donors (non-red cell fraction in centrifuged blood), Epstein-Barr virus transformed B lymphocytes, human fibroblasts, and peripheral blood mononuclear cells. This combination of cell types gave >95% positive signals on the array used, which was a customised array (Corning) with 850 genes made from 70-mer oligonucleotides (Qiagen).

Protocol for Microarrays (No Amplification).

1. Sample Collection.
2. Ambion RNA Extraction.
3. Dnase I Treatment.

4. RT-PCR.
5. Dye Labelling.
6. Probe Quantitation.
7. Probe Hybridisation.
8. Post-Hybridisation Washes.
9. Scanning.

Step 1 – Sample Collection.

- Sample cells taken.
- Cells spun down 1200 rpm 8 mins.
- Pellet resuspended in 200µl lysis buffer.
- Sample frozen at -70°C and transported in dry ice.

(Samples can be stored at -70°C for 3 months)

Step 2 - Ambion RNA Extraction.

- Sample defrosted at room temperature.
- 200µl 64% ethanol added and mixed in by pipetting.
- Mix applied to filter cartridge.
- Spun for 1 min at 13000 rpm.
- Filter washed with 700µl wash solution 1.
- Spun for 1 min at 13000 rpm.
- Filter washed with 500µl wash solution 2/3.
- Spun for 15 secs at 13000 rpm.
- Filter washed with 500µl wash solution 2/3.
- Spun for 2 mins at 13000 rpm.
- Filter cartridges transferred to clean microfuge tubes.
- 35µl elution solution added to each filter cartridge.
- Filters incubated at 70⁰C (on heat-block) for 5 mins.
- Spun for 1 min at 13000 rpm.
- RNA used in Dnase I treatment.

Step 3 – Dnase I Treatment.

- 3.5µl 10x Dnase I buffer added to RNA samples.
- 1µl Dnase I added.
- Samples kept at 37⁰C for 30 mins.
- 5µl Dnase inactivation reagent added to each RNA sample.
- Samples kept at room temperature for 2 mins.
- Spun for 1 min at 13000 rpm.

The RNA is then quantitated by use of a spectrophotometer.

Step 4 – RT-PCR.

At this point it is important that the same quantity of RNA from each sample is used in the labelling reaction. It is hence best to measure the sample with the lowest concentration of RNA, and then use a quantity of RNA from every other sample to match this. The lowest RNA concentration will probably be attained from the –ve control samples, as there should only be “housekeeping” quantities of RNA produced in these.

- Make following reaction mix:

5µg RNA

1µl oligo(dT)₁₂₋₁₈ primer (1µg/µl)

Up to 17ul H₂O

- Heat to 65⁰C for 10mins.
- Cool on ice.
- Add the following to each tube -

8µl 5x first-strand buffer

4µl 0.1M DTT

2µl dATP (10mM)

2µl dCTP (10mM)

2µl dGTP (10mM)

2µl dTTP (10mM)

2µl Superscript II (200U/µl)

1µl H₂O

- Keep at 42⁰C for 2 hours.
- Add 200µl PB buffer to sample.
- Add sample to Qiagen PCR purification column.
- Spin for 1 mins.

- Wash filter twice through with 700µl PE buffer.
- Spin for 1 min extra.
- Place filter into clean tube.
- Add 50µl H₂O to column.
- Spin for 1 min.

Step 5 – Dye Labelling.

- Make following reaction mix:

500ng cDNA

20µl Random primer mix

Up to 43ul H₂O

- Heat to 94⁰C for 5mins.
- Cool on ice.
- Add the following to each tube -

1.2µl dATP (10mM)

1.2µl dTTP (10mM)

1.2µl dGTP (10mM)

0.72µl dCTP (10mM)

1µl Klenow Enzyme (40U/µl)

0.68µl H₂O

1µl Cy3 or Cy5

- Keep at 37⁰C for 2 hours.
- Add 250µl buffer PB to sample.
- Add probe to Qiagen PCR purification column.
- Spin for 1 mins.
- Wash filter twice through with 700µl buffer PE.
- Spin column for extra minute.
- Place filter into clean tube.
- Add 50µl H₂O to column.
- Spin for 1 min.

Step 6 – Probe Quantitation.

- Measure probe concentration by taking measurements at 550nm and 650nm.

Cy3 (550nm) = (Absorbance Measurement x Dilution Factor)/ 0.15

Cy5 (650nm) = (Absorbance Measurement x Dilution Factor)/ 0.25

- Use 40 pmole of each dye.
- Add required volume of each probe to microcon column.
- Spin for 10 mins.
- Invert column into fresh tube.
- Add 30µl H₂O.
- Spin for 1 min.

Step 7 – Probe Hybridisation.

- Pre-hybridise slides in pre-hybridisation solution for 2 hours at 42⁰C.

Pre-hybridisation solution.

12.5ml formamide

12.5ml 20 x SSC

0.25ml 20% SDS

50µl 100mg/µl Fraction V BSA

Up to 50ml H₂O

- Mix 80µl of hybridisation solution for each slide.

Hybridisation Solution.

20µl formamide

20µl 20 x SSC

0.4µl 20% SDS

2.5µl 2mg/ml PolyA DNA

4µl 2mg/ml Cot1 DNA

30µl Cy3 and Cy5 labelled probe

Up to 80µl H₂O

- Denature hybridisation solution at 95⁰C for 5 mins, then keep on ice.
- Add hybridisation solution to each slide and apply cover-slips.
- Place slides in hybridisation chambers.
- Add 10µl H₂O to each hybridisation chamber and moisten seal.
- Seal hybridisation chambers and wrap in dampened paper towels.
- Wrap slide chambers in tinfoil, and keep at 42⁰C overnight.

Step 8 – Post-Hybridisation Washes.

- Remove slides from hybridisation chambers.
- Dip slides in 2 x SSC, 0.1% SDS until cover-slip falls off.
- Wash slides in 2 x SSC, 0.1% SDS at 42⁰C for 2 mins.
- Wash slides in 0.2 x SSC for 2 mins at room temperature.
- Wash slides in 0.05 x SSC for 2 mins at room temperature.
- Wash slides in 0.05 x SSC for 2 mins at room temperature.
- Wash slides in 0.05 x SSC for 2 mins at room temperature.
- Wash slides in 0.05 x SSC for 2 mins at room temperature.
- Dip slides in H₂O.
- Dip slides in 100% ethanol.
- Dry slides by spinning briefly in 50ml falcon tube.

Step 9 – Scanning.

- Scan slides on Axon scanner 4100B scanner and Genepix Pro v5.0 using PMT Power of 600V

Upload files into GEPAS for normalisation

Perform print-tip normalisation (normalising to overall intensities in different regions to account for spotting differences) with background subtraction

2.16.5 TUNEL staining

During apoptosis, endonucleases digest DNA to form short double stranded DNA fragments. These fragments can be detected by attaching biotinylated nucleotides to the 3'-OH ends using a terminal deoxynucleotidyl transferase (TdT) enzyme. These can then be visualised using a streptavidin-horseradish peroxidase conjugate followed by the substrate diaminobenzidine (DAB) which forms a brown precipitate when broken down by peroxidase. Apoptotic cells must be distinguished from necrotic cells morphologically.

Solutions:

3.7% buffered formalin: 5 ml 37% formaldehyde

45 ml PBS

Quenching solution: 5 ml 30% H₂O₂

45 ml methanol

Labelling reaction mix: 1 µl TdT-dNTP kit solution (R&D Systems)

1 µl Co²⁺ kit solution

1 µl TdT enzyme kit solution

50 µl TdT labelling buffer (kit solution diluted 1:9 in dH₂O)

DAB solution: 50 ml PBS
250 µl DAB
50 µl 30% H₂O₂

2.16.5.1 Method

Air dry cryostat sections fully (30 min if fresh or taken from freezer).

Draw round sections with a wax pen, and allow to dry fully (15 min).

Fix in 3.7% buffered formalin for 10 min.

Wash slides for 2 x 5 min in PBS.

Blot slides dry, then immediately add 50 µl proteinase K solution (1 µl kit solution in 50 µl dH₂O per slide) and cover with a coverslip.

Incubate for 15 min at RT, then wash 2 x 2 min in dH₂O.

Place slides into 50 ml of quenching solution for 5 min, then wash 2 x 1 min in PBS.

Incubate slides in 50 ml TdT labelling buffer for 5 min.

Blot slides dry, then immediately add 50 µl labelling reaction mix and cover with a coverslip.

Incubate slides in a 37°C incubator in a humidity chamber for 2 hours.

Transfer slides into 50 ml TdT stop buffer (kit solution diluted 1:9 in dH₂O) and incubate for 5 min.

Wash slides 2 x 2 min in PBS.

Blot slides dry, then add 50 µl streptavidin-HRP detection solution onto each sample (1 µl kit solution in 50 µl dH₂O per slide) and cover with a coverslip. Incubate at RT for 10 min.

Wash slides 2 x 2 min in PBS.

Incubate slides in 50 ml DAB solution for 5 min.

Rinse slides in dH₂O, then transfer to fresh water.

Counterstain by transferring slides to 50 ml methyl green solution for 3 min.

Wash slides by sequentially dipping 10 times each in:

dH₂O

70% ethanol (2 changes)

90% ethanol (2 changes)

100% ethanol (2 changes)

xylenes (2 changes)

Dry xylenes from the back of the slide. Leave xylenes on the surface of the slide to aid the mounting process.

Place two drops of Histomount (Sigma) on the samples and coverslip.

Leave to harden overnight. Store out of direct light.

Notes:

Do not allow samples to dry at any point after they have been fixed.

Use fresh solutions for large numbers of slides (max. 4 slides per 50 ml coplin jar)

Keep TdT enzyme in the freezer – only remove it for long enough to pipette the amounts required, and do this immediately before use.

Empty pipette tip racks with water in the bottom work very well as humidity chambers.

Any flaws in the section will show up brown as they have exposed DNA fragments. This is

not apoptosis, but can be a useful positive control.

If staining mouse tissue, the above protocol overexposes the background. I would suggest using the Mg^{2+} kit solution in the labelling buffer, and incubating for 1 hour instead of 2.

Additionally, the methyl green protocol listed above does not work on mouse muscle. These must be counterstained by incubating in the methyl green solution for 5 min, then dipping in 1-butanol until the sample turns from blue to mainly green, dipping once more in fresh butanol, then dipping 10 times in 2 changes of xylenes and mounting as before.

2.16.6 Dye labelling cDNA

This procedure uses a polymerase reaction to create copies of cDNA that incorporate a cyanine dye into the new copies. This procedure uses Cy3 (green fluorescent; excited at 550nm, emits at 570nm) and Cy5 (red fluorescent; excited at 649nm, emits at 670nm) dyes conjugated to dCTP monomers. As the Cy dyes are quite bulky, they sterically hinder CTP incorporation, so some unlabelled dCTP is added to the reaction mix.

Solutions

Primer mix:

500ng cDNA

20 μ l Random primer mix (Bioprime labeling kit, Invitrogen)

Up to 43 μ l H₂O

Reaction mix (per sample):

1.2µl dATP (10mM)

1.2µl dTTP (10mM)

1.2µl dGTP (10mM)

0.72µl dCTP (10mM) (all from Invitrogen)

1µl Klenow Enzyme (40U/µl) (Invitrogen)

2µl Cy3 (samples) or Cy5 (cDNA reference) (Amersham Biosciences)

2.16.6.1 Method

Heat to 94°C for 5 min to denature dsDNA

Cool on ice

Add 7.3µl reaction mix to each tube

Incubate at 37°C for 2 hours

Add 50µl H₂O to each sample

Add 100µl PB buffer (QIAquick PCR Purification Kit, Qiagen) to sample

Add probe mix to Qiagen PCR purification column

Spin at 13000 rpm for 1 min

Empty collection tube and discard flow-through

Add 700µl PE (QIAquick PCR Purification Kit, Qiagen) buffer to column

Spin at 13000 rpm for 1 min

Empty collection tube and discard flow-through

Spin at 13000 rpm for 2 min

Transfer insert to a fresh 1.5ml microfuge tube

Add 50µl H₂O

Leave at RT for 2 min to allow cDNA to dissolve

Spin at 13000 rpm for 1 min to collect cDNA

Measure probe concentration by taking measurements at 260nm, 535nm and 635nm:

$$\text{Cy3 pmol/}\mu\text{l} = \text{Absorbance (535nm)} / 0.15$$

$$\text{Cy5 pmol/}\mu\text{l} = \text{Absorbance (650nm)} / 0.25$$

$$\text{cDNA yield ng/}\mu\text{l} = \text{Absorbance (260nm)} \times 50$$

Calculate dye concentration per ng cDNA to check efficiency of labelling:

$$\text{CyX pmol/ng} = \text{CyX concentration} / \text{cDNA yield}$$

Ensure at least 50 pmol/ng, otherwise relabel

Add 100 pmol of each dye to a microcon column (Qiagen)

Spin at 13000 rpm for 5 min

Add 10µl H₂O

Invert column into a fresh tube

Spin at 13000 rpm for 3 min

2.16.7 iNOS staining

Optimisation of the iNOS antibody was performed as follows: First, sections were stained according to the previously established protocol for Ki67, but comparing BSA/Triton X-100 wash buffer with normal sheep serum (NSS) with and without antigen retrieval as previously

described. Wash buffer without either the primary or the secondary antibody as controls. No specific staining was seen in either of the antigen retrieval techniques, and little was seen with NSS without antigen retrieval. BSA/Triton without antigen retrieval gave punctate staining, although background was far too high, so the secondary antibody concentration was tested in 5x dilutions from 1:100 to 1:25,000. The optimum secondary concentration was 1:5,000, so the primary concentrations were titrated against this in 5x dilutions from 1:25 to 1:2,500. This gave clear, punctate staining with all concentrations, including the control without any primary. As this is artefact, the protocol was amended to look at different fixation procedures (cold methanol, ethanol and acetone) with no change.

Total protein extracted from polyps and ITs

Protein measured and loaded equally into ELISA

Samples run in duplicate with A450 background correction, quantified against ladder of known concentrations

Diluted protein re-assayed and A650 absorbances corrected for small protein loading differences

2.16.8 RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR, not to be confused with real-time PCR, rtPCR) is a method for creating cDNA chains for analysis. RNA is fairly labile, and the ubiquitous presence of environmental RNases means that it not easy to work with. DNA is much more stable, and is thus used for most analysis.

Solutions

Primer mix:

5µg RNA

1µl oligo (dT)₁₂₋₁₈ primer 1µg/µl (Invitrogen)

Up to 17µl H₂O

Reaction mix (per sample):

8µl 5x first-strand buffer

4µl 0.1M DTT

2µl dATP (10mM)

2µl dCTP (10mM)

2µl dGTP (10mM)

2µl dTTP (10mM)

2µl Superscript II (200U/µl) (all from Superscript II RNase H Reverse Transcriptase kit, Invitrogen)

1µl H₂O

2.16.8.1 Method

Isolate RNA from the samples.

Add 3.5µl DNase I buffer.

Add 1µl DNase I

Incubate samples at 37°C for 30 min

Add 5µl DNase inactivation reagent

Incubate at RT for 2 min

Spin for 1 min at 13000 rpm on a bench centrifuge

Quantify RNA using a spectrophotometer

Use 5µg RNA in the primer mix

Heat to 65°C for 10 min to unfold RNA

Cool on ice

Add 23µl reaction mix to each tube

Incubate at 42°C for 2 hours

Add 200µl PB buffer (QIAquick PCR Purification Kit, Qiagen) to each sample.

Mix well

Make spin column by adding semi-permeable membrane insert (Qiagen) to collection tube

Add sample to spin column

Spin at 13000 rpm for 1 min

Empty collection tube and discard flow-through

Add 700µl PE buffer (QIAquick PCR Purification Kit, Qiagen) to column

Spin at 13000 rpm for 1 min

Empty collection tube and discard flow-through

Spin at 13000 rpm for 2 min

Transfer insert to a fresh 1.5ml microfuge tube

Add 50µl H₂O

Leave at RT for 2 min to allow cDNA to dissolve

Spin at 13000 rpm for 1 min to collect cDNA

Quantify DNA yield via spectrophotometry (Eppendorf Biophotometer)

CHAPTER 3
MEASURING CAPILLARY SUPPLY
PHYSIOLOGICAL ADAPTATION

3 Measuring capillary supply - Physiological adaptation

To understand and refine staining techniques to measure capillarity for use in human tissue, initial experiments were carried out on human skeletal muscle looking at physiological adaptation; how capillary supply is linked to metabolic demand and muscle fibre size and type. We used human muscle biopsies, as these were readily available whilst we awaited ethical committee approval to allow us take human nasal biopsies. We refined the established mouse lectin protocol and tried different lectins to increase the yield. We then compared this physiological adaptation of capillarity to pathological human muscle states.

3.1 Introduction

An accurate quantitative analysis of capillary supply to skeletal muscle is an essential prerequisite for understanding the limits to peripheral oxygen transport. The capillary supply to skeletal muscle is influenced by its metabolic profile, oxidative capacity and fibre size (Hudlicka et al., 1992). Quantitative differences in gross capillary supply are normally expressed as the ratio of capillary number to fibre number (capillary to fibre ratio, C: F) or to fibre area (capillary density, CD). Given that these indices are scale dependent, there is a need for a more discriminatory analysis that takes account of fibre size (Figure 3.1).

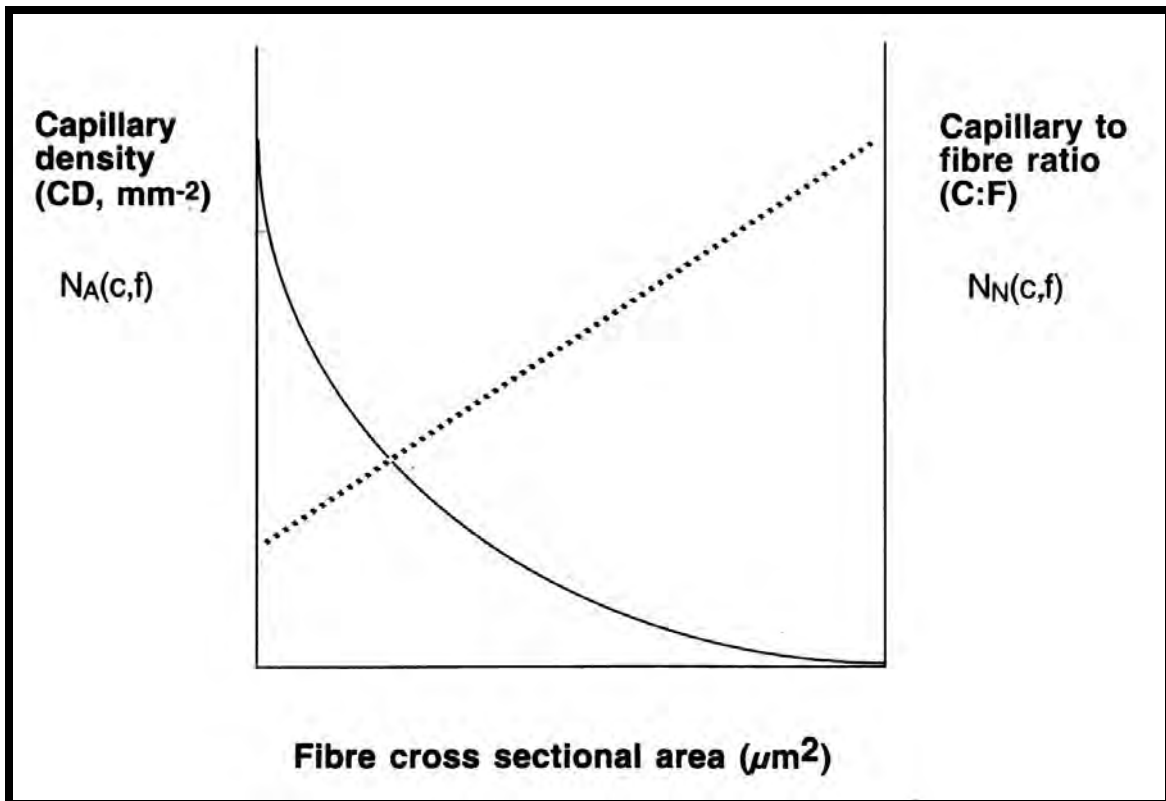


Figure 3.1: Allometry of muscle capillary supply

Changes in muscle capillary supply as a function of fibre cross-sectional area, illustrating the linear positive relationship with capillary to fibre ratio (C:F), and the non-linear negative relationship with capillary density (CD) (Egginton, 2002).

The global analysis was first brought down to a local analysis by Plyley and Groom (1975) when they quantified the mean number of capillaries around a fibre (CAF) for individual categories of fibre type. However, this method does not render information regarding diffusion distances and does not take into account fibre size. This drawback was overcome by relating CAF to fibre area for each category. CAF relative to fibre area gives a slight

overestimate of diffusion capacities of large fibres as a proportionally higher number of capillaries around a fibre cannot compensate for larger diffusion distances. In mixed muscle the influence of neighbouring fibres also needs to be considered and oxygen supply scaled accordingly (Egginton, 1990a). Thus each capillary needs to be weighted according to the metabolic character of its neighbours (Gray and Renkin, 1978; Flood, 1979). Plyley and Groom (1975) also calculated the sharing factor (SF) which is the number of fibres surrounding a capillary. It attempts to focus on the individual capillaries' contributions to the microvascular supply. With both CAF and SF, influence of fibre size, adjacent fibres and proximity of neighbouring capillaries limit their capacity to directly reflect oxygen demand. Gray and Renkin (1978) took this into consideration and used a mean numerical fraction of SF to give a specific capillary to fibre ratio (SCF) and a specific capillary density (SCD) for each fibre group.

Using mathematical models such as Voroni tessellations (Voroni, 1908) it is possible to calculate the domain of influence or area of tissue supplied by individual capillaries (Hoofd et al, 1985; Egginton et al, 1988; Egginton and Ross, 1989; Egginton, 1990). The interaction of capillary domains and individual fibres may be quantified as the sum of partial domains overlapping a fibre profile (local capillary to fibre ratio), being the real equivalent of Gray and Renkin's numerical fraction of SF (Egginton and Ross, 1989)(Figure 3.2)

By calculating the area of muscle tissue supplied by, or the domain of influence of, individual capillaries an unbiased measure of the local capillary to fibre ratio (LCFR) and local capillary

density (LCD) may be derived (Egginton and Ross, 1989)). This novel approach was used to investigate whether the local capillary supply was modelled according to the size or type of individual human skeletal muscle fibres.

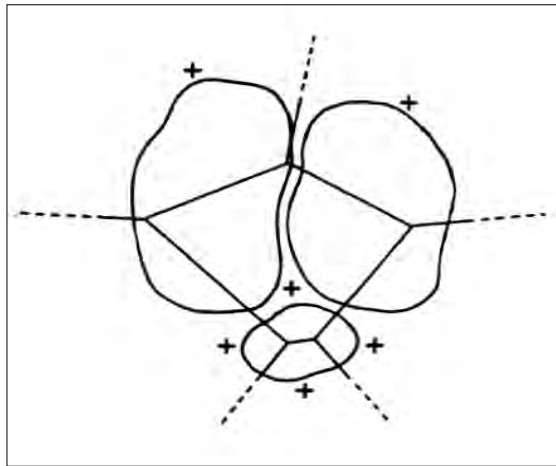


Figure 3.2 Comparison of scoring methods based on the numerical method or areal (Domain method) fraction of a capillary to be ascribed to different fibre types.

Above, the central capillary [+] serves two large glycolytic fibres and a smaller oxidative fibre. The numerical fraction of this capillary serving glycolytic fibres is therefore 0.66 whereas the areal fraction is much greater, around 0.80. In addition to this discrepancy the numerical method only allows for a distribution analysis on the basis of capillary fractions per fibre type while the Domain method provides an additional level of resolution by summing capillary fractions per individual fibre (Egginton and Ross, 1989).

3.2 Methods

With the approval of the Local Ethics Committee and written informed consent, samples from the lateral portion of the quadriceps femoris muscle were obtained by percutaneous needle biopsy from fifteen active young subjects (10 male and 5 female): mean age, 23.3 ± 1.38 years (males) and 21.0 ± 1.73 years (females); height, 1.80 ± 0.02 m (males) and 1.66 ± 0.04 m (females); and body mass, 83.3 ± 3.70 kg (males) and 60.7 ± 1.56 kg (females).



Figure 3.3 *Percutaneous needle biopsy from lateral quadriceps femoris muscle*

Serial sections (10 μ m thick) were prepared for quantitative analysis by histochemical staining of fibre types (Brooke and Kaiser, 1970) and capillaries (Holthofer et al., 1982) and *Ulex europaeus Agglutinin-1* (UEA-1), a lectin for capillaries.

Muscle composition was determined by stereological analysis of fibre area and proportion of fibre types (Egginton, 1990). Digitized images of stained sections were used to determine the

x, y co-ordinates for muscle fibres and associated capillaries. From these data the LCFR and LCD, indices of fibre-specific capillary supply, were calculated (Egginton and Ross, 1989). Briefly, this involves calculating the geometric supply area ('domain') of each capillary, defined as that portion of tissue cross-section closer to one capillary than any other, and the summed fraction of different domains overlapping each muscle fibre (figure 3.1). Under conditions of maximal flow, assuming supply capacity to be similar for all capillaries, the domain size will be inversely proportional to the metabolic demand. Thus, LCFR represents supply capacity in 'capillary equivalents', which may be normalized for the effects of individual fibre size to give the LCD. For comparison, the equivalent number-based indices of local capillary supply, number of capillaries around each fibre (CAF) and its normalized derivative (i.e. CAF per fibre area, CPA), were also calculated. Data were analysed by ANOVA and results expressed as mean values \pm S.E.M.

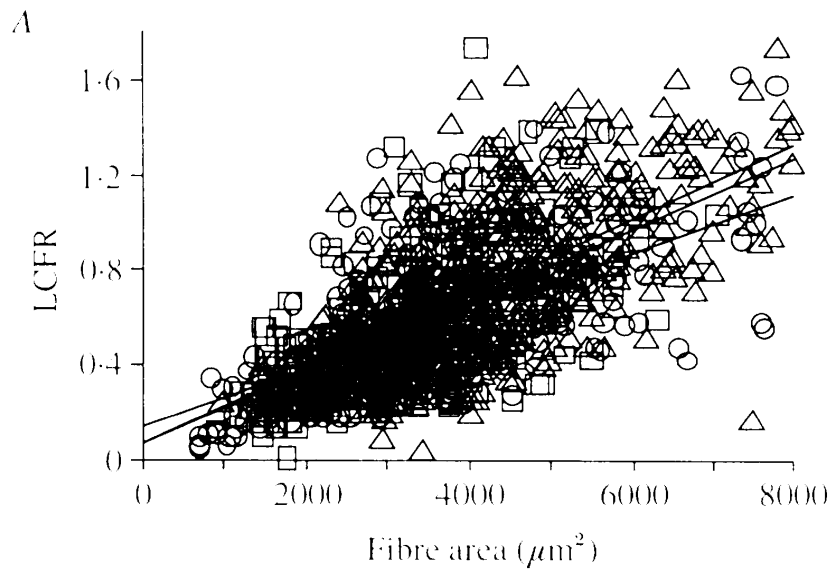
3.3 Results

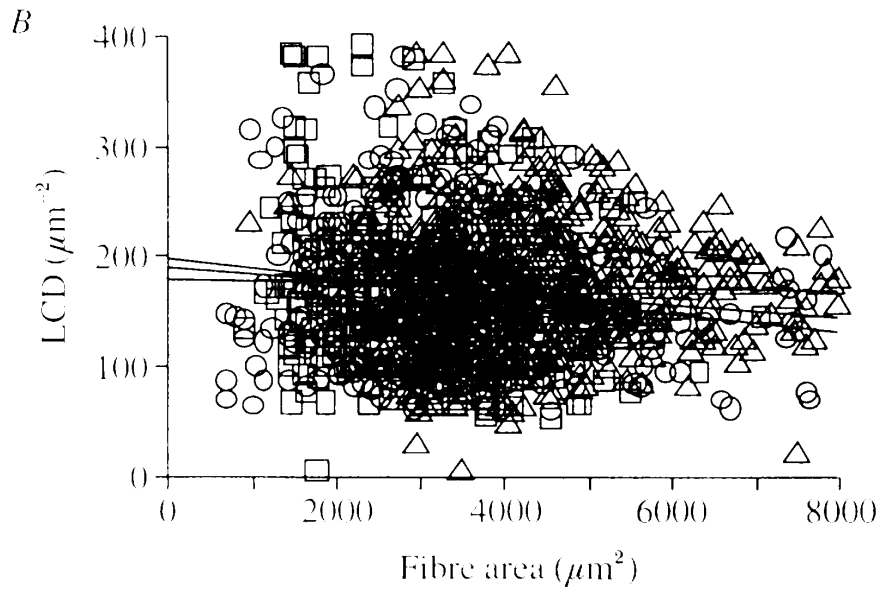
The use of *Ulex europaeus Agglutin-1* (UEA-1) for the identification and computerised quantification of capillaries was not found to be a reliable marker. We found that UEA-1 does not stain all human capillary endothelium equally and differential staining was seen. Kirkeby et al. (Kirkeby et al., 1993), while investigating blood group specific lectins, discovered that UEA-1 does not stain strongly in patients with blood group A. This finding could not be verified by our data as blood group information was not available from patients whose capillary endothelium did not stain strongly, although this was suspected. Previous work using *Ulex europaeus* has only tested the Agglutin-1 (Holthofer et al., 1982, Alroy et al.,

1987). However, data from this investigation suggests that the lectin UEA-II is a more reliable marker for capillary endothelium than the agglutinin-1, staining capillaries more densely especially in sections from patients of blood group A.

Numerical muscle fibre composition was found to be $47.9 \pm 4.2\%$ type I, $31.3 \pm 4.4\%$ type IIa and $21.6 \pm 5.1\%$ type II b fibre types. In relation to muscle area the values were 51.8 ± 3.3 , 27.7 ± 4.4 and $15.7 \pm 4.1\%$, respectively. Mean fibre cross-sectional areas were 4538 ± 240 , 4005 ± 390 and $3957 \pm 814 \mu\text{m}^2$ respectively. Mean C:F was 1.3 ± 0.42 , while CD was $442 \pm 52 \text{ mm}^{-2}$. These values are comparable with those obtained previously for vastus lateralis biopsies from untrained individuals giving a C:F of 1.39-1.79 and a CD of 348-438 mm^{-2} (Ingjer, 1979). A total of 1741 muscle fibres (n=709, 520 and 512 for type I, IIa and IIb, respectively) from these individuals were also matched across serial sections for fibre type, fibre area and capillarity. Capillary supply (domain) area showed a logarithmic-normal distribution. Domain area (μm^2) and \log_{10} (domain area) were 2831 ± 150 and 3.41 ± 0.024 , respectively (n= 6 biopsies and 944 domains). LCFR showed a positive correlation with fibre area (LCFR = $0.075 + 0.0015 \times \text{area}$; $r^2 = 0.47$) that was independent of fibre type (Figure 3.1 A). Slopes for the regression with fibre type were very close (0.0012-0.0016; $r^2 = 0.40$ -0.45), although mean values scale with oxidative capacity i.e. type I > IIa > IIb. Normalizing the local capillary supply to give LCD produces a slope very close to zero (LCD = $186.8 + 0.0042 \times \text{area}$; $r^2 = 0.009$), effectively removing the scaling effect of fibre size for both slope and mean values, which is consistent with the observed lack of any significant difference among fibre types (Figure 3.1 B).

Figure 3.4: Local capillary indices in the lateral quadriceps, split according to fibre type: type I (triangle), type IIa(square) and type IIb(circle). A, local capillary to fibre ratio (LCFR); B, local capillary density (LCD).





Fibre type	LCFR	LCD (μm^{-2})	CAF (μm^{-2})	CPA	Fibre Area (μm^2)
Type I	0.723 ± 0.084	174.1 ± 10.71	1.323 ± 0.168	329.7 ± 34.9	4176 ± 340
Type II a	0.569 ± 0.056	172.1 ± 13.30	1.027 ± 0.120	338.2 ± 50.8	3399 ± 366
Type II b	0.532 ± 0.047	169.8 ± 8.34	0.912 ± 0.076	311.6 ± 23.7	3216 ± 366

Table 3.1: Fibre type specificity of local capillary supply in the lateral quadriceps

Figure 3.5: Muscle stained to show capillaries (lectin) and sequential section stained to co-localise muscle fibre types (Myosin ATPase) x 20 magnification.

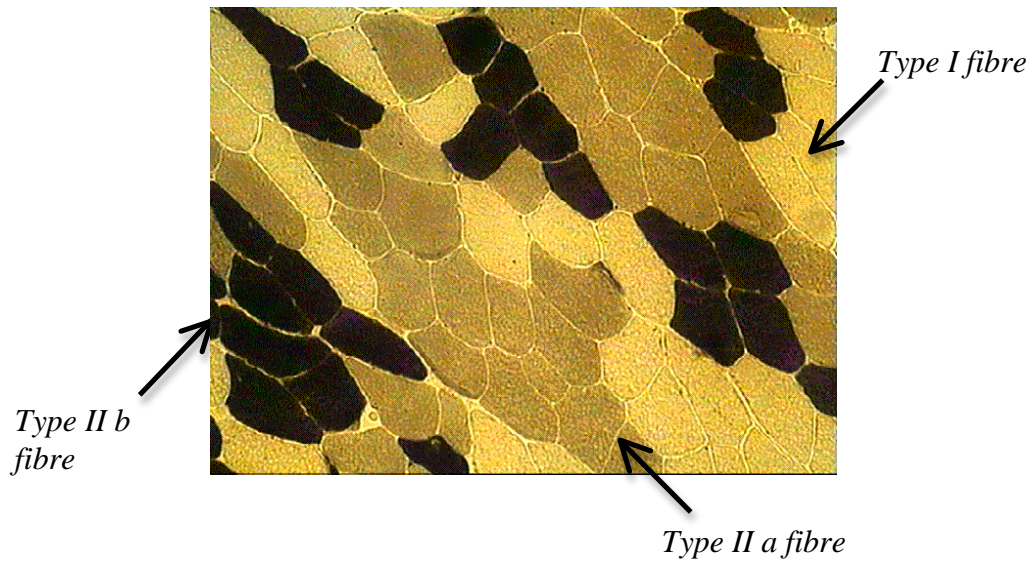
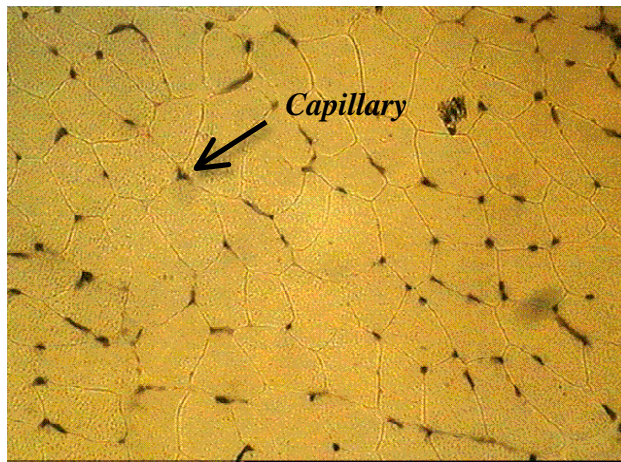
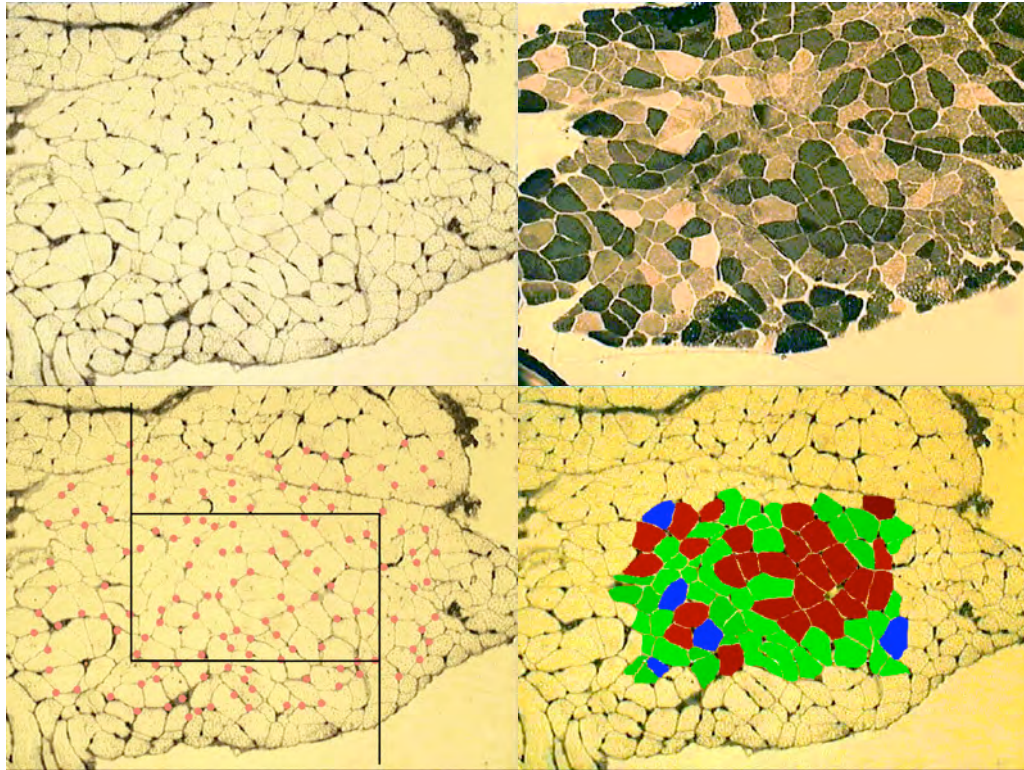


Figure 3.6: *Digitised images of lectin localised capillaries and mATPase localised muscle fibres using Image J for domain analysis.*



Green = type I; Blue = type IIa; Red = type IIb

3.4 Discussion

Ulex europeaus agglutinin I is not a valid marker of human capillary endothelium, as differential staining due to blood group specificity was found. The agglutinin II did not show any differential staining and is therefore superior to the agglutinin I. However, more investigation needs to be carried out using UEA II in muscle from humans with different blood groups.

Many studies have sought to determine whether the capillary bed of skeletal muscle is limiting for aerobic capacity via its role in setting the level of functional hyperaemia. While capillarity

is increased following a period of training, a number of variables may complicate the implicit relationship between supply and demand. For example, in a longitudinal training study Andersen & Henriksson (Andersen and Henriksson, 1977) showed that an expansion of the capillary bed was accompanied by fibre type transformation in vastus lateralis. Both the duration of training and size of fibres were also important factors, with C:F increasing significantly after 5 weeks, but CD only after 8 weeks due to the accompanying fibre hypertrophy. From biopsies taken before and following 24 weeks of endurance training, Ingjer (Ingjer, 1979) found a significant increase in C:F, with the CAF for slow oxidative fibres increasing more than that for fast glycolytic fibres. Indeed, Saltin & Gollnick (Saltin and Gollnick, 1983) suggested that the supply area for capillaries would be 20-30% (type Ib) or 10-20% (type Ia) greater than that for type I fibres, which is similar to the differences shown by our measured values of local capillary supply (LCFR; Table 3.1). These and other studies are in agreement with the more extensive literature from animal experiments showing that local demand (in terms of fibre type) may influence capillary growth, and that CD often varies inversely with fibre size (Hudlicka et al., 1992). Such considerations may explain reported discrepancies in CD among human training studies.

Using a specific marker for capillaries and a discriminatory analysis of local capillary supply, we have examined over 1700 human muscle fibres. By comparison with the discontinuous distribution of values from any of the integer-based indices of capillary supply, which convey little information regarding muscle composition, the continuous indices can be used to describe the local capillary supply and have the potential to discriminate between more subtle changes in fibre composition and/ or size. This may be of particular benefit where fibre size

changes between samples, e.g. hypertrophy following high resistance exercise (Abernethy et al., 1994), or where an increase in CD does not reflect capillary growth, but rather the atrophy of muscle fibres (Hoppeler and Desplanches, 1992). In particular, while both fibre size and capillary supply are reduced with age, the apparent specificity of training-induced increases in capillary supply to type I fibres in elderly subjects (Proctor et al., 1995) deserves closer attention. Indeed, in a pilot study we showed a regression of LCFR against fibre area in trained muscle which was merely offset from that of controls ($LCFR = 0798 + 00017 \times \text{area}$; $r^2 = 0.17$), with a similar slope and clustering for fibre types, although LCD showed a slight negative slope against fibre size ($LCD = 501.2 - 0031 \times \text{area}$; $r^2 = 0.15$), suggesting that additional factors have some influence on local capillary supply (Ahmed et al., 1995) . We therefore conclude that the capillary supply to human skeletal muscle is scaled according to fibre size and is relatively independent of fibre type. The method of capillary domains offers a reproducible technique, with the potential to discriminate between subtle adjustments in local capillary supply at the level of individual human muscle fibres.

CHAPTER 4
MEASURING CAPILLARY SUPPLY
PATHOLOGICAL ADAPTATION

4 Measuring capillary supply - Pathological adaptation

Skeletal muscle readily undergoes adaptive remodelling, and its capillary supply responds to metabolic demand but scales primarily according to fibre size not fibre type (Chapter 3) (Ahmed et al., 1997b). More than 70 diseases may have an angiogenic component, with either excessive or insufficient capillary supply (Egginton, 2010). Pathological conditions, such as diabetic retinopathy or psoriasis, are classed as angiogenic disorders as they uncouple the capillary supply of a tissue from its metabolic needs. Uncoupling of capillary supply from metabolism can also be performed experimentally with *in vivo* models, through increasing shear stress (by vasodilators) and passive stretch (by overload)(Egginton, 2009). We examined how different pathologies affect the capillary supply of human skeletal muscle by a process of adaptive remodelling through: 1) examination of biopsies from patients with muscular atrophy, hypertrophy or peripheral vascular disease; 2) a meta-analysis of the available literature.

4.1 Methods

Percutaneous needle biopsies of vastus lateralis were taken from control volunteers (n=5), and patients with muscle fibre atrophy (n=2), fibre hypertrophy (n=4) or peripheral vascular disease (n=7) with informed consent and local Ethical Committee approval. Biopsies were snap-frozen in liquid nitrogen-cooled isopentane, and capillaries stained using *Ulex europeaus* lectin-1 on 10µm cryostat sections. Capillaries were counted and fibre areas measured by stereology of digitised images, using 2-4 fields of view from random sections (Fig. 4.1). A graticule was used to measure the sample area, and capillary density (CD, mm⁻²), fibre size

(a(f), μm^2) and the capillary to fibre ratio (C:F) was then calculated from this.

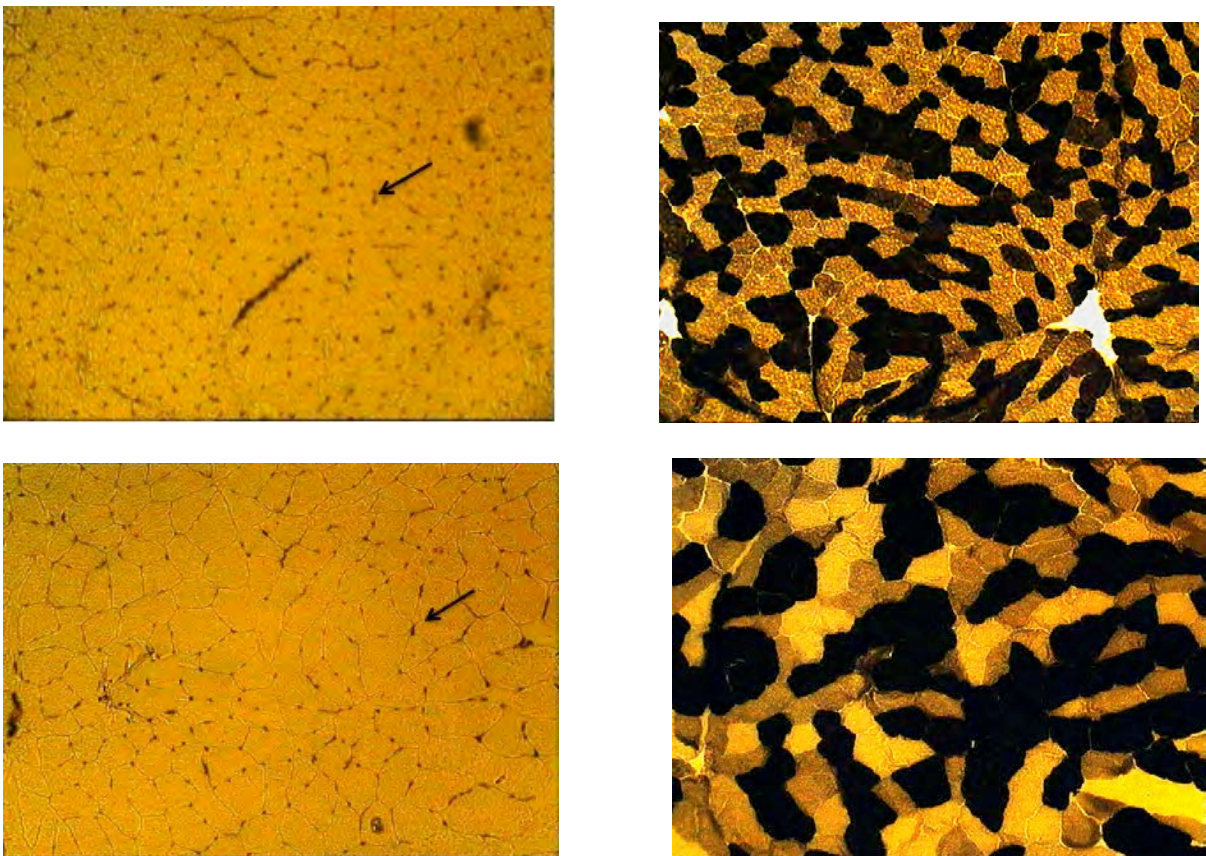
For meta-analysis, literature searches using combinations of relevant terms (muscle, capillary, vasculature, fibre, human) were supplemented by reference mining all papers found. Only adult human studies were used. 304 papers were checked to find those containing data on capillary density and mean fibre size, or data from which capillary density and fibre size could be calculated. Data from 44 papers was collated and analysed using C:F, CD, fibre size, methodology, muscle type and experimental protocols as separate variables.

Formalin/ gluteraldehyde fixed samples had their average fibre size increased by 30% to correct for muscle shrinkage (Goldspink, 1961).

4.2 Results

Figure 4.1 Muscle biopsies stained for capillaries and muscle fibre types

Photographs taken at x 10 magnification of 10 μ m sections of muscle biopsies taken from patients with idiopathic muscle atrophy (top) and hypertrophy (bottom) stained with *Ulex europaeus* lectin-1 (left), showing the position of capillaries (black arrows) and mATPase to stain muscle fibre types (right).



The top panels demonstrate smaller muscle fibres in keeping with muscle atrophy and the bottom panels larger fibres as seen in muscle hypertrophy.

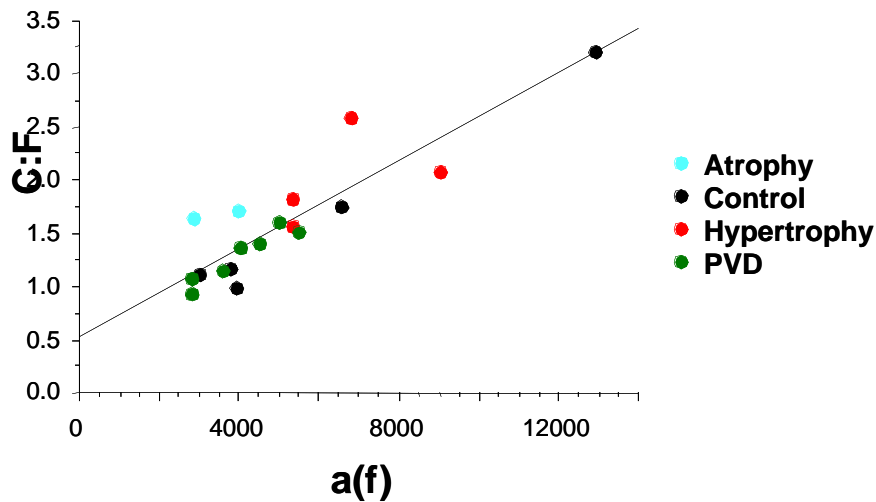
Table 4.1: Stereological analysis

Capillary to fibre ratio (C:F), mean fibre area ($\bar{a}(f)$), and capillary density (CD) shown for control volunteers, patients with muscle hypertrophy, muscle atrophy, or peripheral vascular disease (PVD). Mean \pm SEM.

	C:F	$\bar{a}(f) \mu\text{m}^2$	CD mm^{-2}
Control	1.64 \pm 0.21	6040 \pm 870	293 \pm 15
Hypertrophy	2.01 \pm 0.03*	6630 \pm 570	319 \pm 30
Atrophy	1.68 \pm 0.41	3440 \pm 1160*	434 \pm 34
PVD	1.29 \pm 0.09*	4040 \pm 730	323 \pm 19

*P <0.05 vs. control

Figure 4.2 Regression analysis of experimental data



Linear regression of capillary to fibre ratio (C:F) vs. mean fibre area ($\bar{a}(f) \mu\text{m}^2$) for control volunteers, patients with muscle hypertrophy or atrophy, of peripheral vascular disease demonstrates that scaling of capillarity is independent of the magnitude of capillary supply.

The overall R^2 increased from 4.05 to 4.43 when formalin/ gluteraldehyde fixed samples had their average fibre size increased by 30% to correct for muscle shrinkage (Goldspink, 1961).

Figure 4.3 Regression of meta-analysis data

Linear regression of capillary to fibre ratio (C:F) vs. mean fibre area ($\bar{a}(f) \mu\text{m}^2$) for all control, experimental and pathological conditions.

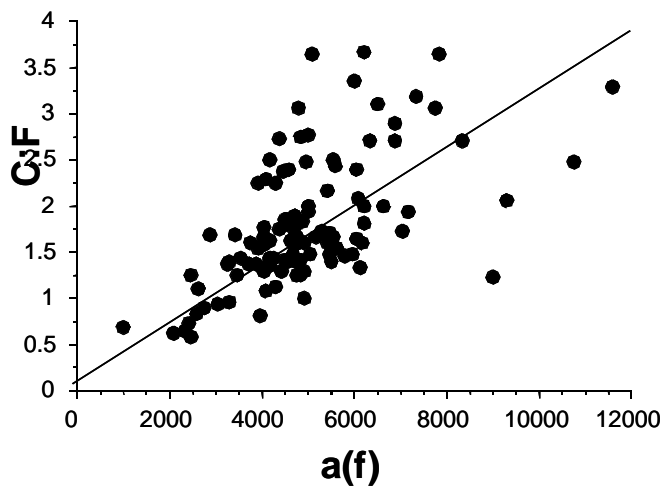


Figure 4.4 Regression of grouped meta-analysis data

Linear regression of capillary to fibre ratio (C:F) vs. mean fibre area ($\bar{a}(f) \mu\text{m}^2$) for controls, endurance trained muscles, acute muscle atrophy and chronic muscle atrophy.



4.3 Discussion

Mean fibre area confirmed the clinical diagnoses of atrophy and hypertrophy. Hypertrophy maintained capillary density (CD), but increased capillary to fibre ratio (C:F), whereas atrophy maintained C:F but increased CD (Table 3.1). Regression analysis of C:F vs. mean fibre size showed a single line for controls, hypertrophy and peripheral vascular disease, with a trend for idiopathic muscle atrophy to lie above this line (Fig. 3.2). This suggests active remodelling of the capillary bed by feedback control. Evidence from this comes from analysis of distribution with similar geometric patterns seen across muscle with 2 orders of magnitude difference in CD (Egginton and Gaffney, 2010), suggesting that the growth process is a fixed genetic program. This may be driven by oxygen consumption via some feedback control mechanism however we have shown in chapter three that muscle fibre size is the key determinant to capillarity (Ahmed et al., 1997a). This has significant clinical implications and may mean a different approach to therapy. Of this were the case then we would see a similar trend in other published studies in the literature. We then undertook a meta-analysis approach to investigate this.

Following meta-analysis, there was no significant difference in the regression lines when studies were split into groups for gender, age, or muscle type (Fig. 4.3). There was a significant difference in data points for muscles that had undergone significant endurance training, which had a higher C:F for a given fibre size than other muscles (Fig. 4.4). The regression for atrophic muscles was only slightly above that for control muscles, unless the

atrophy was split into chronic (>6 weeks) and acute atrophy (<6 weeks). Chronic atrophy has a regression line similar to controls, whereas acute atrophy showed vascular maintenance.

4.4 Conclusions

Capillary supply to human skeletal muscle is primarily determined by muscle fibre size, with adaptive remodelling occurring in response to resistance training or chronic muscle atrophies. Endurance training leads to a higher C:F than would be predicted by fibre size, showing angiogenesis in response to increased metabolic demand, and acute atrophy shows a maintenance of the vascular supply that is not seen in chronic atrophy, indicating that vascular regression occurs subsequent to the atrophy. In pathological processes there appears to be a decoupling of angiogenesis from metabolic demand, for example capillarity is mainly dependant on fibre size not fibre type, suggesting that the preponderance of angiogenesis-based diseases may be dependent on the tissue involved.

CHAPTER 5
MEASURING HUMAN NASAL CAPILLARY SUPPLY
METHOD DEVELOPMENT

5 Measuring Human Nasal Capillary Supply - Method development

5.1 Staining of nasal mucosa to examine remodelling

5.1.1 Introduction

Immunohistochemical examination of nasal mucosal tissue is essential for studying the aetiology of conditions such as chronic sinusitis, polyposis or airway remodelling. Multiple labelling of different molecules on the same section is highly desirable as it allows co-localisation of different factors, however, most established protocols use paraffin-embedded human tissue (Mumbuc et al., 2007), which limits the available tissue markers, as many antibodies will not recognise epitopes after paraffin embedding. Using fresh tissue allows whole mount staining (using relatively large tissue samples); the sample can then be examined with confocal microscopy and analysed in three dimensions. This approach could yield a wider range of information, avoiding problems with heterogeneity of structure by examining a larger mass of tissue, and correcting for the large changes in tissue volume typically seen in the nasal mucosa due to the nasal cycle. This has been successfully applied to the examination of blood vessels with fluorescent lectins in animal models (Williams et al., 2006b), and we have demonstrated in chapters 3 and 4 the staining of capillaries in human muscle. Hamid et al., has also demonstrated the use of lectins in a proof-of-principle human study (Hamid et al., 2003), but the use of lectins can be problematic in large human studies as no single lectin will stain a population with heterogeneous blood groups (Capaldi et al., 1985).

As different lectins bind to different sugar moieties, we reasoned that a mixture of the three

different lectins reported to stain human blood vessels (Capaldi et al., 1985) would successfully stain a far greater proportion of patient samples, and would therefore provide a more robust staining protocol for visualising capillary beds in three dimensions, allowing further investigation of vasculature changes during tissue remodelling in patients.

5.2 Methods

Subjects

Patients were recruited from University Hospitals Birmingham NHS Trust, UK, with local ethical committee approval (numbers 0692 and 06/Q2702/15). Patients' informed consent was obtained. Polyp and ipsilateral inferior turbinate specimens, 1 cm back from the anterior edge, were obtained from patients undergoing routine polypectomy. Previous studies have found no difference in vascularity between the different areas of the lateral nasal wall (Philpott et al., 2005). Inferior turbinate specimens from the same anatomical site were also taken from patients undergoing routine septoplasty or septorhinoplasty.

Fixation

First, 16 μm sections were observed under fluorescent illumination for red, green and blue filters to check for autofluorescence after different fixation methods. 4% buffered formalin (20 min), cold acetone (2 min), and 100% ethanol (5 min) were used, then washed in PBS for 3 x 5 min, and mounted. Dry sections and sections left in PBS for 5 min were also examined. Acetone and ethanol fixation resulted in substantial autofluorescence under green illumination and a small, but observable, amount of autofluorescence was seen under all channels for all

fixation methods. Formalin fixation resulted in the lowest autofluorescence under all channels and was therefore chosen for subsequent optimisation.

Immunohistochemical staining

The different stains were tested separately, both for efficacy of staining and for checking non-specific fluorescence in other channels. DAPI staining was performed using Vectashield + DAPI. This successfully stained cell nuclei with no fluorescence in the other two channels, but the 16 μm sections contained too many nuclei in different layers to allow quantification. Different thicknesses of sections were cut and stained, with 10 μm sections being the thinnest that good quality sections could be reproducibly obtained.

Staining for capillaries was attempted with fluorescein-conjugated lectins, with a comparison made between *Ulex europaeus*, *Euonymus europaeus* and *Griffonia simplicifolia* lectins. All three lectins gave a similar pattern of staining on test sections, however as individual lectins can fail to stain individuals with different blood groups (Capaldi et al., 1985), a 1:1:1 combination of the lectins was used at a concentration of 10 $\mu\text{l/ml}$ for 60 min. This gave good staining with a lower background than the individual stains at that concentration. Proliferation staining was performed using a stain for PCNA that is well established in the mouse.

Due to the problems with PCNA (Table 5.2) staining, Ki67 was chosen as a proliferation marker instead (Mumbuc et al., 2007), using the same protocol, but changing to the primary (Ki-67, Dako) and secondary (clone, Dako). This gave some staining, but with a very poor signal-to-noise ratio, and after consultation with the manufacturers, an antigen retrieval procedure was performed. This uses citrate and heat to break down the protein cross-links

caused by formalin fixation. The standard protocol uses buffered citrate heated to 95°C, then the slides are kept at 95°C for 20 min and allowed to cool in the buffer for another 20 min. An alternative protocol suggests that a microwave is used to simmer the citrate buffer for 5 min before allowing the slides to cool for 20 min. A comparison of these two procedures showed no difference between them, so the microwave procedure was chosen for subsequent use due to its shorter duration. Antigen retrieval gave good staining for Ki67, and had no effect on either lectin or DAPI staining. The stains were then combined in pairs and all three together to check for any unforeseen interactions. The lectin stain was found to show some fluorescence in the blue channel, so the dilution was lowered to 5 µl/ml. The order of staining was checked with the lectin being applied before and after the Ki67 stain, and with the secondary antibody. Whichever stain was applied first lost signal intensity, so simultaneous application of the secondary antibody with the lectin gave best results. Signal intensity for the DAPI stain was much higher than the other stains, necessitating changes in camera exposure times, so this was rectified by diluting the Vectashield + DAPI 1:1 in Vectashield.

5.2.1 Results

Samples from a total of 60 patients were stained using *Ulex europeaus lectin*, and compared to samples from 50 patients stained with a combination of *Ulex europeaus*, *Griffonia simplicifolia* and *Euonymus europeaus*. Samples that showed stained blood vessels were designated as positive, and samples in which blood vessels were not visualised were restained. Repeated failure to stain capillaries was seen in 23 of the single-lectin samples and in eight of the triple-stained samples ($p < 0.005$; Pearson's chi-square test), representing a significant

difference between single and triple lectin methods

Our method of using a mixture of three lectins stained a significantly higher proportion of samples than using *Ulex europeaus* lectin alone (84% vs 62%, $P < 0.005$ Student's t-test).

Comparison of different proliferation markers showed that Ki67 is a more suitable marker than PCNA for use in frozen sections.

	Single	Triple
+ve	37	42
-ve	23	8
% +ve	62%	84%

Table 5.1: Comparison of single *Ulex europeaus* lectin staining vs triple lectin protocol.

Data are shown for samples that stained well after up to three attempts (+ve) and for those that repeatedly failed to stain (-ve).

Samples from a total of sixty patients were stained using *Ulex europeaus* lectin, and compared to samples from fifty patients stained with a combination of *Ulex europeaus*, *Griffonia simplicifolia* and *Euonymus europeaus*. Samples that stained blood vessels clearly were designated as positive, samples that did not show any blood vessels were redone. Repeated failure to stain capillaries was seen in 23 of the single-lectin samples and 8 of the triple-stained samples ($P < 0.005$, Pearson's chi squared test), representing a significant difference between single and triple lectin methods (Table 5.1, Figure 5.1).

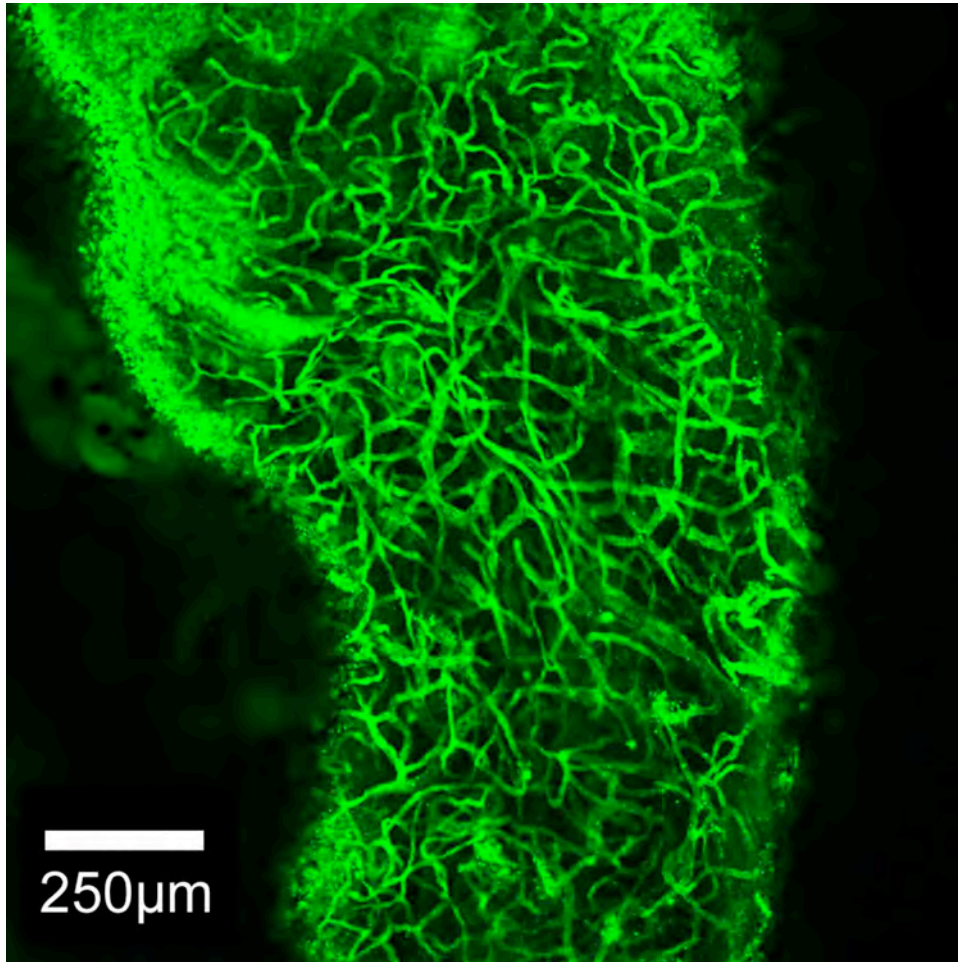


Figure 5.1 Inferior turbinate mucosa stained with whole mount protocol using three lectins.

Both PCNA and Ki67 staining gave best results using poly-L-lysine coated slides, and fixing in 4% buffered formalin for 20 minutes. Both stains gave best results with a wash buffer containing Triton X-100. Ki67 staining required antigen retrieval, and optimal antibody concentrations were 1:100 for both the primary and secondary antibodies. PCNA staining required antibody concentrations of 1:50 for primary and 1:100 for secondary.

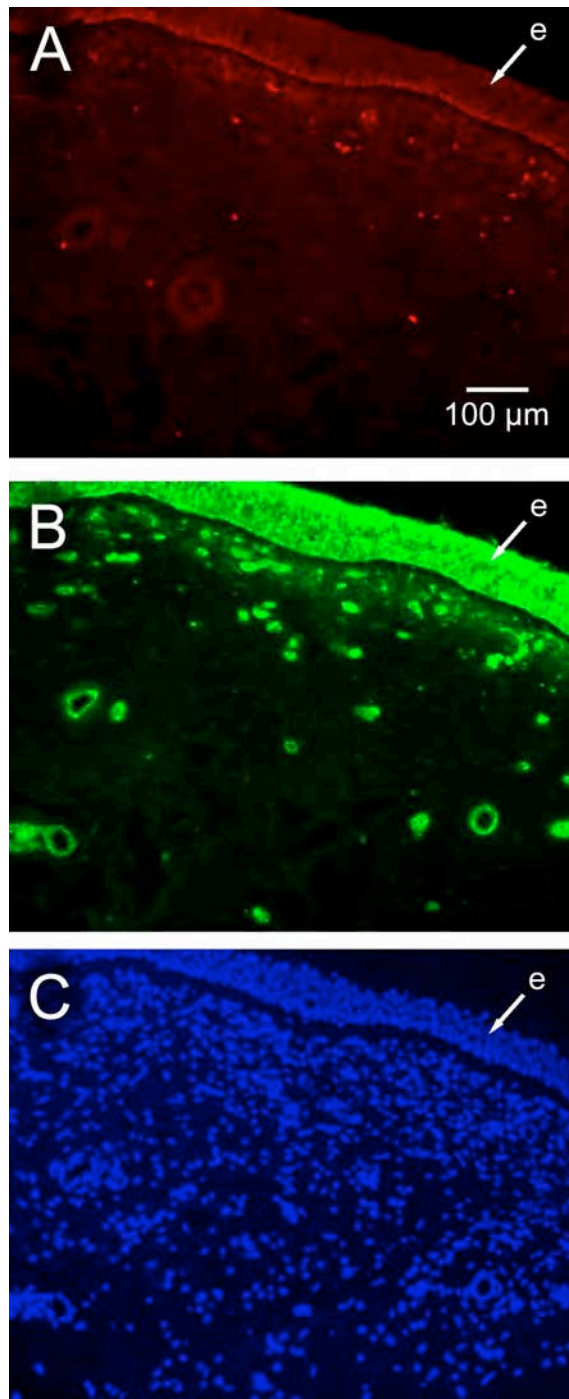


Figure 5.2: Triple stain of a single section of inferior turbinate mucosa under different fluorescent illuminations. e: epithelial layer. A: Rhodamine-conjugated Ki67 stain. B: Fluorescein-conjugated lectin stain. C: DAPI stain for nuclei.

	PCNA	Ki67
Proliferation Count/mm ²	1127 ± 201	39 ± 9*
Proliferation Index	34.3 ± 5.6	1.4 ± 0.3*

Table 5.2: Comparison of PCNA and Ki67 as measures of proliferation.

Data are shown for the total proliferation count (expressed as positive counts per mm²) and proliferation index (expressed as percentage of total nuclei. Mean ± SEM, n=12 *P<0.05 Student's t-test).

A significant difference was seen in the level of proliferation marked by PCNA and Ki67 staining (Table 5.2). Both the proliferation count per mm² and proliferation index was significantly higher when measured using PCNA staining compared to Ki67.

5.2.2 Discussion

The objective of these experiments was to provide a robust multiple staining protocol using samples that have not been embedded in paraffin, so that a broader range of antibodies can be used. The use of lectins to stain blood vessels is well established in animal models, but is less widely used in humans due to variability in staining between individuals with different blood groups (Capaldi et al., 1985). Our data show a marked improvement when using a mixture of the three most commonly used human-specific lectins (which bind to different sugar moieties present on the endothelium), compared with use of a single lectin. Lectins were also seen to bind to nasal mucosa epithelium (figure 5.2), as previously described; (Hassid et al., 1997, Huang et al., 1993) however, as this layer is avascular, it does not interfere with capillary

visualisation. Lectins have several advantages over other immunohistochemical methods in the staining of blood vessels. They can be readily conjugated to several fluorophores, allowing multiple stains to be used on the same section. The one-step protocol reduces the signal degradation associated with other multiple labelling methods, as well as saving time. Lectins are relatively inexpensive, allowing whole-mount stains to be performed economically. Finally, using lectins avoids the problems commonly associated with the use of two primary antibodies raised in the same host species.

Proliferating cell nuclear antigen (PCNA, also termed cyclin) is a protein that acts as a processivity factor for deoxyribonucleic acid polymerase delta in eukaryotic cells, and the staining of this antigen is well documented for paraffin-embedded sections. However, we have demonstrated that staining for PCNA is not appropriate for frozen sections, as the calculated proliferation index is unreasonably high. Normal mucosal tissue is highly unlikely to have 34 per cent of total nuclei undergoing mitosis at any one time (Table 5.2). It is likely that this antibody stains proliferating cell nuclear antigen epitopes in fresh human cells that are not in their biologically active form, as we have also seen unrealistically high labelling indices in serum-starved Human Umbilical Vein Endothelial Cell (HUVEC) monolayers where mitosis is arrested (J. Williams, S. Egginton, G. Nash, unpublished observations). Staining for Ki67 is far more successful after a brief formalin fixation with subsequent antigen retrieval. Results for proliferation indices for fresh tissue using this method broadly agree with those published for tissue prepared with paraffin-embedding (Hiroshima et al., 2002).

5.3 Conclusion

This is a novel and robust method for analysing the capillary bed and the extent of cellular proliferation in unfixed samples of human respiratory mucosa. This method will allow further investigation into the vascular response during tissue remodelling in human biopsies, and could thus help elucidate the aetiology and pathogenesis of chronic sinusitis, nasal polyposis, allergic rhinitis and asthma.

CHAPTER 6
ANGIOGENESIS IN NASAL POLYPOSIS

6 Angiogenesis in Nasal Polyposis

6.1 Introduction

Nasal polyps are oedematous, semi translucent, benign masses that develop from the mucosal linings of the paranasal cavity, usually originating from the mucosa in the osteomeatal complex.(Larsen and Tos, 2004) The aetiology and pathogenesis of nasal polyposis are far from clear, with suggestions of them being adenoma formations,(Billroth, 1885) arising from inflammation,(Bachert et al., 2000a, Jenkins, 1932) oedema,(Krajina, 1963) or epithelial rupture.(Tos, 1990) Recently, a number of studies have proposed a role for angiogenesis, based on upregulation of pro-angiogenic factors in polyps.(Caye-Thomasen et al., 2004, Ito et al., 1995b, Coste et al., 2000a)

The vascular endothelial growth factors (VEGFs) are a family of glycoproteins that are constitutively expressed in tissue and have an essential involvement in angiogenesis.(Ferrara, 1999) Five members have been identified so far, of which VEGF-A is the predominant angiogenic factor through binding to VEGFR2. VEGFR-2 binding initiates a large number of signalling pathways that stimulate endothelial cells to proliferate, migrate and/or survive, as well as leading to an increase in vascular permeability.(Gerber et al., 1998, Bates and Curry, 1997a, Rousseau et al., 2000, Takahashi et al., 1999) VEGF mRNA(Ito et al., 1995b) and protein(Yang et al., 2002) are up-regulated in nasal polyps compared to inferior turbinates, and localised to both epithelial and inflammatory cells, while primary cultures of epithelial cells from nasal polyps secrete VEGF.(Coste et al., 2000a) Other researchers have reported VEGF

expression in vascular endothelium and glandular cells in nasal polyps,(Guo et al., 2001b) so the expression and role of VEGF in polyposis is unclear, with both paracrine and autocrine functions a possibility.

In order for angiogenesis to occur, endothelial cells need to migrate, an action that is dependent on up-regulation of matrix metalloproteinases (MMPs).(Rundhaug, 2005) MMP expression is involved in many forms of angiogenesis,(Rundhaug, 2005, Haas et al., 2000) MMP expression has been observed in nasal polyps,(Lechapt-Zalcman et al., 2001, Watelet et al., 2004) and it has been suggested that the success of treatment with macrolide antibiotics is mediated by suppression of MMP activity, and hence angiogenesis.(Kanai et al., 2004)

All this evidence is circumstantial, and the presence of pro-angiogenic molecules does not automatically lead to angiogenesis(Milkiewicz et al., 2004). Angiogenesis is controlled by the balance of pro- and anti- angiogenic factors,(Folkman, 1995) so measurement of angiogenesis has to examine actual capillary growth. Angiogenesis is uniquely difficult to study in the nose, as the cycle of congestion and decongestion leads to large changes in tissue volume, which makes normal measures of capillary density meaningless without confirmation by other methods. In addition, polyposis frequently occurs alongside other airway diseases, such as asthma or chronic sinusitis, which often cause tissue remodelling. Any tissue remodelling will cause a restructuring of the vascular supply for that tissue, which would confound attempts to measure any angiogenesis associated with polyp formation alone.

Based on the current literature if polyposis is indeed driven by angiogenesis, there should be

changes in the morphology and architecture of the capillary bed, alongside proliferation of endothelial cells, similar to that seen in bowel polyps.(Balazs, 1990) If angiogenesis is not a major factor in the aetiology of polyp formation, then the capillary bed should expand in line with the metabolic needs of the tissue, and be morphologically similar to non-diseased tissue.(Papetti and Herman, 2002) The architecture and proliferation of the capillary bed was analysed, as well as capillary density, to provide unambiguous measures of the extent of angiogenesis in nasal polyposis.

6.2 Subjects

This study involved patients recruited from University Hospitals Birmingham NHS Trust, UK. Following local ethical committee approval (Numbers 0692 and 06/Q2702/15) and informed consent, polyp (P) and ipsilateral inferior turbinate specimens (PIT), 1 cm back from the anterior edge, were obtained from patients undergoing routine polypectomy (n=5, allowing at least 80% power for 95% confidence intervals in half the biological effect we would expect to see(Pritchard et al., 1995, Li and Wilson, 1997), based on power calculations of pilot data). Previous studies have shown there is no difference in vascularity in different areas of the lateral nasal wall(Philpott et al., 2005). Control inferior turbinate specimens (CIT) were taken from patients undergoing routine septoplasty or septorhinoplasty (n=5). Neither the control nor the polyp group had asthma, aspirin sensitivity or diagnosed allergies. All polyp patients had been previously given oral or nasal corticosteroids in accordance with best clinical practice. None were on oral corticosteroids within two weeks of surgery.

6.2.1 Tissue Preparation

Samples taken for confocal analysis were immediately fixed in 4% buffered formalin and left for 24 hours. Samples were then washed in 3 changes of phosphate buffered saline (PBS) for a further 24 hours before staining with 1:100 dilution of a fluorescein-conjugated lectin mixture (1:1:1 *Ulex europeaus*, *Griffonia simplicifolia*, *Euonymus europeaus*, Vector Labs) to account for different affinities associated with blood groups.(Capaldi et al., 1985) Samples were then washed and stored in PBS until being viewed under a confocal Leica DM IRE2 microscope. Vertical stacks of images (Z-stacks) were taken from multiple orientations, and single images measuring 1300 x 880 μm were taken from the same Z-stack separated by 50 μm . These were analysed by standard point and intercept counts(Egginton et al., 1993) with a stereological grid using squares of 64 μm^2 . Sample area, microvascular surface density (S_V , the surface area of capillaries compared to volume of the sample) or volume density (V_V , the total volume of capillaries compared to volume of the sample) were calculated using standard stereological counting procedures (Egginton et al., 1993). These were averaged for multiple levels in each Z stack and then averaged for different orientations of the same sample.

6.2.2 Immunohistochemical Staining

Samples taken for immunostaining were immediately snap frozen in liquid nitrogen-cooled isopentane (in theatre) and 8 μm sections cut on a cryostat. Sections were allowed to air dry before being fixed in 4% buffered formalin for 20 min. Antigen retrieval was performed in citrate buffer (10 mM citric acid adjusted to pH 6.0 with NaOH, 0.05% Tween 20) heated to 95°C for 5 min then allowed to cool to room temperature. Slides were then blocked for 30

min at room temperature in wash buffer (1.5% w/v BSA, 0.6% v/v Triton X-100 in PBS), then incubated for 2 hours with primary antibody (Ki-67, Dako) diluted 1:100 in wash buffer to label proliferating nuclei. Sections were then washed in PBS and incubated for 1 hour with secondary antibody (TRITC polyclonal rabbit anti-mouse, Dako) diluted 1:100 in wash buffer. Sections were rinsed in PBS and then incubated for 30 min with the lectin mixture to stain blood vessels. Sections were washed in PBS, rinsed with dH₂O and mounted using Vectashield containing DAPI (4',6-diamidino-2-phenylindole) to label all nuclei, and a proliferation index calculated as (Ki-67 count / DAPI count) for capillaries, interstitium and the epithelial layer.

Stained sections were viewed under fluorescent microscopy, with the number of blood vessels and nuclei in a 500 x 500 μm sample recorded. Two samples were taken randomly from each patient. Ki-67 staining was recorded as being either associated with blood vessels, or the interstitial space.

6.2.3 Statistical analysis

All data are presented as mean \pm S.E.M. Statistical significance between groups was performed using factorial ANOVA with post-hoc comparisons among groups using a 5% significance level. A self-blinded researcher took all measurements.

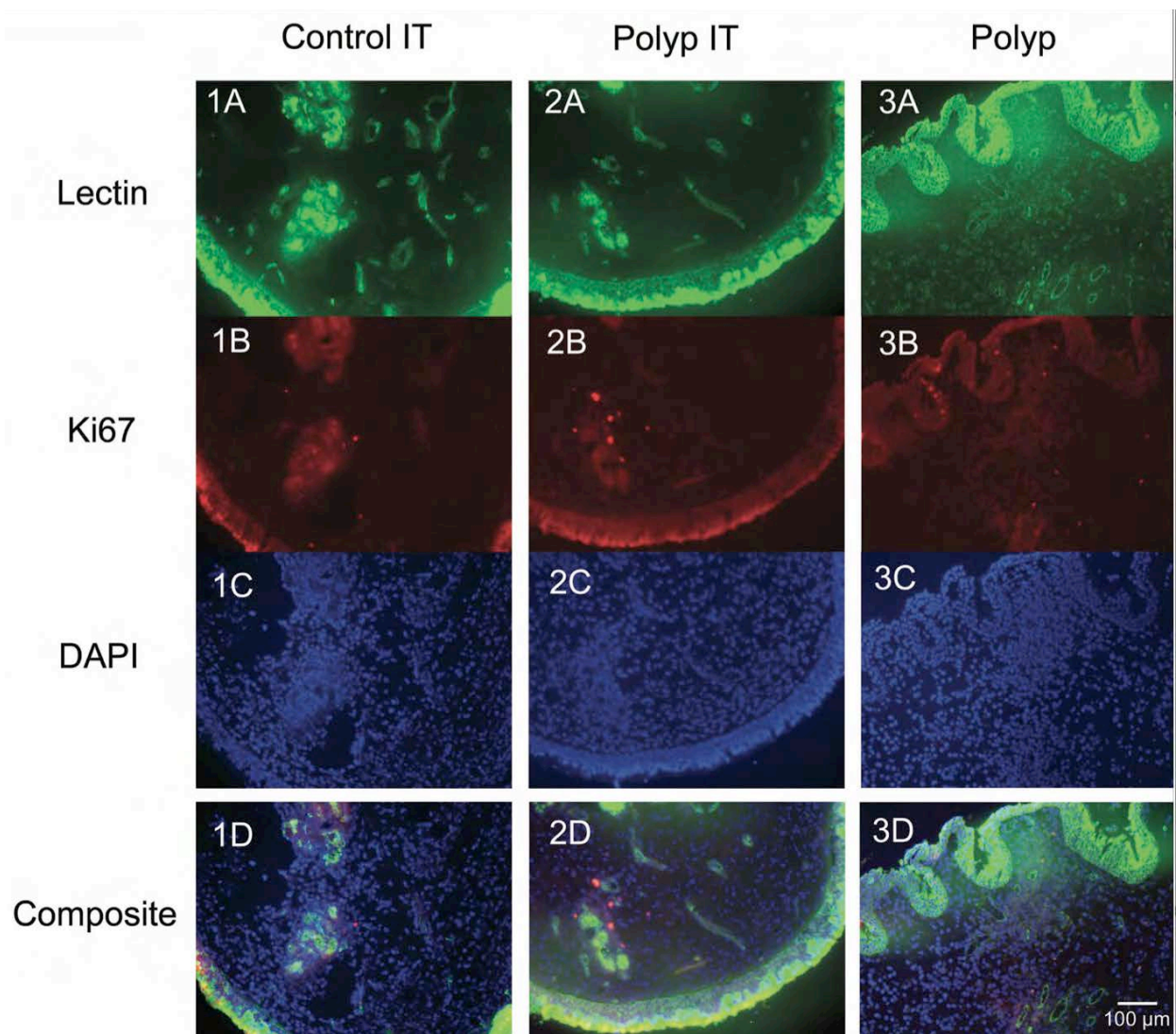
6.3 Results

6.3.1 Inferior turbinate samples

Histological sections showed no overt differences in inferior turbinate samples from patients with polyposis (PIT) to those from control patients (CIT) (Figure 6.1). Vessel density was not significantly different from control values of $35.2 \pm 4.4 \text{ mm}^{-2}$ (Figure 6.2). Proliferation indices for capillaries ($1.8 \pm 0.4 \times 10^{-3}$), the epithelium ($9.9 \pm 3.2 \times 10^{-3}$) and the interstitium ($76.3 \pm 26.4 \times 10^{-3}$) also showed no significant differences between inferior turbinate samples from the two groups (Figure 6.2, Table 6.I). Confocal microscopy showed no differences in the morphology of the capillary bed (Figure 6.3, Table 6.2).

Figure 6.1 Lectin, Ki-67 and DAPI staining showing capillary density and sites of proliferation

Panels show sections stained with a fluorescein-labelled lectin staining capillaries (A), rhodamine-labelled Ki-67 antibody staining proliferating nuclei (B), DAPI staining all nuclei (C), and an overlaid composite image (D)



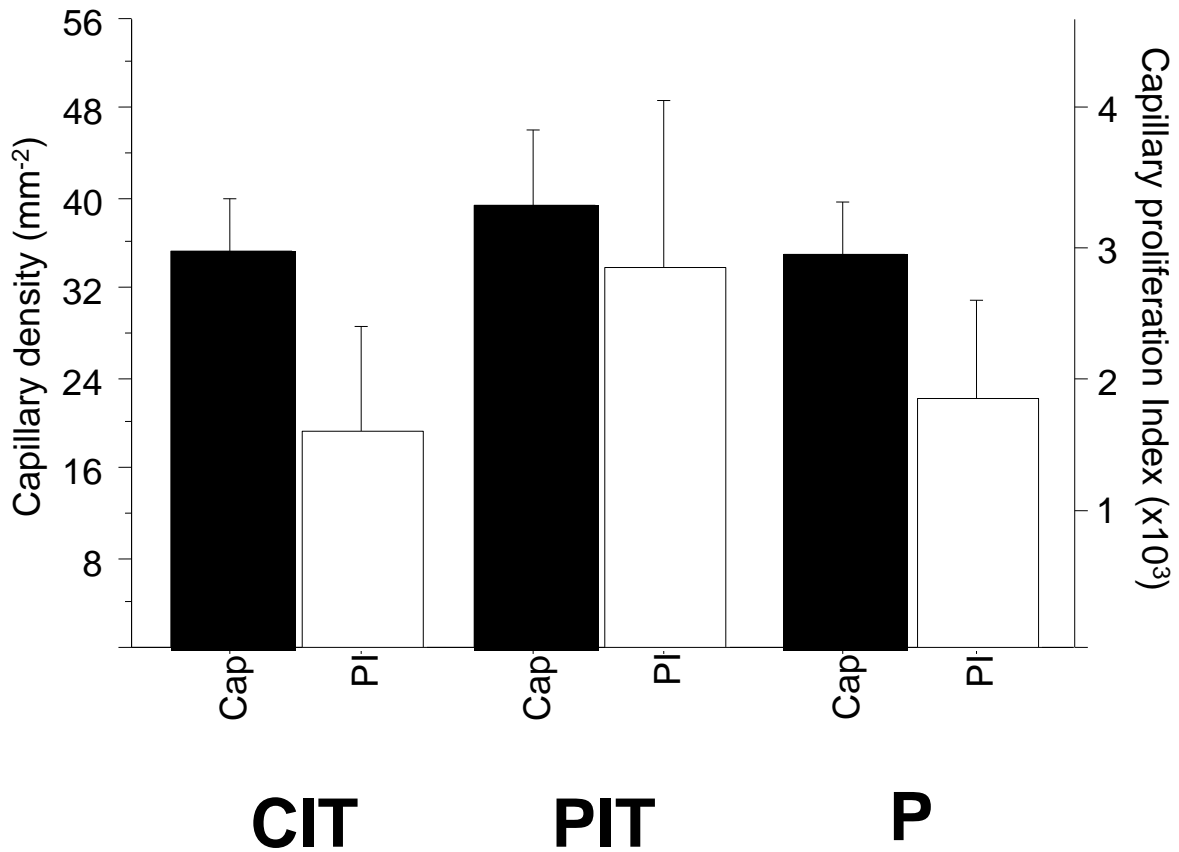


Figure 6.2 Capillary Density and Proliferation Index

Capillary density (Cap, black bars) and proliferation index (PI, white bars) are shown for inferior turbinates from control patients (CIT) and inferior turbinates (PIT) and polyps (P) from patients with nasal polyposis. Mean \pm SEM (n=4). *P<0.05 vs. control

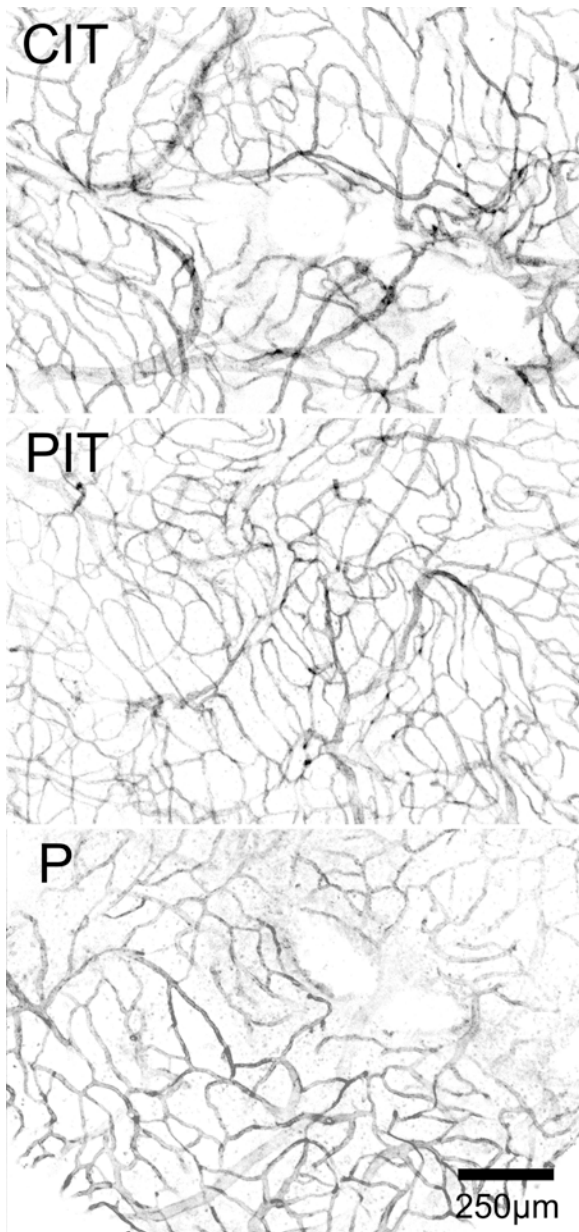


Figure 6.3 Morphological features of the capillary bed

Extended focus (30µm), colour inverted images of the capillary bed from inferior turbinates from control patients (CIT) and inferior turbinates (PIT) and polyps (P) from patients with nasal polyposis stained with fluorescein-conjugated lectin.

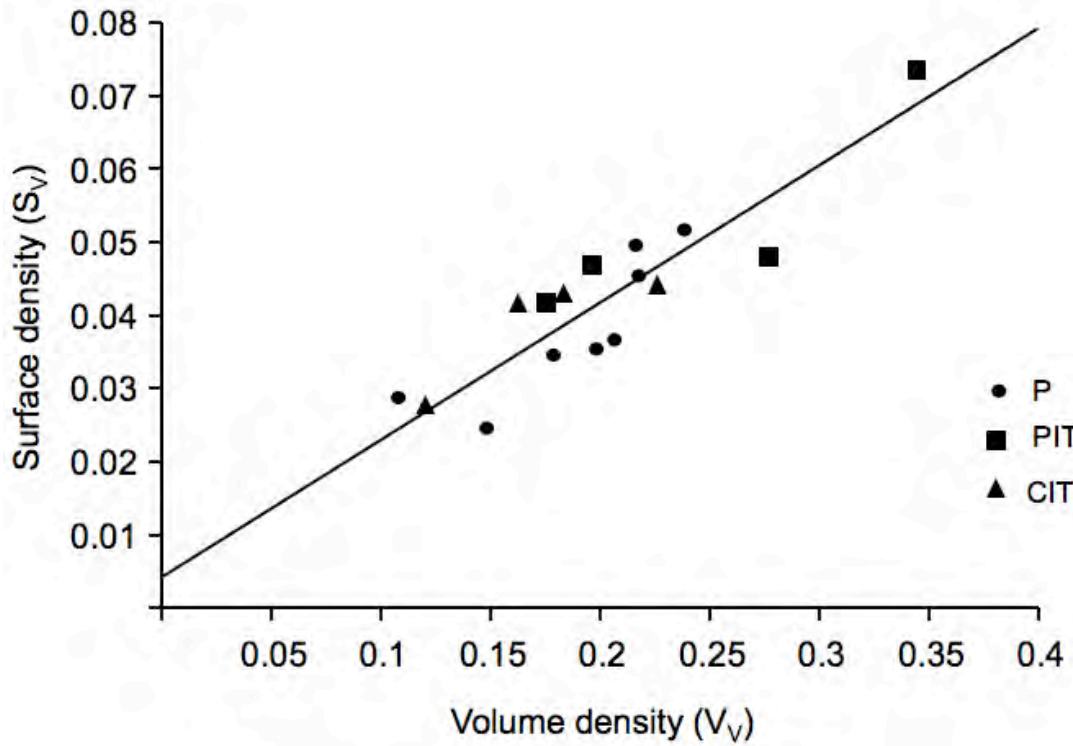


Figure 6.5: Comparison of mean capillary surface density vs capillary volume density

Regression of vascular surface and volume densities across groups. $S_V = 0.006 + 0.177 * V_V$;

$R^2 = 0.811$

Table 6.1: Proliferation indices for the epithelial and interstitial compartments

Interstitial and epithelial proliferation indices are shown for inferior turbinates from control patients (CIT) and inferior turbinates (PIT) and polyps (P) from patients with nasal polyposis. Mean \pm SEM (n=4). *P<0.05 vs. control

	Interstitial proliferation index ($\times 10^3$)	Epithelial proliferation index ($\times 10^3$)
CIT	9.9 \pm 3.2	76.3 \pm 26.4
PIT	13.9 \pm 5.2	73.2 \pm 31.3
P	86.8 \pm 20.8*	84.3 \pm 22.2

Table 6.2 : Stereological analysis of the capillary bed

Capillary diameter, volume density (volume of capillaries per unit area of sample) and surface density (surface area of capillaries per unit area of sample) are shown for control patients (CIT) and inferior turbinates (PIT) and polyps (P) from patients with nasal polyposis. Mean \pm SEM (n=4). *P<0.05 vs. control

	Capillary Diameter (μm)	Surface density (S_V) (μm^{-1})	Volume density (V_V)
CIT	12.4 \pm 0.3	0.042 \pm 0.003	0.184 \pm 0.016
PIT	13.0 \pm 0.5	0.052 \pm 0.014	0.243 \pm 0.038
P	16.5 \pm 0.6 *	0.037 \pm 0.010	0.189 \pm 0.019

6.3.2 Polyp samples

The vascular bed of the polyp was grossly different from the matched inferior turbinate sample, having distended vessels (Figure 6.1, Figure 6.3) with a greater mean diameter: 16.5 ± 0.6 vs $12.4 \pm 0.3 \mu\text{m}$ in P vs CIT, respectively; $P < 0.05$ (Table 6.2). However, vessel density and capillary associated cellular proliferation, commonly accepted measures of angiogenesis (Hudlicka et al., 1992), showed no change from the levels seen in the inferior turbinate (Figure 6.2), despite an increase in interstitial proliferation [$86 \pm 21 \times 10^{-3}$ vs $99 \pm 32 \times 10^{-3}$ (Table 6.1)]. Proliferation in the epithelial layer was unchanged from controls (Table 6.1). There was no significant difference in the polyp or matched inferior turbinate compared to control patients in either microvascular surface density (S_V) or volume density (V_V), although capillaries within polyps were dilated (Table 6.2). Importantly, the regression of volume density against surface density lies on the same line as that for the inferior turbinate samples, indicating no change in the architecture of the capillary bed (Figure 6.4).

6.4 Discussion

Angiogenesis is a complex process relying on a balance between pro- and anti- angiogenic factors, and can manifest in different ways in the same tissue. (Williams et al., 2006c) As angiogenesis can be a heterogeneous process, any assay of angiogenesis *in vivo* must rely on the final outcome of angiogenesis, namely an expansion of the vascular bed. This is uniquely difficult to perform in the nose, as the continuous cycle of congestion and decongestion makes

standard measures of capillary density difficult to interpret.

Consequently, this study used polyp samples (P) with matched inferior turbinate (PIT) samples from the same side of the same patient, at the same time point. These samples were also compared with inferior turbinate biopsies taken from healthy control patients (CIT), allowing comparison of polyps with both internal and external controls. None of the patients included in this study suffered from asthma, allergies or aspirin sensitivity. Airway remodelling in response to asthma and allergy may have angiogenesis or mucosal layer proliferation that is completely unrelated to the process of polyp formation (Watelet et al., 2006, Homer and Elias, 2005), which would have potentially confounded our results. Using a 'clean' patient group without these conditions allows us to examine whether angiogenesis is part of the aetiology of polyposis, or merely occurring as part of a separate pathology. Multiple measures of angiogenesis (capillary density, capillary-associated cell proliferation, capillary surface- and volume density) were averaged from multiple images, taken from multiple orientations (for parameters measured by confocal microscopy) in order to overcome any potential analytical bias, and provide a definitive measurement of angiogenesis in nasal polyps.

None of these parameters suggested that there was any abnormal or pathogenically active angiogenesis occurring in nasal polyps compared with either matched IT or healthy IT.

Capillary density was almost constant between the three groups of samples, suggesting that whilst angiogenesis must be taking place to allow growth of the polyp, the angiogenesis is only sufficient to maintain the capillary density required for nasal mucosal integrity and

function.

Capillary surface and volume density were similarly unchanged in the three groups, again showing that the angiogenesis seen in nasal polyposis results in a capillary bed with the same surface area and percentage volume as the normal mucosal network. Moreover, the regression of individual surface densities versus volume densities (Figure 6.4) is adequately described by a single line for all groups. Such a plot provides a way of measuring the overall architecture of the capillary bed. If nasal polyposis were a condition dependent upon active angiogenesis, the capillary sprouts and smaller daughter capillaries would give the sample higher surface density for a given volume density, resulting in a steeper regression line for the polyp samples. In addition, a single sample exhibiting a change in capillary bed architecture would show as an outlier, which would obviously show any heterogeneity in angiogenic response.

Sprouting angiogenesis requires proliferation of endothelial and perivascular cells to form new capillaries, therefore measurements of capillary-associated proliferation signals are often taken as an indirect measure of angiogenesis. In this study, capillaries from nasal polyps showed no increase in proliferation from the very low values seen in either matched IT or healthy controls. As mucosal epithelium has a high cell turnover rate this was used as a positive control, and also showed no significant change between our study groups (Table 6.1). Interestingly, interstitial proliferation (not associated with capillaries) was greatly increased in certain polyps, showing heterogeneity of cell division among samples. However, these highly proliferative polyps showed no increase in capillary-associated proliferation compared to other polyps (data not shown).

The initial hypothesis that angiogenesis is important in nasal polyposis was derived mainly from semi-quantitative immunohistochemical studies showing upregulation of VEGF,(Yang et al., 2002) MMPs,(Lechapt-Zalcman et al., 2001) bFGF (FGF-2),(Powers et al., 1998) and TGF- β (Elovic et al., 1994a) in nasal polyps compared to controls, with elevated bFGF also seen with a quantitative ELISA.(Norlander et al., 2001) Whilst this evidence supports a role for angiogenesis, it is far from conclusive, especially as upregulation of different factors is only seen in a proportion of cases. MMPs, bFGF and TGF- β have pleiotropic effects on various cell types,(Okada-Ban et al., 2000, Mott and Werb, 2004, Kim et al., 2005) and may well be important in the growth of the polyp itself. Indeed, our results show a significant level of proliferation in the polyp. However, the lack of active angiogenesis seen in this study suggests they are not acting to promote vessel growth over that required to support tissue expansion. VEGF is far more specific for endothelial cells, however it is constitutively expressed and changes in VEGF levels do not necessarily correspond to changes in capillarity,(Gavin et al., 2005, Milkiewicz et al., 2004) given that angiogenesis is a regulated, multifactorial phenomenon. VEGF can also have other effects: it was originally described as vascular permeability factor (VPF), and is 50 times more effective than histamine at dilating vessels.(Bates and Curry, 1997b) The 33% increase in capillary diameter seen in this study, and the oedematous nature of polyps is likely to be at least partially a response to the observed levels of VEGF. However, a threshold level of VEGF is required to initiate angiogenesis,(Williams et al., 2006a) and the semi-quantitative measures used to examine VEGF expression in polyposis give no indication that this threshold has been reached.

The maintenance of a normal vascular density and shape in a growing structure clearly requires some degree of angiogenesis. This, along with the angiogenesis seen in response to airway remodelling, may have led to the hypothesis that angiogenesis is part of the pathogenesis of polyposis. In nasal polyposis the rate of angiogenesis required to meet the physiological needs of the tissue is low and can be driven by metabolic or mechanical factors,(Hudlicka et al., 1992) rather than being an integral part of the pathology as it is in solid tumours, bowel polyps, psoriasis or retinopathy. Therefore, treatment of nasal polyps with anti-angiogenic therapies may reduce symptoms, but will not provide an effective treatment for polyposis.

6.5 Conclusion

No significant differences in capillary density, capillary-associated proliferation, capillary surface density or capillary volume density were seen between the three study groups, and the regression of surface density versus volume density described a linear relationship. Polyp samples showed increases in capillary diameter and interstitial proliferation.

These results show no active angiogenesis occurring in the polyp, or changes in capillary bed architecture, although capillaries seem more oedematous in the polyp. As the capillary supply increases in line with the physiological needs of the growing polyp, we conclude that angiogenesis is not a driving force in the aetiology of nasal polyposis.

This study examined capillary density, capillary-associated proliferation, and capillary bed morphology in nasal polyps and control tissue. None of these common measures of

angiogenesis have shown any difference between nasal polyps, matched inferior turbinate or control inferior turbinate samples. Thus, we conclude that angiogenesis is not a driving force in the aetiology of nasal polyposis, and research for an effective treatment should focus elsewhere.

CHAPTER 7
GENE EXPRESSION IN NASAL POLYPS

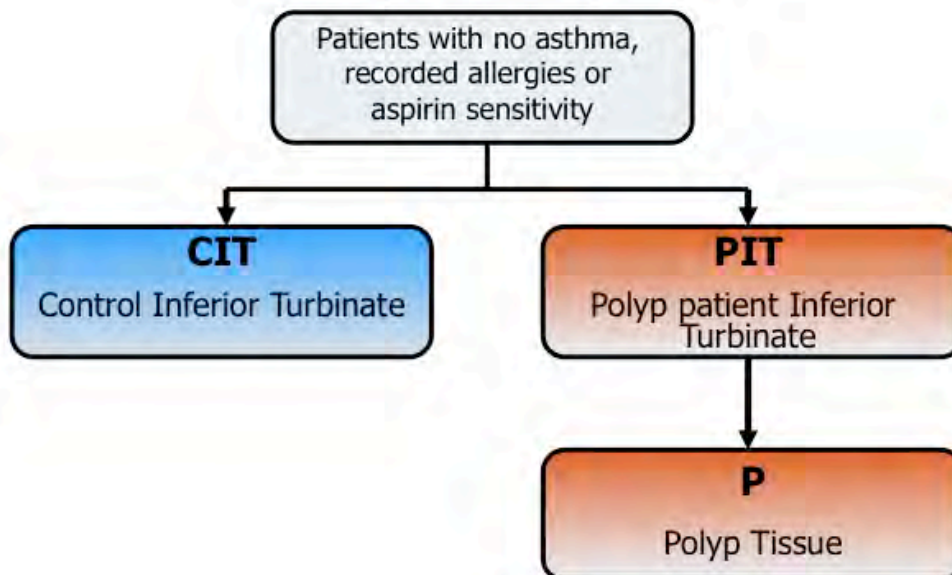
7 Gene expression in nasal polyps

In the work described in chapter 6 it became evident that angiogenesis is not the driving force in the aetiology of nasal polyposis. However, there remains a body of evidence that supports high levels of pro-angiogenic factors in nasal polyps with other authors having specifically identified higher levels of vascular endothelial growth factor (VEGF) – commonly thought to be the most important growth factor in angiogenesis (Ferrara, 2002, Ito et al., 1995b, Yang et al., 1998a, Coste et al., 2000a). However, this compound has multiple roles such as increasing vessel permeability (originally called Vascular Permeability Factor (Senger et al., 1983); and vasodilatation (Georgi et al., 2011). To investigate further the evidence for angiogenesis in nasal polyposis experiments were designed to look at gene expression, specifically investigating for known inflammatory and pro and anti angiogenic genes. There are different methods for screening for unknown genes that change their expression pattern, such as 2D protein gels, multiplex beads and mass spectrometry. They all have their own advantages but the simplest and fastest method is using gene microarrays, allowing insight into the potential biological response available by transcriptional regulation. A targeted microarray gene chip system was used containing 850 of the most common inflammatory, angiogenic and neoplastic genes. The study design was meticulous in having carefully selected internally matched samples with polyp and inferior turbinate specimens from the same patient from the ipsilateral nasal cavity compared with control inferior turbinate mucosa from patients without polyposis. None of the patients had a history of asthma or aspirin sensitivity as both are known to affect polyposis (Drake-Lee, 1987, Parikh et al., 2002). RNA was extracted from the tissue and converted to cDNA which was then used for the analysis.

7.1 Study Design

Nasal polyp and inferior turbinate biopsies were taken from each side of patients with bilateral polyposis, with the ipsilateral inferior turbinate acting as an internal control. Inferior turbinate samples were also taken from patients with no asthma or allergy undergoing routine septorhinoplasty, as an external control. Polyp patients with a history of asthma and/or allergy, or Samter's triad were excluded. These same groups were used for the microarray, microfluidics and the luminex experiments. N=5 in each of the 3 groups.

Sample Groups



7.2 cDNA production

A large polyp and control inferior turbinate biopsy was homogenised and RNA extracted by TRIzol (see Methods). The purity of the RNA was confirmed using Beta actin on an agarose gel (Figure 7.1) The quality of the cDNA was analysed by Northern blot after end-point primer amplification Figure 7.1

A variety of methods were attempted to get good-quality cDNA for analysis. Three different kits were used: Sensiscript reverse transcription (Sens), High capacity cDNA reverse transcriptase(HC) and Superscript reverse transcription as a control (Sup).

Sens and HC were tested with random hexamers (H) and oligo d(T) primers and a combination of the two (HT).

RNA was redissolved in 25 μ l ddH₂O, the RNA concentration was measured using a biophotometer (Eppendorf) and was found to be 1147 μ g/ml, which is 1147ng/ μ l.

2 μ l (2294ng) of this RNA solution was used in each RT reaction. Amplification of cDNA (see Methods) was performed on 1 μ l aliquots of Sens/HT, HC/HT and Sup (Sens/HT/Amp, HC/HT/Amp, Sup/Amp).

RT reaction products were purified using Qiaquick PCR purification kit before having their concentration measured using a biophotometer (eppendorf) (Table 7.1). 5 μ l aliquots were then run on a denaturing agarose gel (see Methods) and densitometrically analysed.

Figure 7.1 Agarose gel for Beta actin

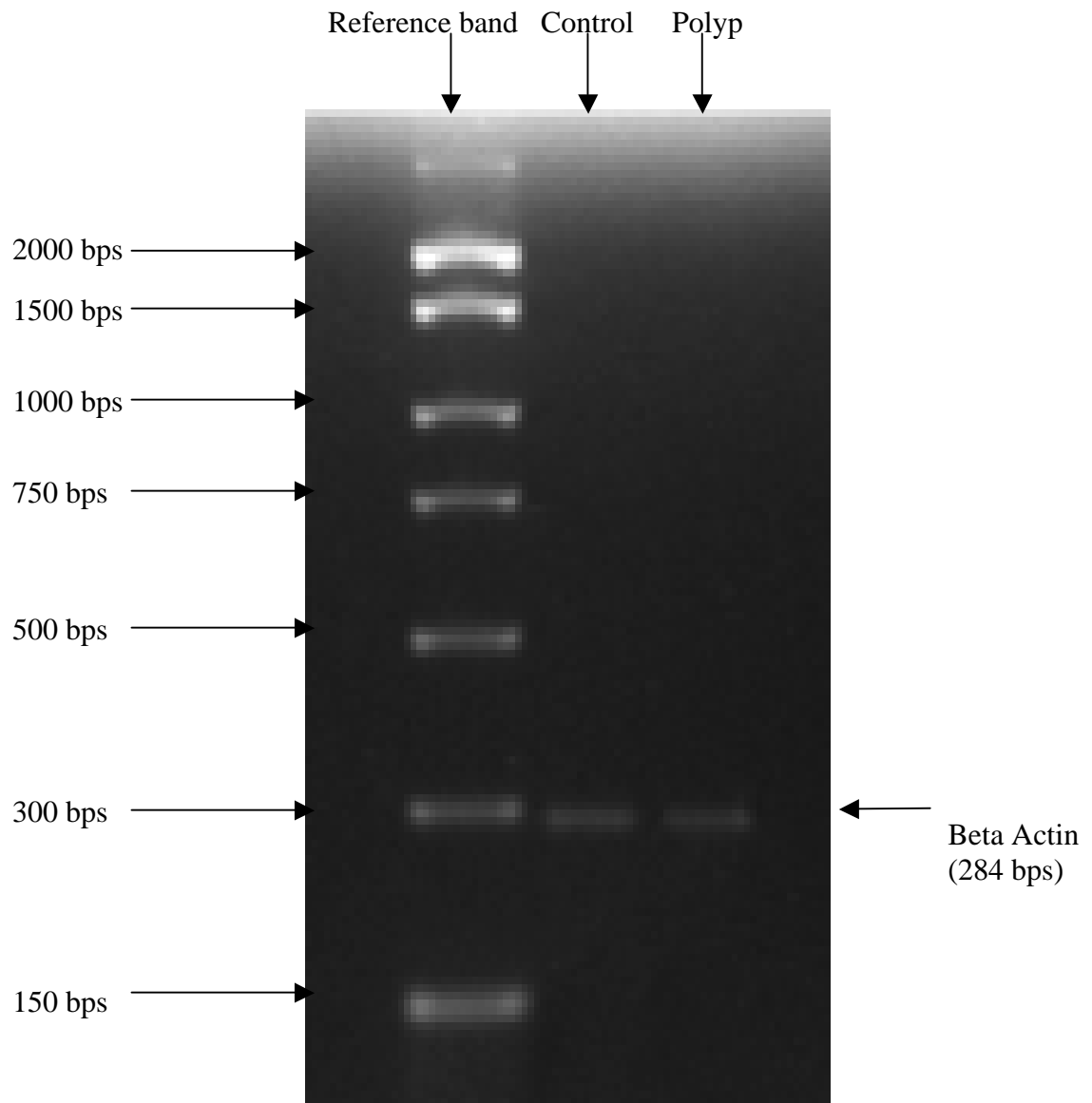


Table 7.1: cDNA Production

Sensiscript reverse transcription (Sens), High capacity cDNA reverse transcriptase(HC) and Superscript reverse transcription as a control (Sup).

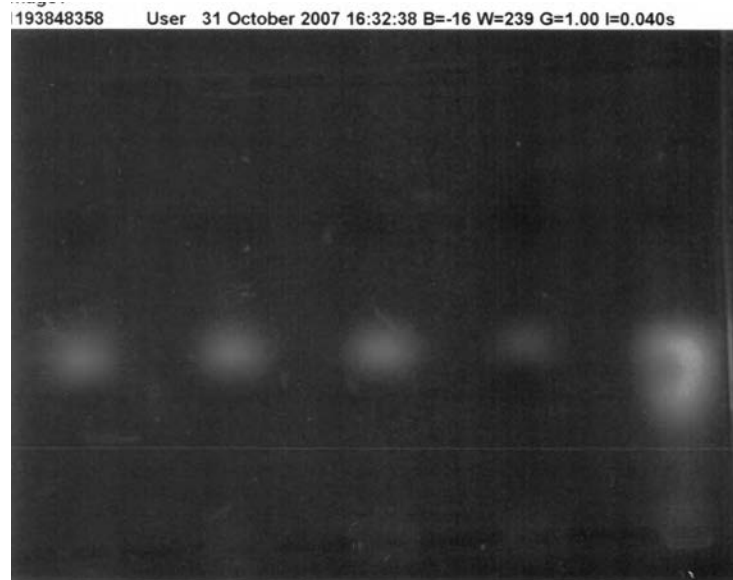
Sens and HC were tested with random hexamers (H) and oligo d(T) primers and a combination of the two (HT).

(Amp) is amplification using the Qiagen amplification kit.

Sample	ssDNA ($\mu\text{l/ml}$)
Sens/T	26.6
Sens/H	22.4
Sens/HT	15.6
HC/T	36.8
HC/H	38.8
HC/HT	23.9
Sup	15.6
Sens/HT/Amp	3.9
HC/HT/Amp	4.7
Sup/HT/Amp	76.4

As cDNA concentration gives a crude measure of how much PCR product has been obtained, the size and purity of the PCR products were assessed by running 5 μl aliquots on a denaturing agarose gel (see Methods) and densitometrically analysing the results.

Figure 7.2 Native electrophoresis gel – Total cDNA from primer amplification in Table 7.1



All the different methods of reverse transcription yielded bands at roughly the same point on the agarose gel, indicating that the PCR products obtained were of similar size, so there was little difference in cDNA fragment length with the different methods of cDNA synthesis.

There was a good correlation between relative cDNA concentration measured densitometrically from the gel, and cDNA concentration measured photometrically.

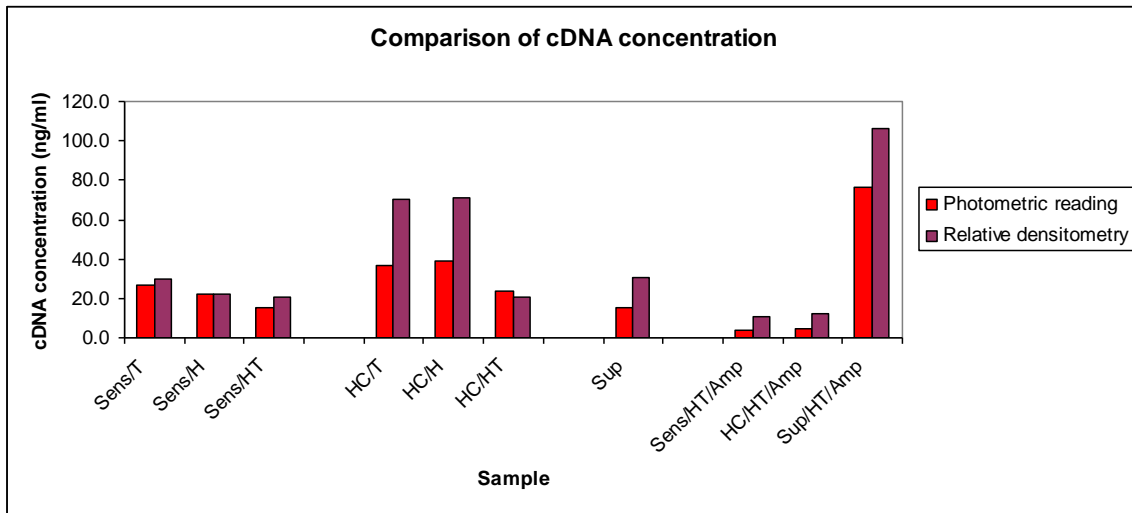


Figure 7.3: Comparison of cDNA concentration

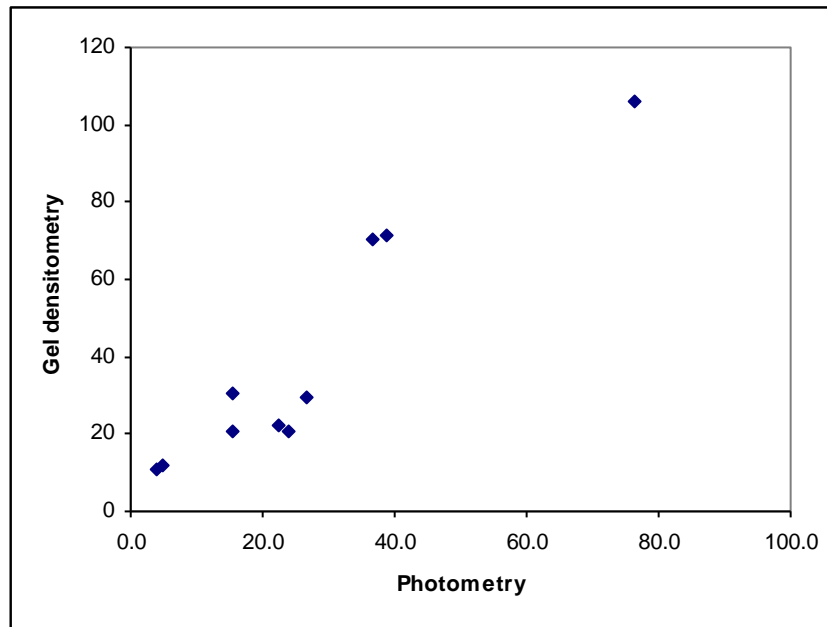


Figure 7.4: Gel densitometry vs. Photometry

Gel densitometry is a way of measuring the relative density of the electrophoresis bands. Photometry is from cDNA samples loaded giving a measure of sample concentration before electrophoresis. The graph confirms fairly good correlation.

7.2.1 Primer efficiency test

The quality of cDNA was assessed by measuring how well the samples amplified TGF-beta primers (known to be found in nasal polyps (Guo et al., 2001a, Ahn, 2001, Elovic et al., 1994b)). This was done using end-point primer amplification (see Methods), with PCR products purified using the Qiaquick PCR purification kit. Samples were run on a denaturing electrophoresis gel to check product size and band integrity.

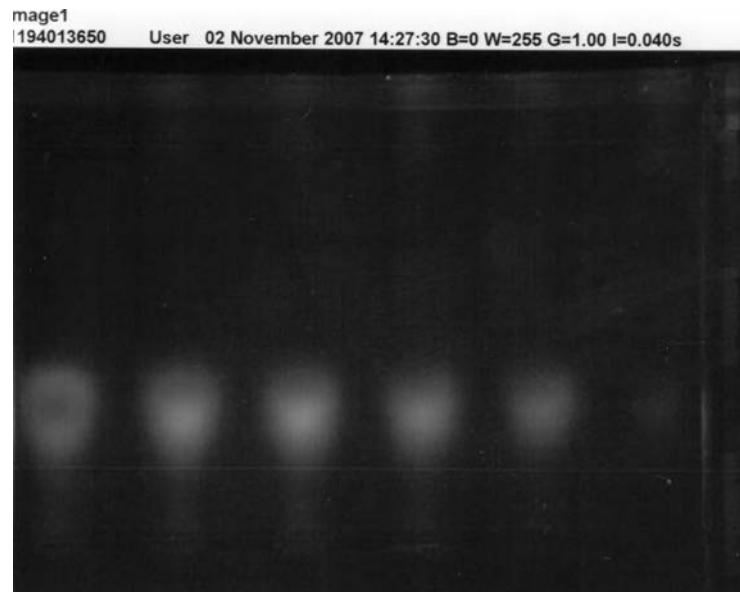


Figure 7.5: Denaturing electrophoresis gel – TGF beta

Table 7.2: TGF-Beta densitometry yield

Sensiscript reverse transcription (Sens), High capacity cDNA reverse transcriptase(HC) and Superscript reverse transcription as a control (Sup).

Sens and HC were tested with random hexamers (H) and oligo d(T) primers and a combination of the two (HT).

(Amp) is amplification using the Qiagen amplification kit.

Sample	TGF-beta Densitometry
Sens/T	6403
Sens/H	6940
Sens/HT	8201
HC/T	4927
HC/H	847
HC/HT	5260
Sup	4240
Sens/HT/Amp	6982
HC/HT/Amp	4830
Sup/HT/Amp	7023

On the basis of these results Sens/HT, HC/T, HC/HT and Sup/HT/Amp were run on a microfluidics card.

7.3 Gene Microarray

Gene microarrays:

RNA was extracted from samples using the TRIZOL method. 1st strand synthesis was performed using the SMART system kit with PCR product clean up. For the gene arrays, DNA was dye-labelled using a cyanine dye and spotted on the arrays. Positive control gene values were obtained from human buffy coat fraction.

Raw data is in 1 vs 1 list. Primary component analysis (PCA) plots are measures of similarity to the overall mean of all the samples. The groups are different colours with CIT blue, PIT green and P red. The x-axis represents 90% of the variability, with the y-axis showing 90% of the remaining, and the z the final. This is basically to give a visual separation akin to a logarithmic scale.

We used Significance of Analysis of Microarrays (SAM, Stanford) and Prediction Analysis of Microarrays (PAM, Stanford) statistical analysis software packages. PAM is a technique for class prediction from gene expression data using nearest shrunken centroids (Tibshirani et al., 2002). The method of nearest shrunken centroids identifies subsets of genes that best characterise each class.

From the data we produced Lorenz plots and cluster heatmaps of the data.

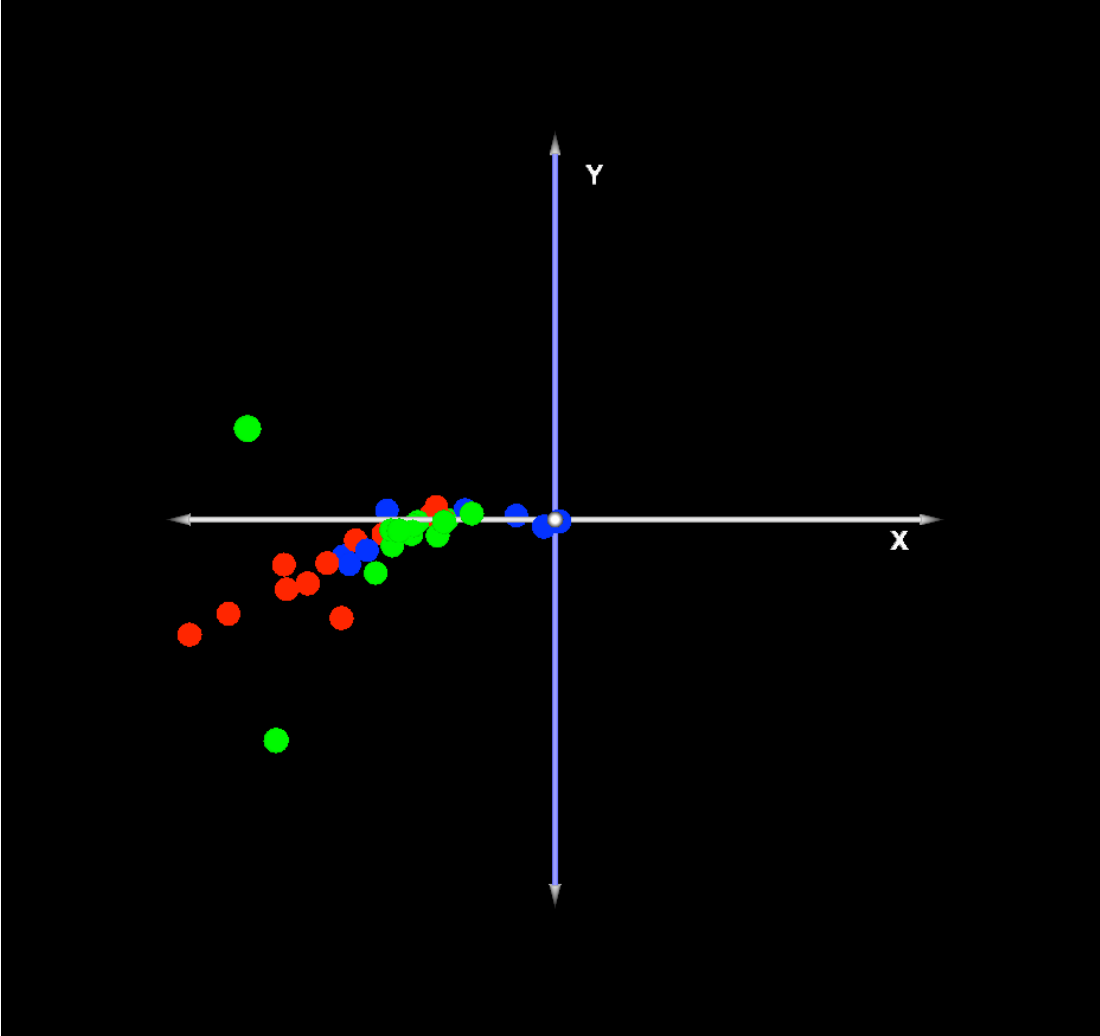


Figure 7.6: Primary Component Analysis plots angle 1. The groups are different colours with CIT blue, PIT green and P red

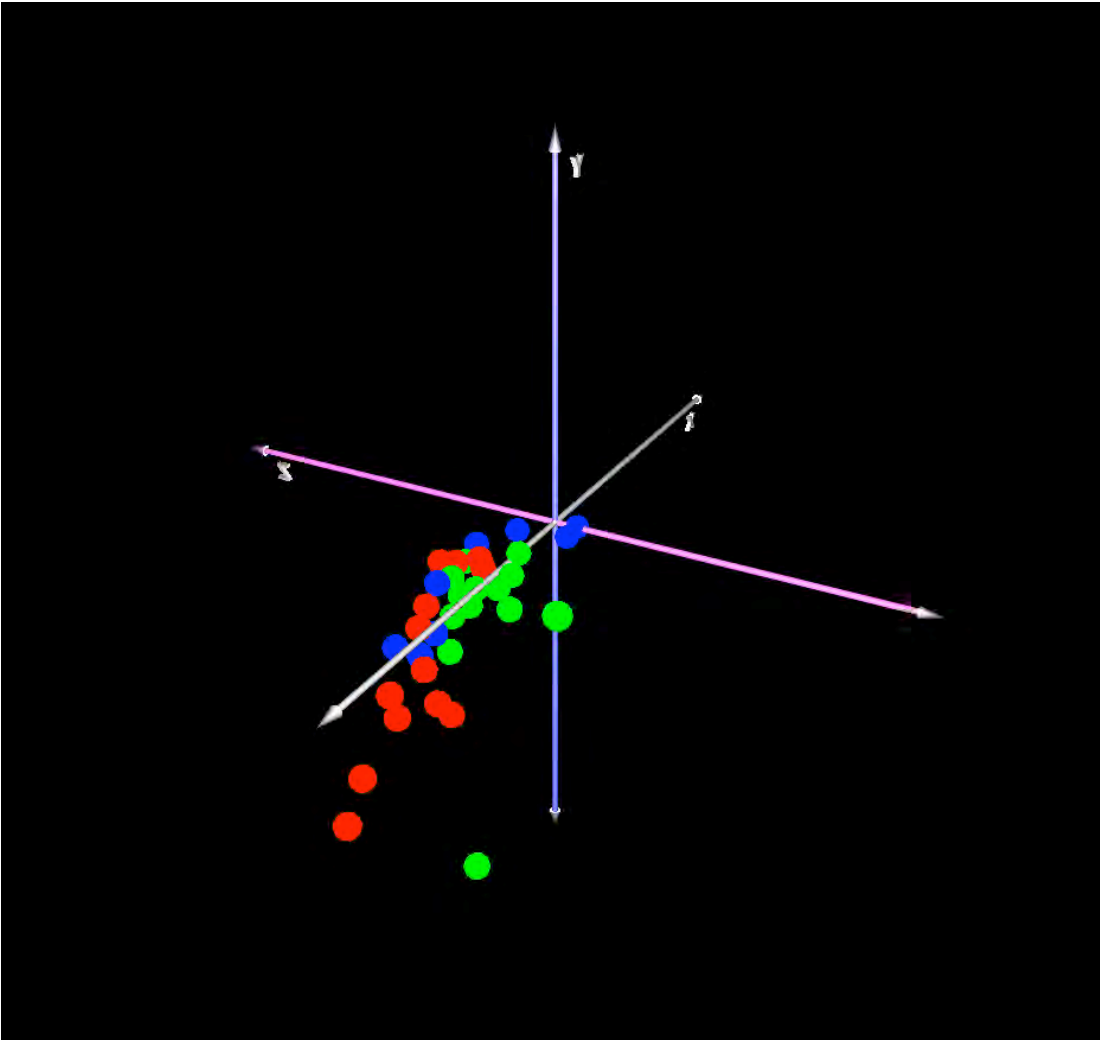


Figure 7.7: Primary Component Analysis plots angle 2. The groups are different colours with CIT blue, PIT green and P red

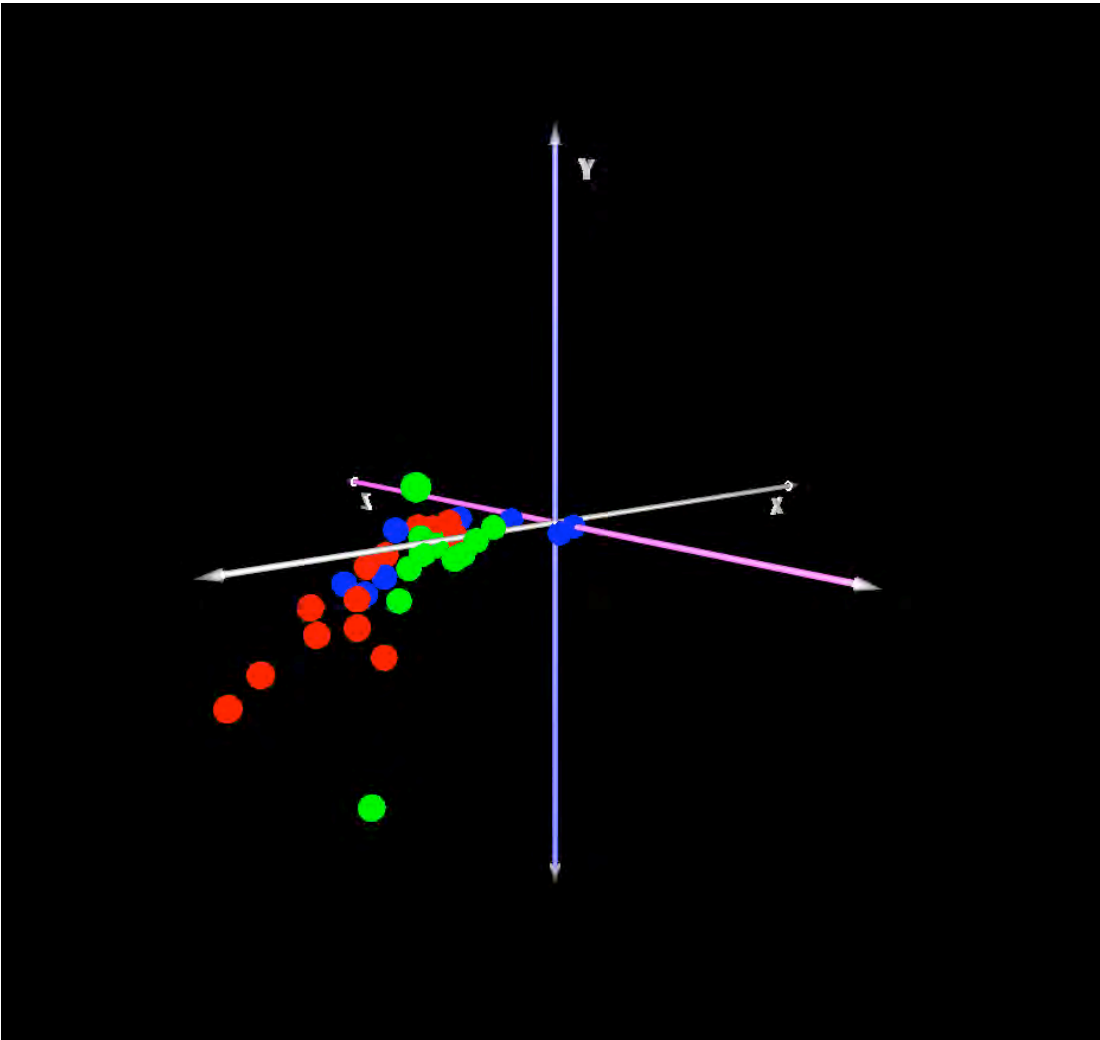
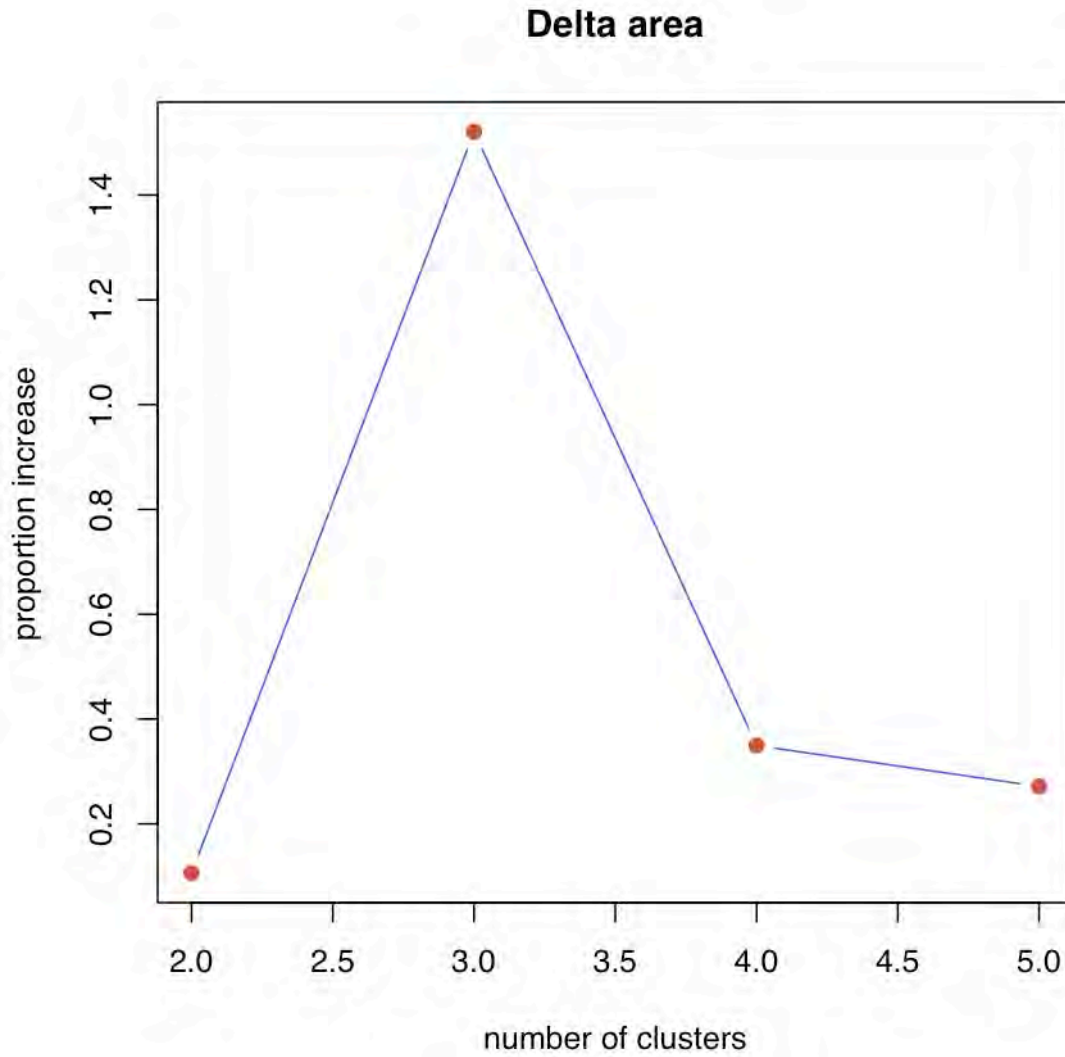


Figure 7.8: Primary Component Analysis plots angle 3. The groups are different colours with CIT blue, PIT green and P red

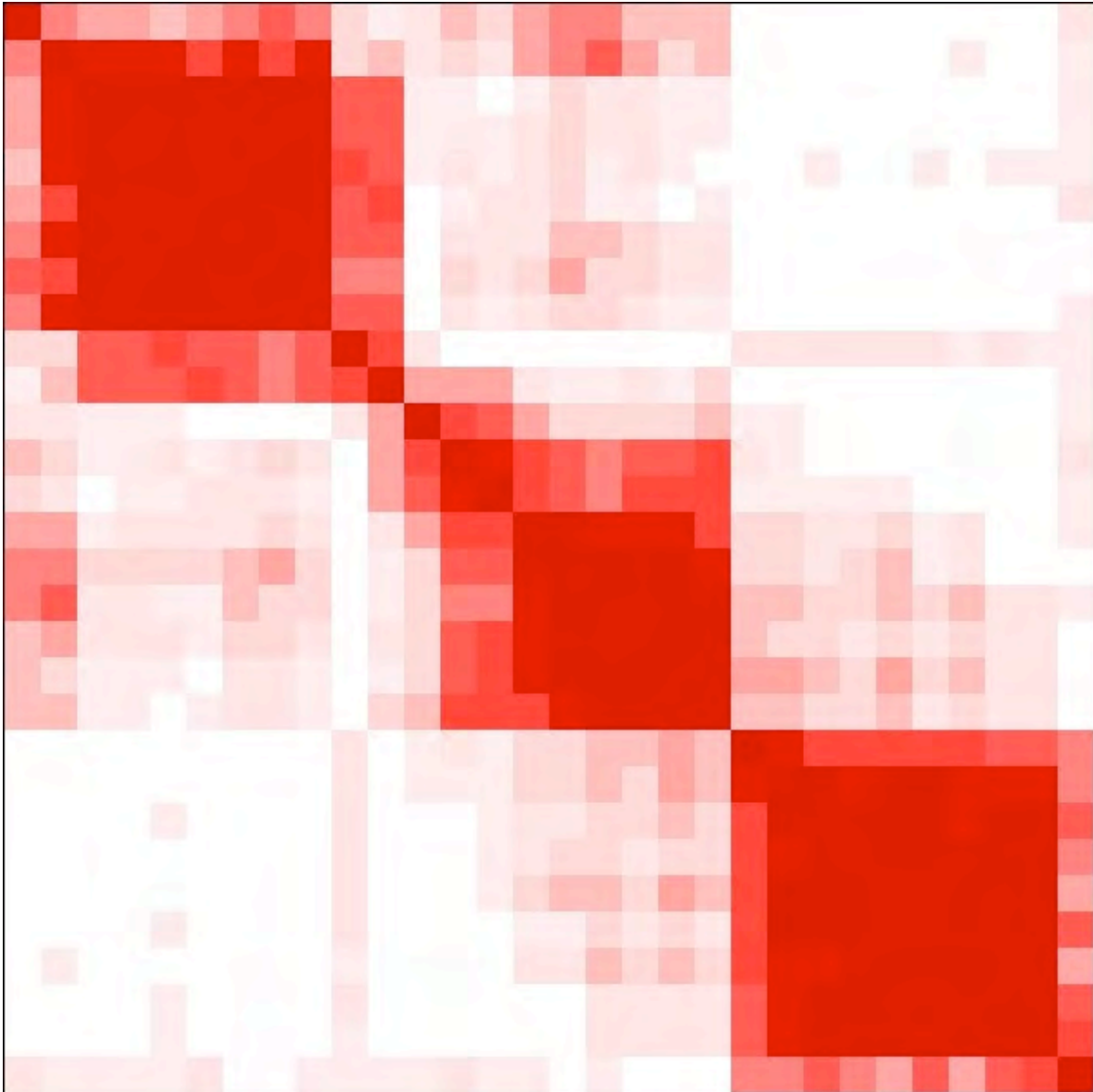
Approximate clustering hints that they are fairly distinct, but there is a definite inter-patient variability, as one would expect. A Lorenz plot was drawn to analyse how the samples grouped together.

Figure 7.9: Lorenz plot – measure of cluster



The peak in delta area at 3 clusters confirms that the samples group into 3 and confirms quality of the samples used in the gene array.

Figure 7.10: Cluster heatmap of predictive analysis of the gene array data.



This demonstrates that the samples from the 3 groups in the gene array (P, PIT and CIT) all cluster separately confirming the quality of our samples and data suggesting a single mechanism for polyposis as the clusters are fairly tight.

It is not possible to determine which group is which.

Table 7.3: PAM analysis showing genes above threshold in order of the highest magnitude of change in the three groups from microarray data.

P vs PIT	P vs CIT	CIT vs PIT
iNOS	iNOS	TACSTD2
iNOS	IRS2	TACSTD2
iNOS	PARC	TRA1
PARC	EMP3	RPS3A
TACSTD2	TNFRSF21	HSPCA
TP53		VDAC1
	Beta-	
VDAC1	2_Microglobulin	VDAC2
IFNAR1	PRKCA	CYC1
TNFRSF14	PARC	HLA-A_0201
TERF2IP	EP300	FKSG2
RBL2	IAP-1	GALECTIN_3
IL-1_R_antagonist	CLU	HLA-A_0201
PDCD6	L19	NSMAF
Transferin_R	CBLB	TRANK
PARC	CLU	RbAp48
IAP-1	FRACTALKINE	PIG7
TACSTD2	IGF1	TRA1

The repeats are different spots and should be seen several times above our (arbitrary and semi-quantitative) threshold level

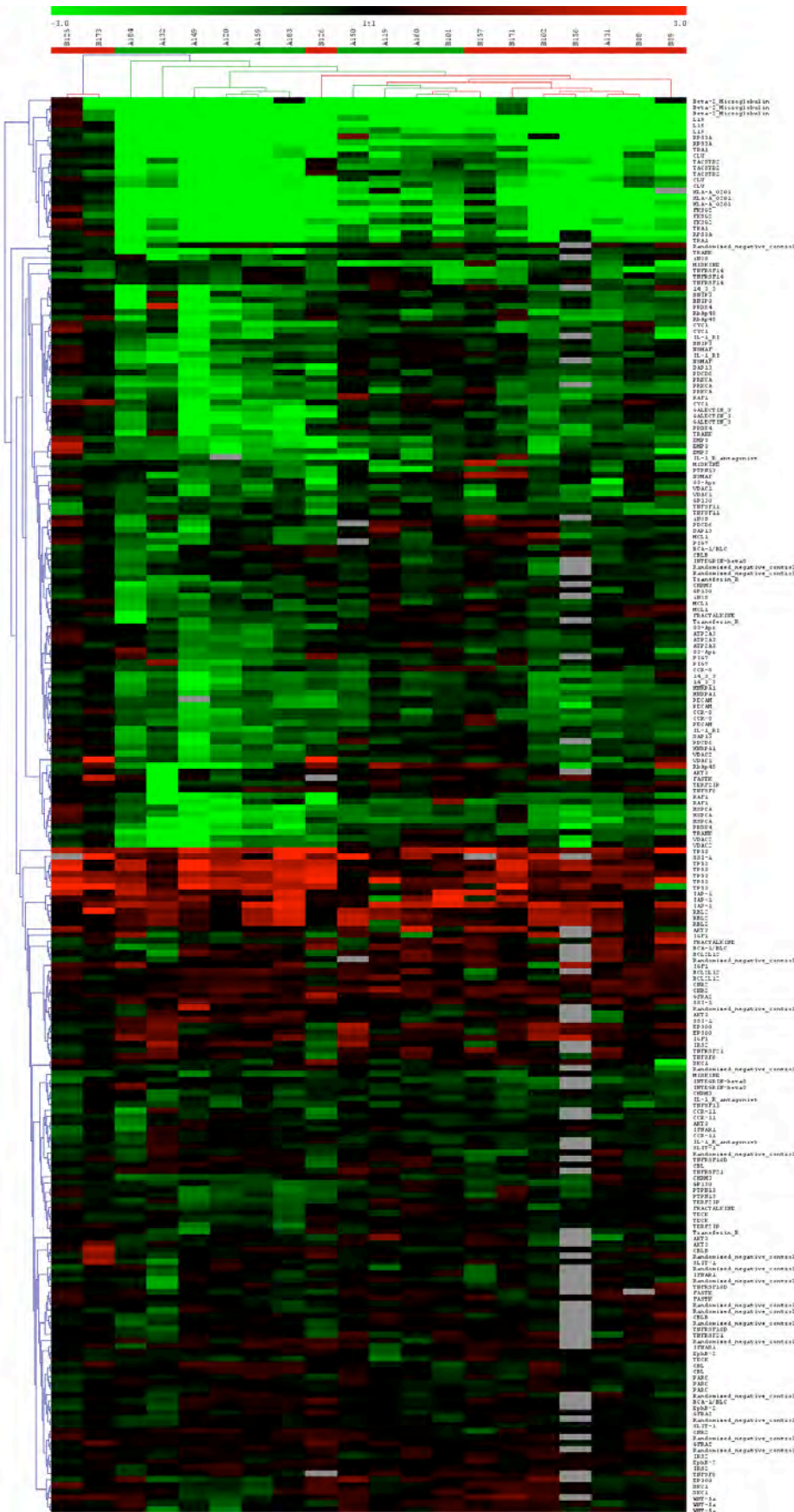


Figure 7.11:
Stanford's standard analysis of microarrays (SAM) and predictive analysis of microarrays (PAM) of CIT v PIT

Key to gene colours

Black = unchanged
 Red = PIT downregulated
 Green = PIT upregulated

Figures 7.11 and 7.12 are graphical representations of changes in gene expression between inferior turbinates from control patients (CIT) and inferior turbinates (PIT) with matched polyps from patients with nasal polyposis. Genes are shown more green where the expression is higher in the second named group, more red where the expression is lower, and black where no change is detected.

There were significant differences between all sample groups, however there was a greater difference between the control and polyp patient inferior turbinate tissue than there was between the polyp patient inferior turbinate and the matched polyps. The greatest changes were seen in genes associated with cell cycle control, apoptosis, cell signalling, inflammation, and tissue remodelling. The greatest change between the polyp tissue and matched inferior turbinate tissue was in the level of inducible nitric oxide synthase (iNOS, NOS2) (See Tables 7.4 and 7.5).

Table 7.4: From the PAM analysis (Table 7.3) we identified genes that showed the most change, listed in order of magnitude - PIT v Polyp group.

Changed in Polyp	Name	Other Names
iNOS		iNOS/NOS2
PARC		CKb7; PARC; AMAC1; DCCK1; MIP-4;
PDCD6		AMAC-1; DC-CK1; SCYA18
TNFRSF14	Programmed cell death 6	ALG-2; MGC9123
		LTg; TR2; HVEM; LIGHT
SREBF2	Sterol regulatory element binding transcription factor 2	SREBP2
TP53		P53; p53; TRP53
MadCAM-1		CD34
REELIN		
AKT3		
AKT3		
CAD-8		CDH8
		HM89; LAP3; NPYR; WHIM; LESTR;
		NPY3R; fusin; HSY3RR; NPYY3R;
CXCR-4		D2S201E
		LEC; LMC; NCC4; CKb12; HCC-4;
		LCC-1; Mtn-1; NCC-4; SCYL4;
HCC-4		ILINCK; SCYA16; CCL16
	Insulin-like growth factor binding protein 4	
IGFBP4		
ILF1	Interleukin enhancer binding factor 1	FOXK
TNFRSF21		DR6; BM-018
BAK1	BCL2-antagonist/killer 1	BAK; CDN1; BCL2L7
	BCL2/adenovirus E1B 19kD	
	interacting protein 2	NIP2; BNIP-2
BNIP2		CDK1
CDC2		HGFR; RCCP2
C-MET		
C-MYC		
EphA_4		
EphrinB_2		
IRS2		

Table 7.5: From the PAM analysis (table 7.3) we identified genes that showed the most change, listed in order of magnitude – CIT v PIT group.

Changed in PIT	Name	Other Names
EMP3	Epithelial membrane protein 3	YMP
HLA-A_0201		HLA-A
MPL_R		MPLV; TPOR; C-MPL; CD110
PRKCA		PKCA
PTPN13	Protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)	PNP1; FAP-1; PTP1E; PTPL1; PTPLE; PTP-BL; PTP-BAS
Beta-2_Microglobulin		B2M
FRACTALKIN		NTN; NTT; CXC3; CXC3C; SCYD1;
E		ABCD-3; C3Xkine
GMF-beta	Glia maturation factor beta	
TRANK	?see PRDX4	AOE37-2
CLU	Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed pro	CLI; APOJ; SGP2; SGP-2; SP-40; TRPM2; TRPM-2
ENA-78		ENA78; SCYB5; ENA-78; CXCL5
NAIP		BIRC1
TNFSF6		FAS; APT1; CD95; APO-1; FASTM GAL3; MAC2; CBP35; GALBP;
GALECTIN_3		LGALS2; LGALS3
HSPCA		HSPN; LAP2; HSP86; HSPC1;
L19		Hsp89Hsp90; HSP90A; HSPCAL1;
PRDX4		FLJ31884
RPL7		RPL19
SRP72	Signal recognition particle 72kD	AOE37-2
TRA1	Tumor rejection antigen (gp96) 1	RPL7
BAG5	BCL2-associated athanogene 5	ECGP; GP96; GRP94
BECN1	Beclin 1 (coiled-coil, myosin-like BCL2 interacting protein)	BAG-5; KIAA0873
Cyclophilin_A		CYPA; CYPH
DAP13	13kDa differentiation-associated protein	NDUFA12
DAP3	Death associated protein 3	DAP-3; MRPS29; MRP-S29; bMRP-10

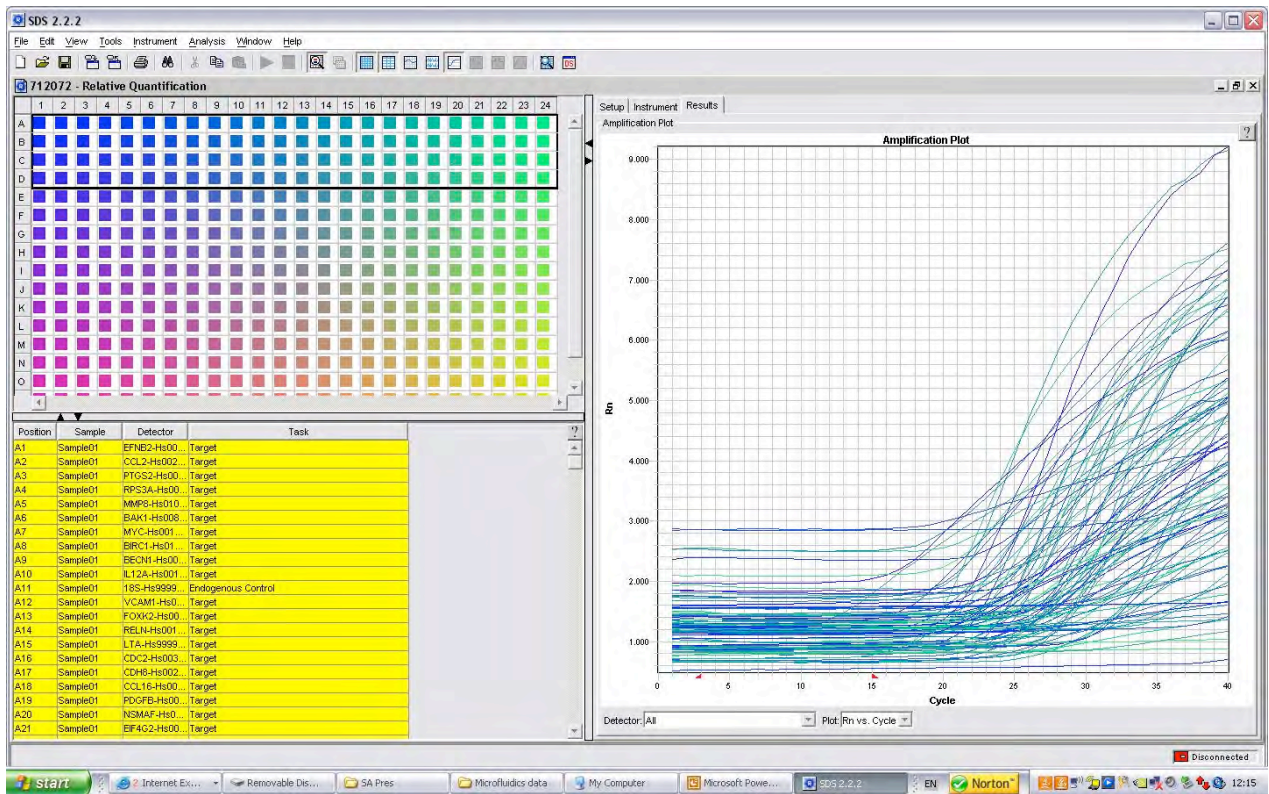
7.4 Microfluidics

Multiplex, real-time PCR (RT-PCR) is a powerful tool that enables simultaneous quantification of control and target genes in the same reaction. The technique not only allows reliable normalization of target gene levels, but also conserves sample material.

In real-time PCR, labelled nucleotides are attached to a quenching molecule that gets detached when the nucleotide is incorporated into RNA, releasing the fluorescent label. Thus, the more RNA there is in a sample, the greater the fluorescence in the well. This initially increases exponentially as each new copy gets replicated along with the existing copies. A threshold value is either manually chosen, or generated by the software, and the cycle number where the curve crosses this value is called the threshold cycle, or C_T . Comparison of expression profiles of genes passing C_T allows quantitation relative to an endogenous control. The difference between the C_T value of the sample, and that of the reference is called the ΔC_T . ΔC_T is directly proportional to number of copies of mRNA in the original sample.

The first analysis used computer-generated C_T values, compared to C_T for the 18S RNA control on the array. This was cleaned up with all blank values deleted, then put into Statview. Unfortunately, Statview was unable to handle this much data, so genes were split into 6 families, and an ANOVA performed using a Bonferroni correction with an alpha value calculated from the Bonferroni equation. There were no statistically significant results. Other post-hoc ANOVA tests, such as Scheffe's also gave no significant result.

Figure 7.13: Curves of fluorescence vs cycle number – RT-PCR



Real-time quantitative PCR data from microfluidics

Panel shows the raw data from a 96-gene custom-made microfluidics card, showing PCR cycle number (x-axis) vs fluorescence intensity (y-axis). The copy number of each gene increases with each PCR cycle, and this increases the fluorescence in the corresponding well. The earlier the cycle at which the fluorescence hits a threshold level, the greater the number of copies of the gene in the original sample.

The genes with the greatest magnitude of change in the gene array (iNOS, TNF, IL-5) were given manual C_T thresholds and reanalysed using t-tests. This confirmed a trend with a P value of < 0.1 but again not being statistically significant.

The software supplied with the PCR machine can also generate “Average ΔC_T values” which can calculate a ΔC_T value by extrapolating curves, and thus generate information where a clear curve crossing a threshold value is difficult to obtain.

The data was analysed using both techniques described above, with very similar non-significant results.

As the 18S RNA results could have been the problem, all the C_T values were taken, and the data for each plate averaged, and this was used as the control (centroids technique). Data was analysed as before, with much the same results.

Finally, the data was put through Stanford’s significance of microarrays software (SAM), which we used for the gene arrays. These gave similar non-significant results despite the samples all being paired with the microarrays.

7.5 Discussion

The gene array SAM and PAM analysis (Figures 7.11 and 7.12) showed more green coloured squares in figure 7.11 (CIT vs. PIT) than Figure 7.12 (PIT vs. Polyp), indicating that polyposis is a more global phenomenon affecting the mucosa with some additional changes causing polyposis. This in part is expected, as the PIT and CIT are different patients whereas the gene changes detected between PIT and Polyp are within the same patient with the gene changes representing the change mucosa over the inferior turbinate to nasal polyp just a centimetre or so away in the ipsilateral nasal cavity. The CIT vs. PIT analysis showed changes with greatest magnitude in genes associated with cell cycle control, apoptosis, cell signalling, inflammation and tissue remodelling. The PIT vs. Polyp analysis showed changes in similar families of genes, however, the largest change seen was in the gene for iNOS.

This changes the view that nasal polyposis is an angiogenic driven disease.

This data were then used to design a microfluidics array to measure the changes in gene expression, in order to quantitatively validate the broader perspective given by the microarrays.

The lack of significance of any difference in the common angiogenesis genes in the microarray may be due to biological variability (inter-patient variance) or confounding issues: age, gender, other morbidities.

CHAPTER 8
PROTEOMICS

8 Proteomics

8.1 Method development: TRIZOL protocol

TRIZOL reagent allows simultaneous extraction of RNA, DNA and protein from the same samples, however the traditional method (recommended by the manufacturer) of extracting protein then redissolving with 1% SDS is very inefficient, and leaves traces of SDS that may interfere with subsequent analysis. Redissolving proteins with 9.5M Urea with 2% CHAPS ([3-[(3-cholamidepropyl)-dimethylammonio] propane sulfonate]) has been reported to greatly improve the both quantity of protein recovered and number of proteins detectable by SELDI (Surface Enhanced Laser Desorption/ Ionization) analysis, compared to 1% SDS, 10% acetonitrile, and 1% Triton X-100 (Man et al., 2006). Precipitating the protein using acetone rather than isopropyl alcohol increases protein recovery by about 15% according to the manufacturer of a similar product (<http://www.mrcgene.com/tri.htm>), however this has not been shown with TRIZOL reagent. A comparison of acetone and isopropyl alcohol was therefore performed on 2 equal aliquots (100 μ l) of the same sample with resuspension in urea/CHAPS, repeated once.

	1st Sample	2nd Sample
Isopropyl alcohol	0.341	0.354
Acetone	0.385	0.367

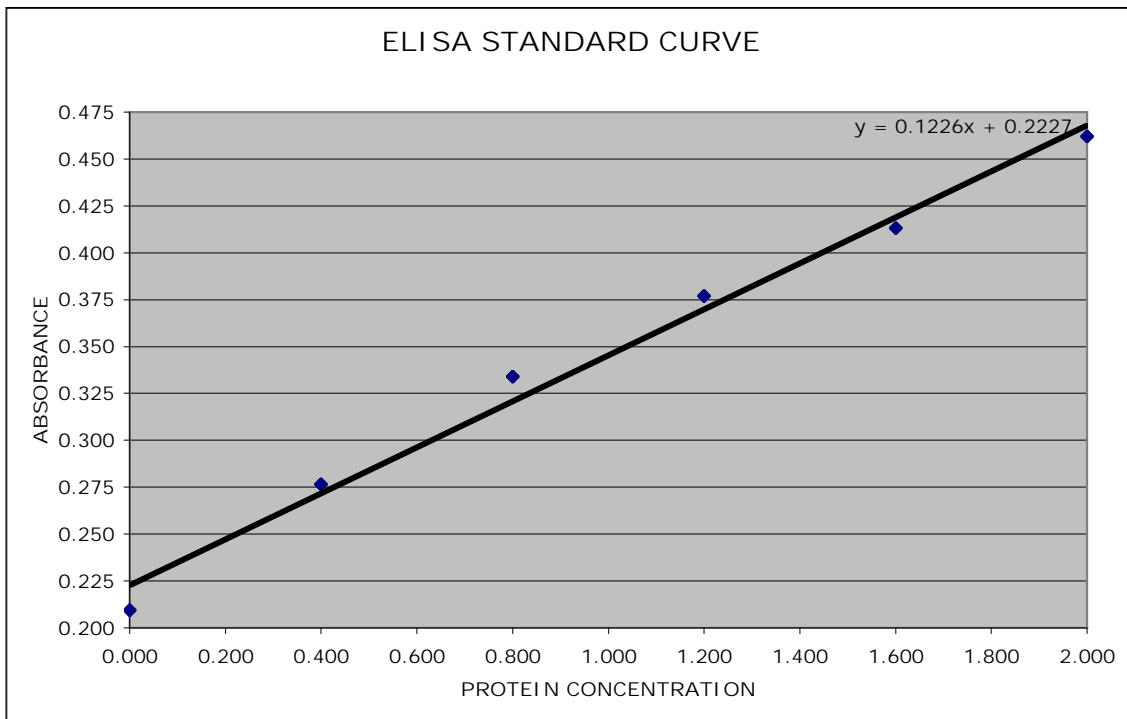
Table 8.1: Comparison of acetone and isopropyl alcohol

After resuspension, protein content was measured using the DC compatible protein measurement kit, which is based on the Lowry protein assay (Lowry et al., 1951) and is not affected by low concentrations of SDS.

Absorbances were higher for acetone extraction, indicating that a greater quantity of protein was extracted by this method, although if we assume a linear relationship between absorbance and protein concentration, the protein recovery is only increased by 8%.

8.2 ELISA

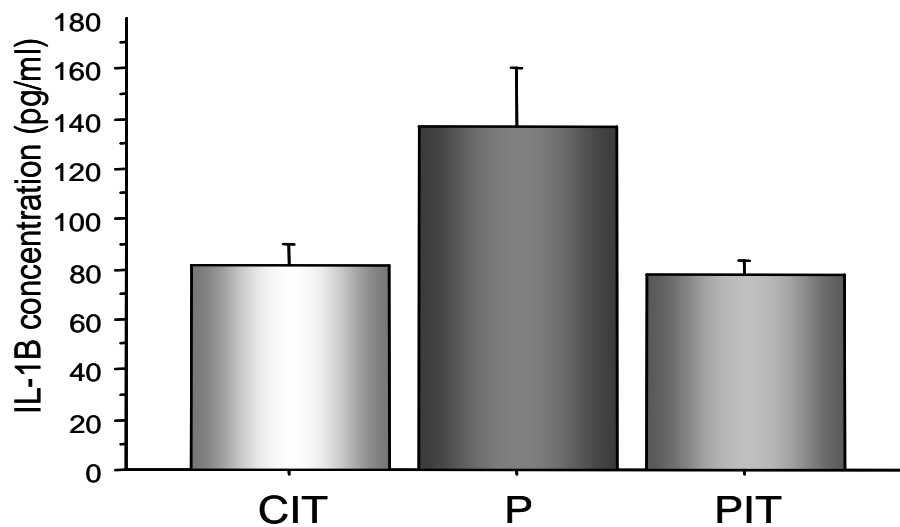
In preparation for proteomic studies I have extracted protein and created a VEGF ELISA standard curve.

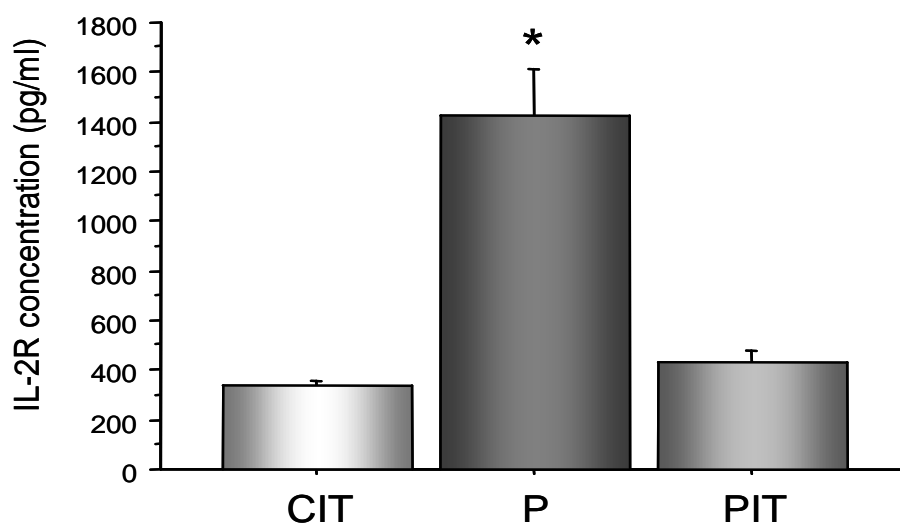
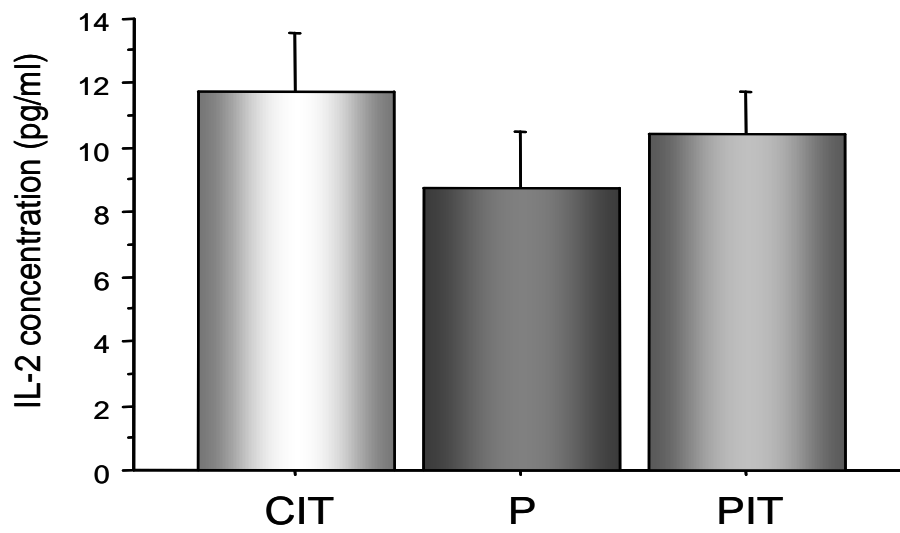
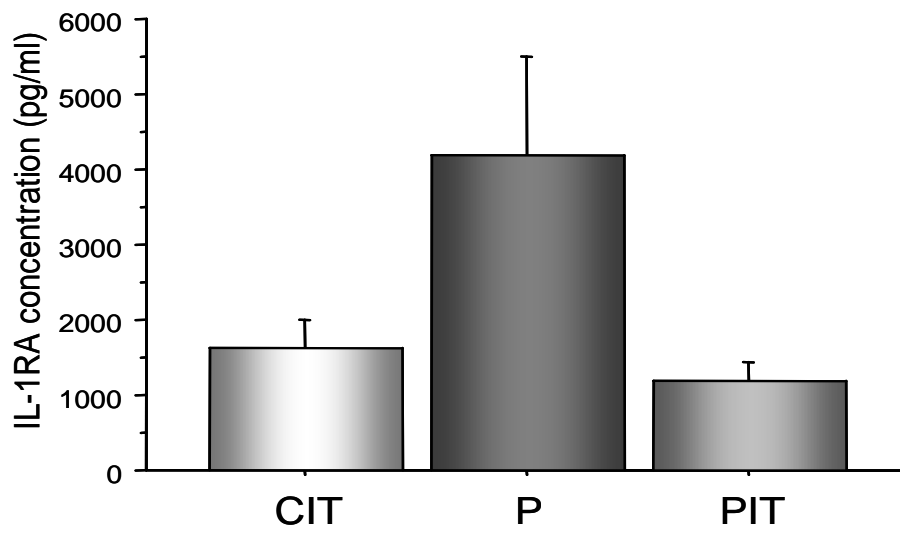


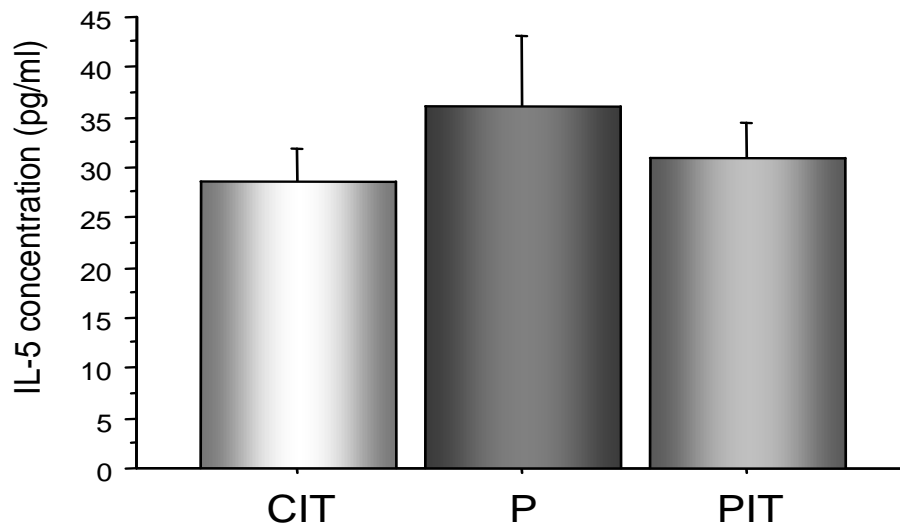
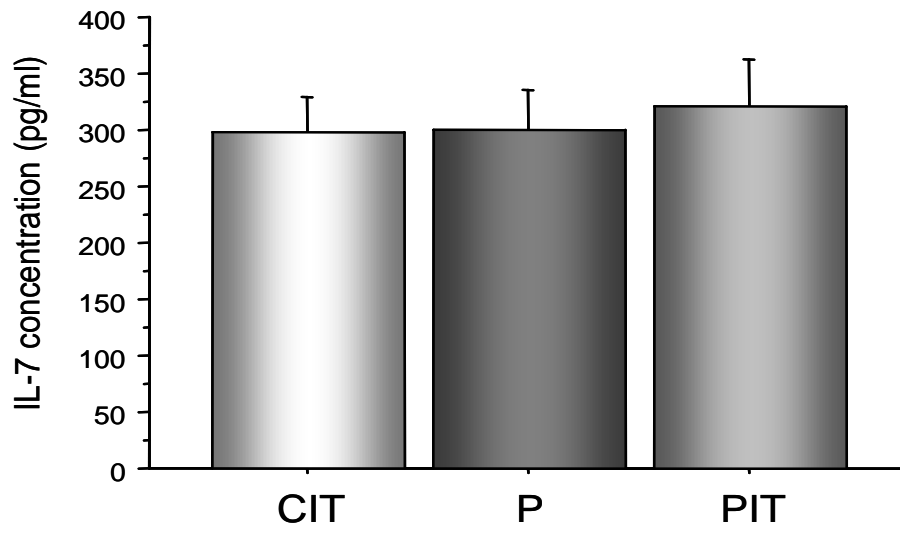
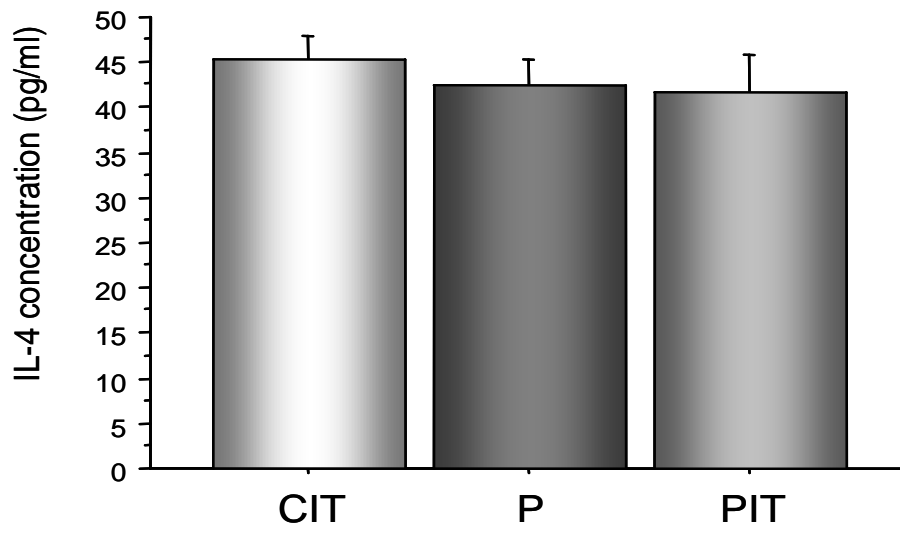
8.3 Luminex

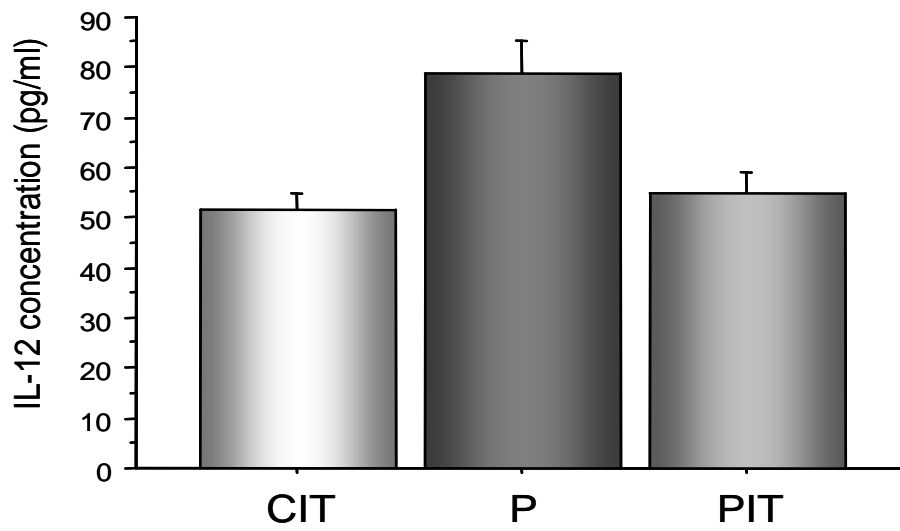
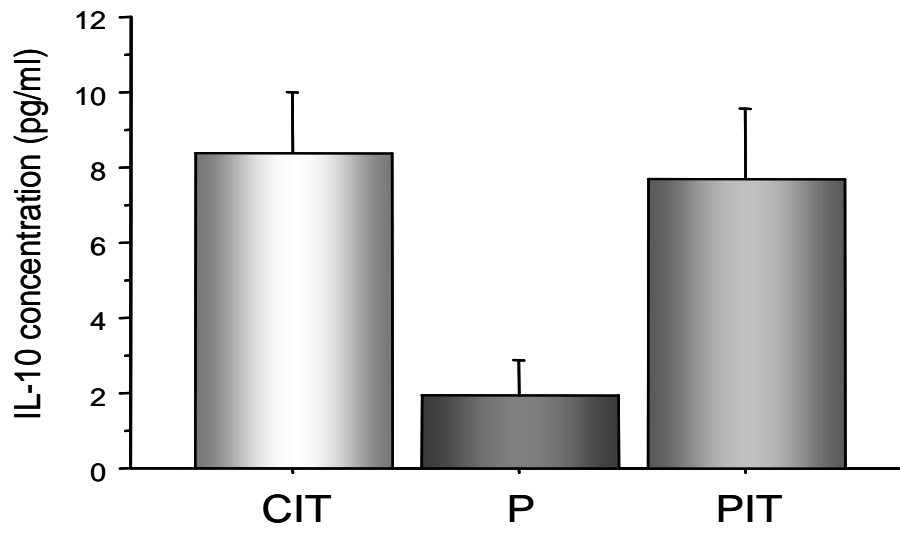
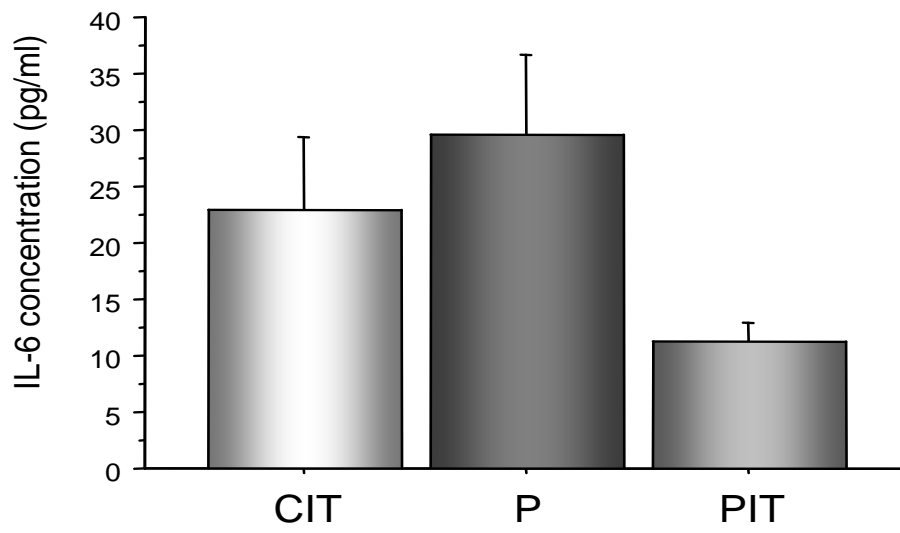
Gene expression data will provide a snapshot of the pathological response, identifying pathways with potentially altered activity. However, there may be post-transcriptional modification of signalling pathways that may not be adequately identified using just this approach. We therefore also needed to examine the gene product, i.e. quantify protein levels within biopsies. The Luminex system measures water-soluble proteins, typically cytokines and growth factors. We used a custom array with 34 cytokines:

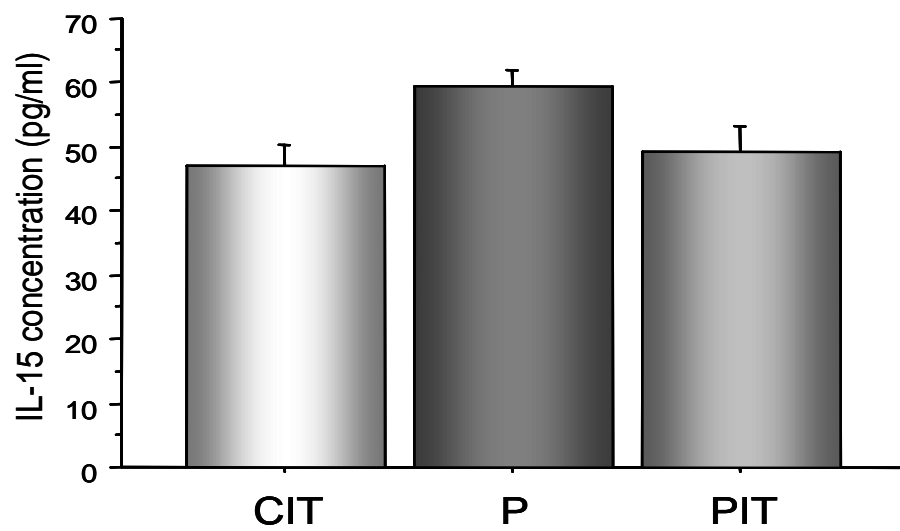
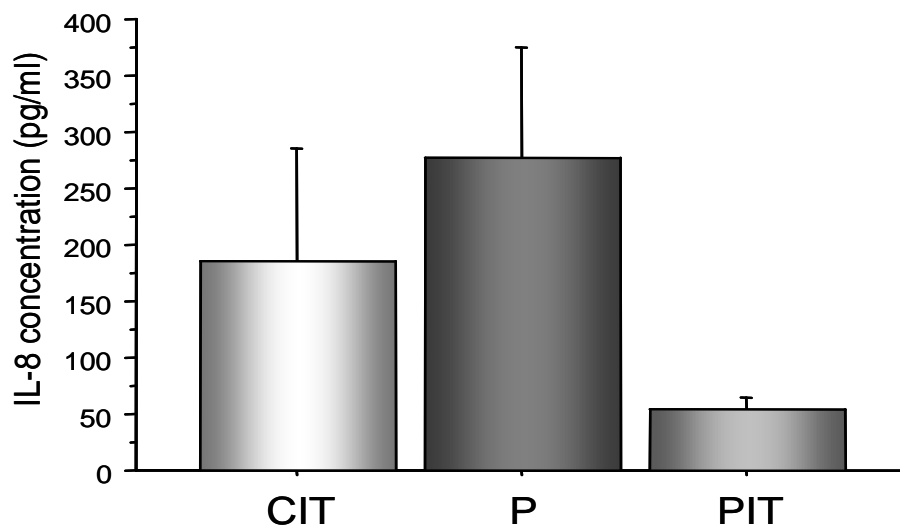
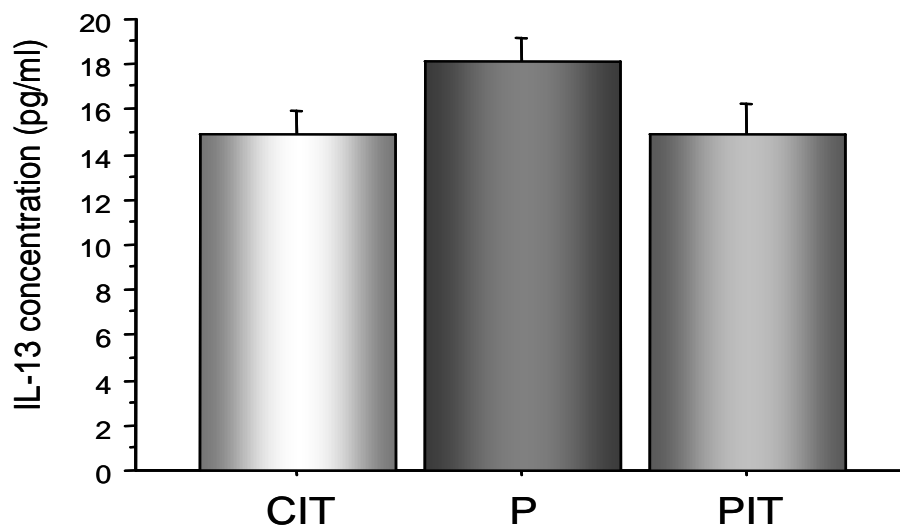
8.3.1 Results:

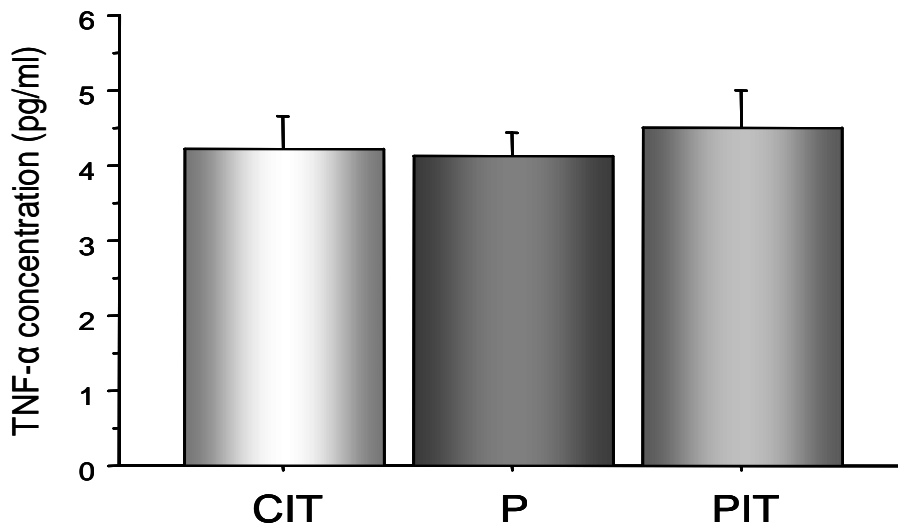
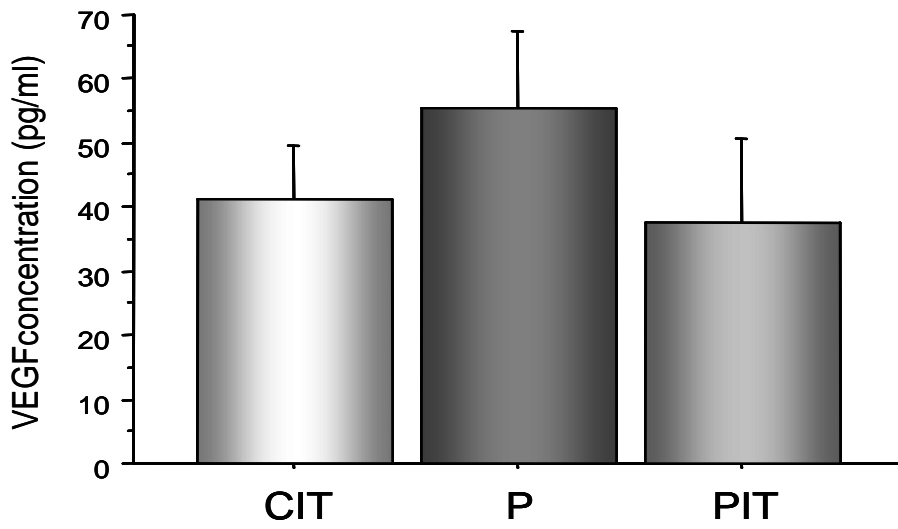
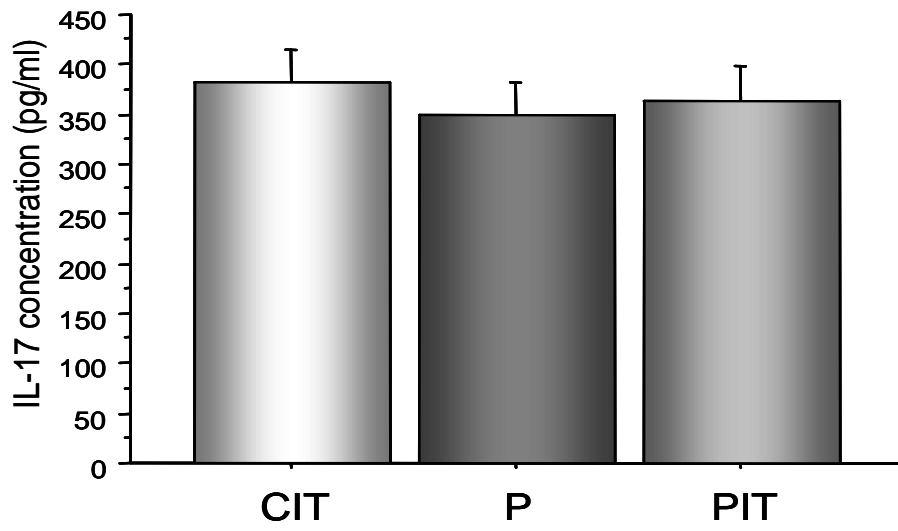


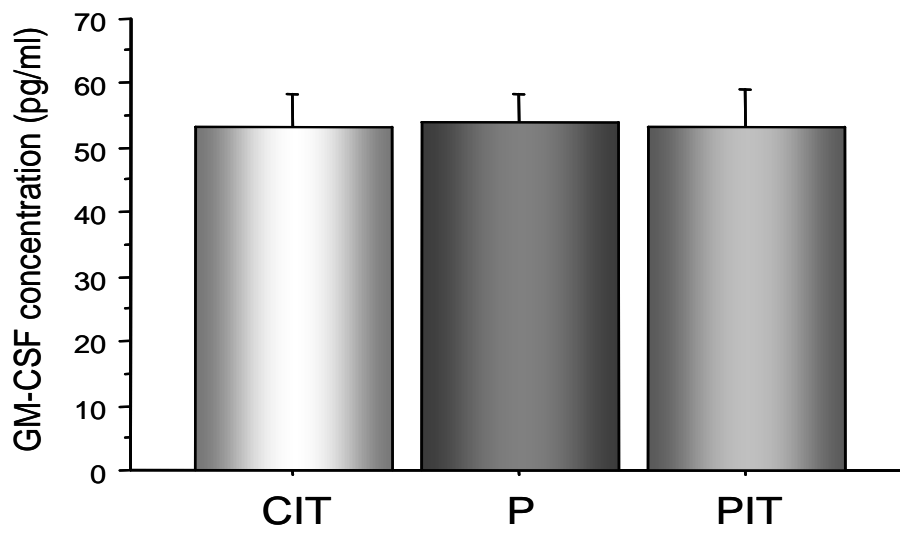
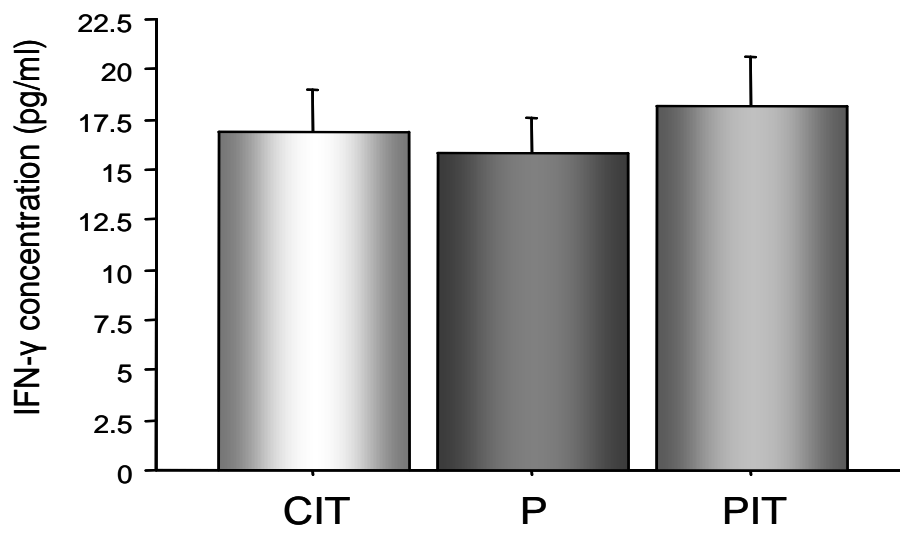
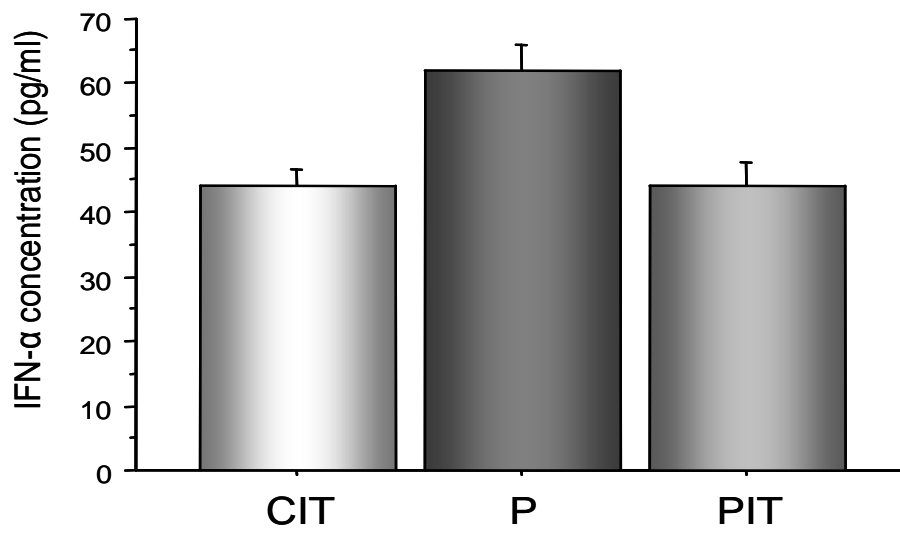


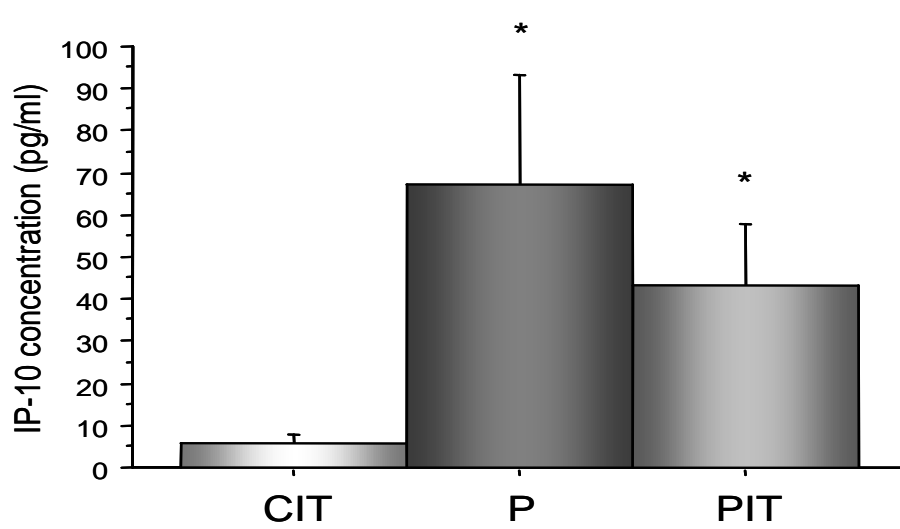
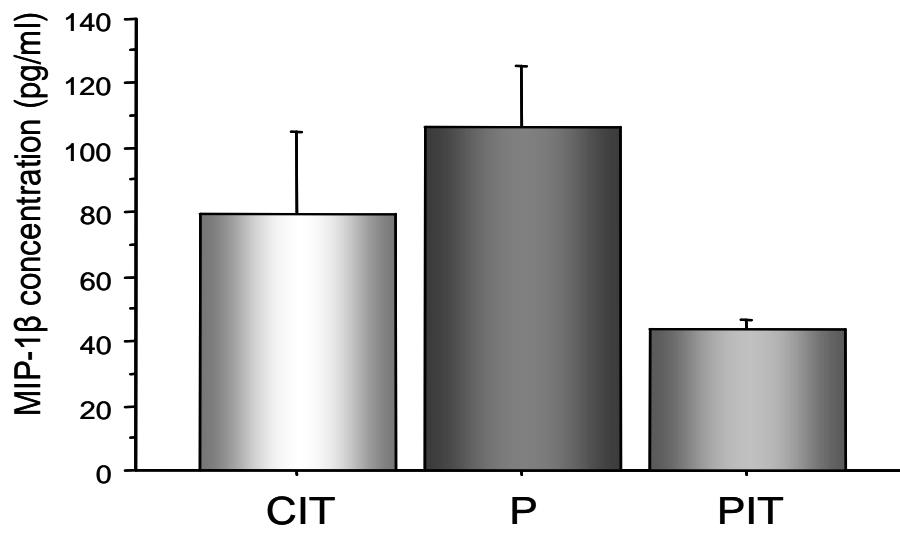
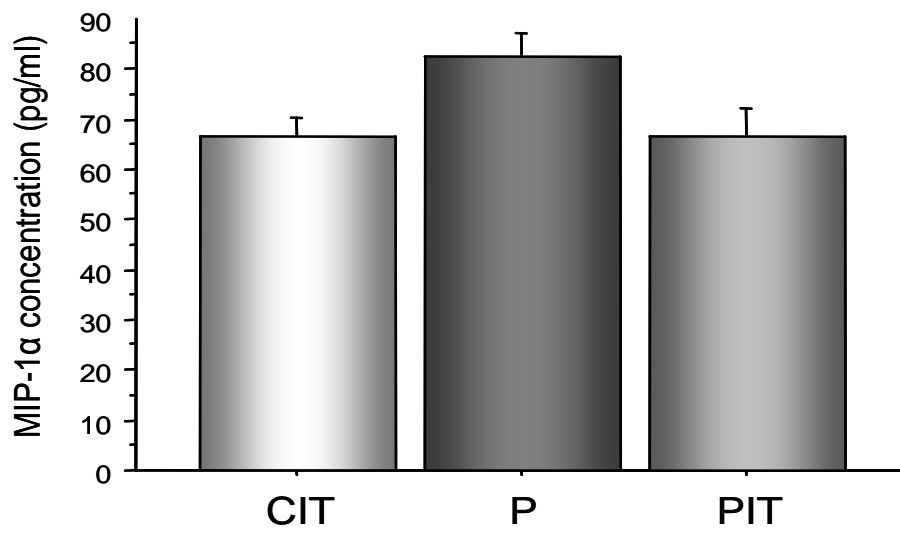


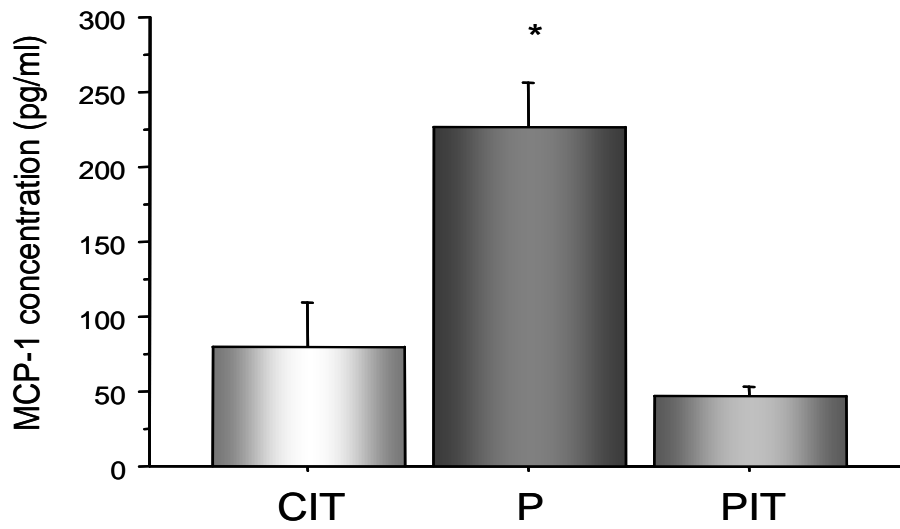
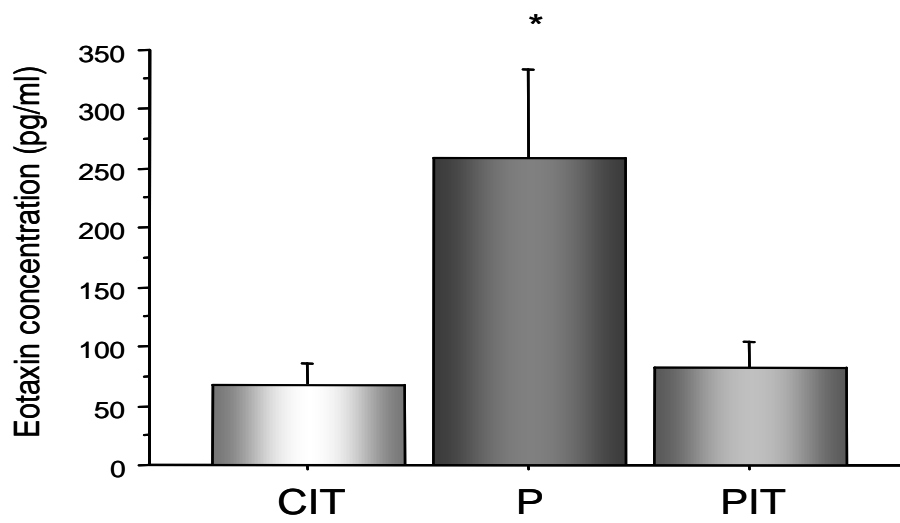
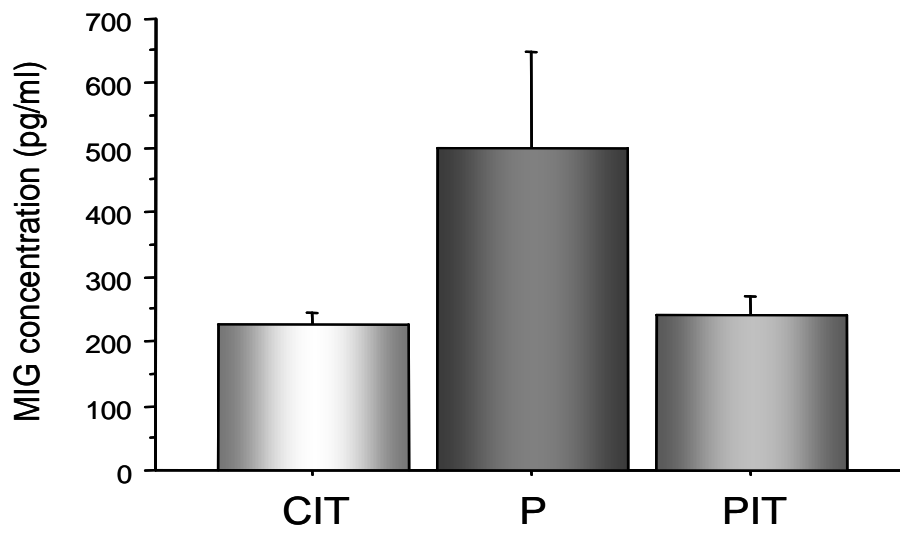


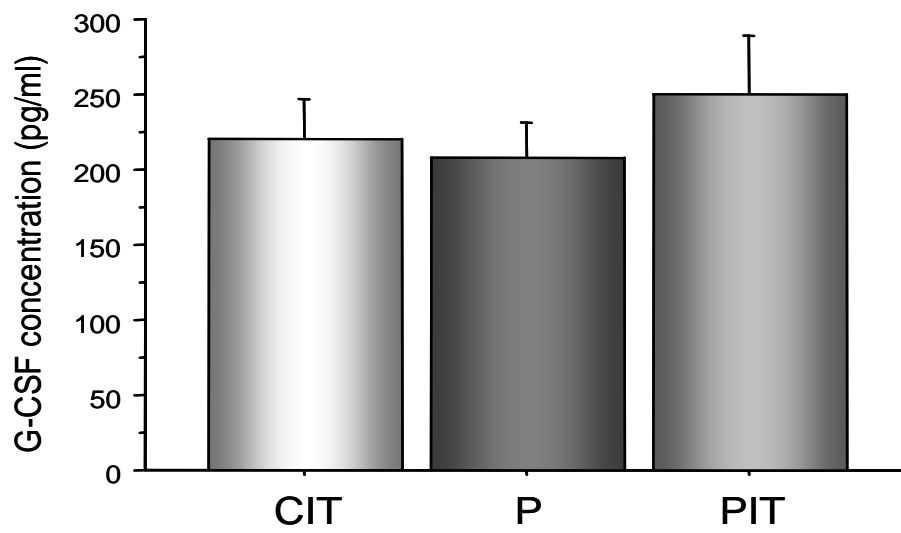
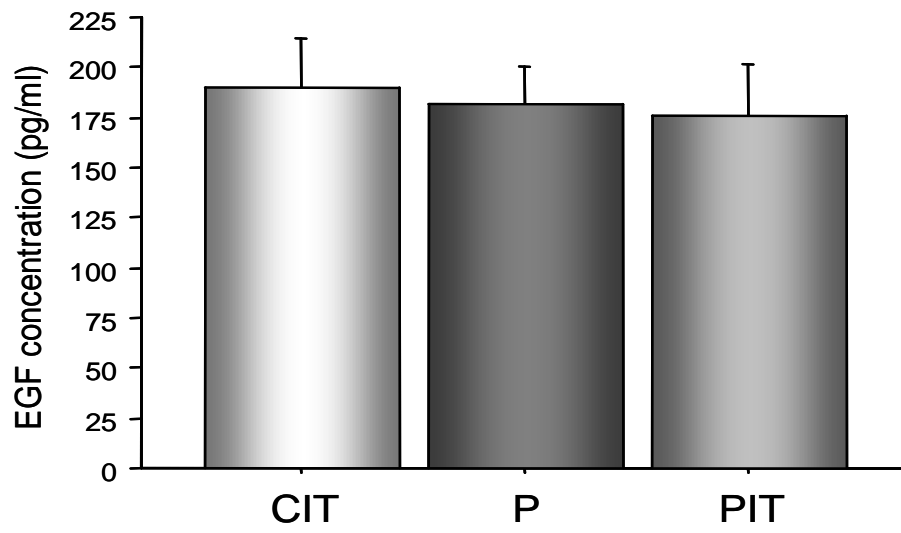
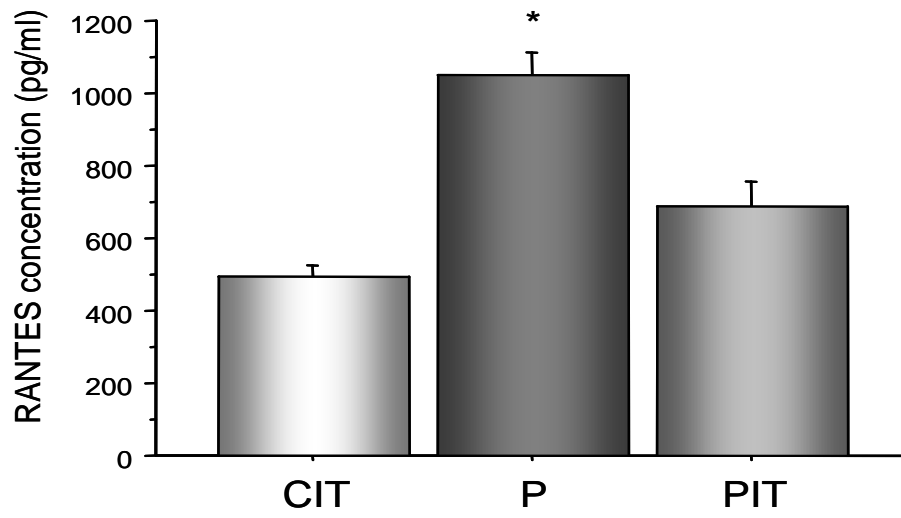


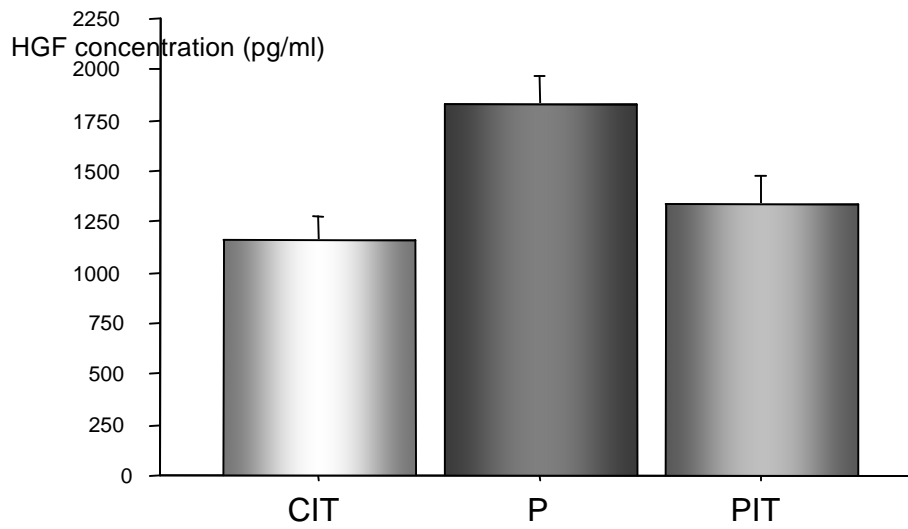
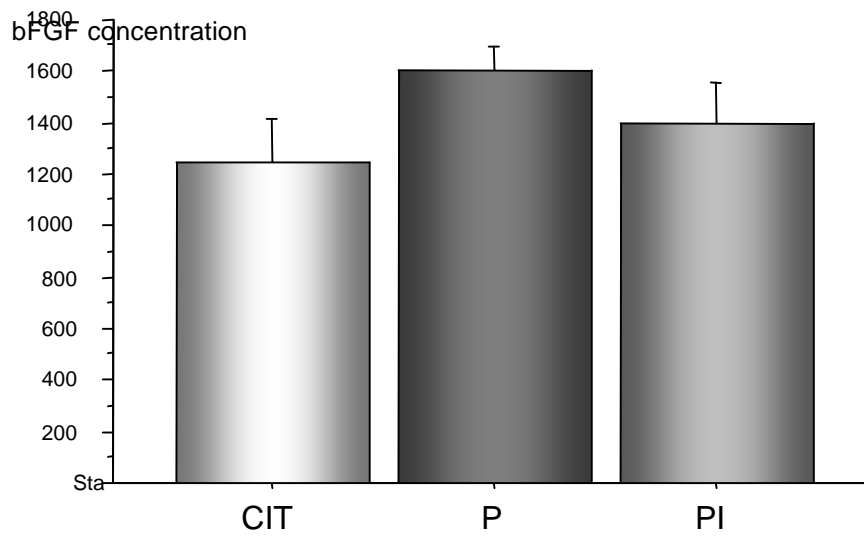












Statistically significant results:

- (i) IP-10 (CXCL10) upregulated in both polyps and PIT
- (ii) MCP-1
- (iii) Eotaxin & RANTES
- (iv) IL-2R –

8.4 iNOS Enzyme Linked Immunosorbent Assay (ELISA)

The gene array analysis demonstrated that the greatest magnitude of change between Polyp and inferior turbinate from the same patient was in the gene for iNOS which is upregulated in polyps. This was not commercially available in a microfluidics card so we used an iNOS ELISA (see methods chapter). Total protein was extracted from polyps and inferior turbinates. The protein was measured and loaded equally into ELISA. The samples were run in duplicate with A450 background correction, quantified against a ladder of known concentrations. Diluted protein was re-assayed and A650 background was used. Absorbances were corrected diluted for small protein loading differences.

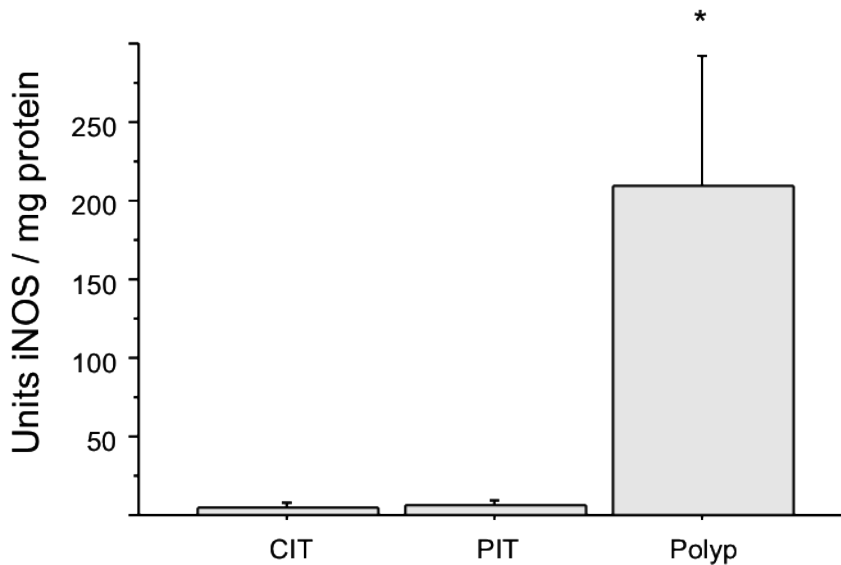


Figure 8.1 *Inducible Nitric Oxide Synthase ELISA*

*Units of iNOS per mg of protein are shown for inferior turbinates from control patients (CIT) and inferior turbinates (PIT) and polyps (P) from patients with nasal polyposis. Mean ± SEM (n=4). *P<0.05 vs. control*

8.5 Discussion

Quantitative PCR broadly confirmed the results of the gene array, with changes in genes involved in cell cycle control, apoptosis, cell signalling and inflammation, although greater patient numbers are needed for good statistical significance. Examination of protein concentration of iNOS with ELISA confirmed a clear and significant upregulation of iNOS in polyp tissue. Gene microarrays show differences in a number of genes involved in cell cycle progression and apoptosis. We have previously shown an increase in cell proliferation in nasal polyps (Chapter 6 (Ahmed et al., 2008)), but the slow, chronic growth of nasal polyps suggests that this growth must be tempered by other factors. The luminex data shows significantly higher levels of genes involved in chemoattraction of eosinophils and macrophages. Specific cytokines identified include Eotaxin and RANTES, which are both chemoattractant cytokines for macrophages and eosinophils. Both have been identified in higher concentration in nasal polyps (Chao et al., 2012, Shin et al., 2000) with a suggestion of a possible involvement in eosinophil activation. Similarly, IP-10 (CXCL10) is also upregulated in both polyps and PIT. This is a chemoattractant for macrophages & monocytes, and reported to be pro-angiogenic (Morelli et al., 2011). MCP-1 is a macrophage chemoattractant and IL-2R – Interleukin 2 receptor is involved in signalling and T cell proliferation.

Growth factors (HGF, VEGF) appear to be upregulated, but these results are non-significant. iNOS protein is highly upregulated in polyps.

These changes between the polyp and PIT may point to the mechanism by which polyps are formed.

CHAPTER 9
APOPTOSIS IN NASAL POLYPS

9 Apoptosis in nasal polyps

9.1.1 Introduction:

Nasal polyps are oedematous, semi translucent, benign masses that develop from the mucosal linings of the paranasal cavity, usually originating from the mucosa in the osteomeatal complex(Larsen and Tos, 2004). Nasal polyposis is a heterogenous condition associated with chronic sinus inflammation, allergy and asthma. A proportion of patients with asthma have Samter's triad where they have aspirin (or salicylate) hypersensitivity as well as asthma and nasal polyps(Samter and Beers, 1968, Bateman et al., 2003, Caplin et al., 1971a). The aetiology and pathogenesis of nasal polyposis are far from clear, with suggestions of them being adenoma formations(Billroth, 1885), arising from inflammation(Bachert et al., 2000a, Jenkins, 1932), oedema(Krajina, 1963) or epithelial rupture(Tos, 1990). Recently, a number of studies have proposed a role for angiogenesis(Caye-Thomasen et al., 2004, Ito et al., 1995a, Yang et al., 1998b, Coste et al., 2000b) with polyps having a higher rate of cell proliferation and tissue remodelling than normal nasal tissue. We have previously shown an increase in cell proliferation in nasal polyps (Chapter 6 (Ahmed et al., 2008)) and the aim of this study was to examine whether the conditions of asthma, allergy or aspirin sensitivity cause more active nasal polyposis and to what extent.

9.1.2 Methods:

Nasal polyp and inferior turbinate biopsies were taken from each side of patients with bilateral

polyposis. Inferior turbinate samples were also taken from patients with no asthma or allergy undergoing routine septorhinoplasty, as an external control. Polyp patients with a history of asthma and/or allergy, or Samter’s triad were grouped accordingly. Frozen sections were TUNEL stained to label apoptotic cells, and parallel sections stained for Ki-67 to label proliferating nuclei.

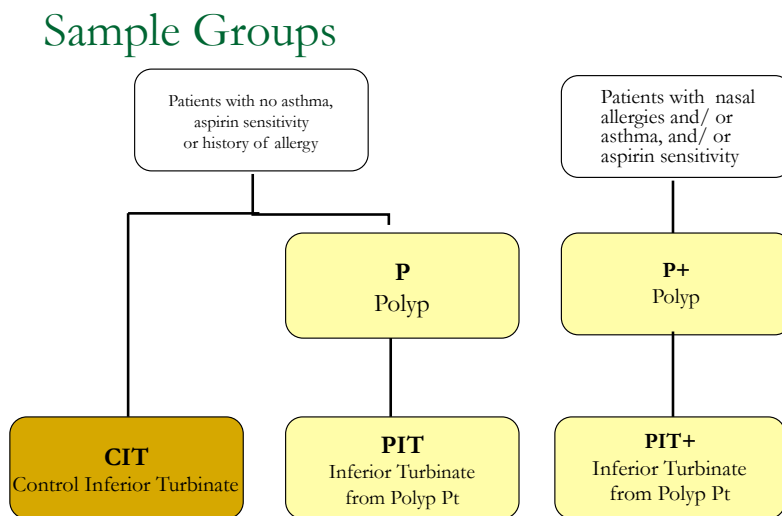


Table 9.1: experimental design (n=5 per group).

9.1.3 Results:

Patients with a history of allergy and/or asthma had significantly higher apoptotic indices than patients with neither. There appeared to be an additive effect of asthma and allergy with more apoptosis occurring in patients with both or Samter’s triad compared to patients with one condition only. Proliferation was higher in patients with asthma and/or allergy than in patients with neither.

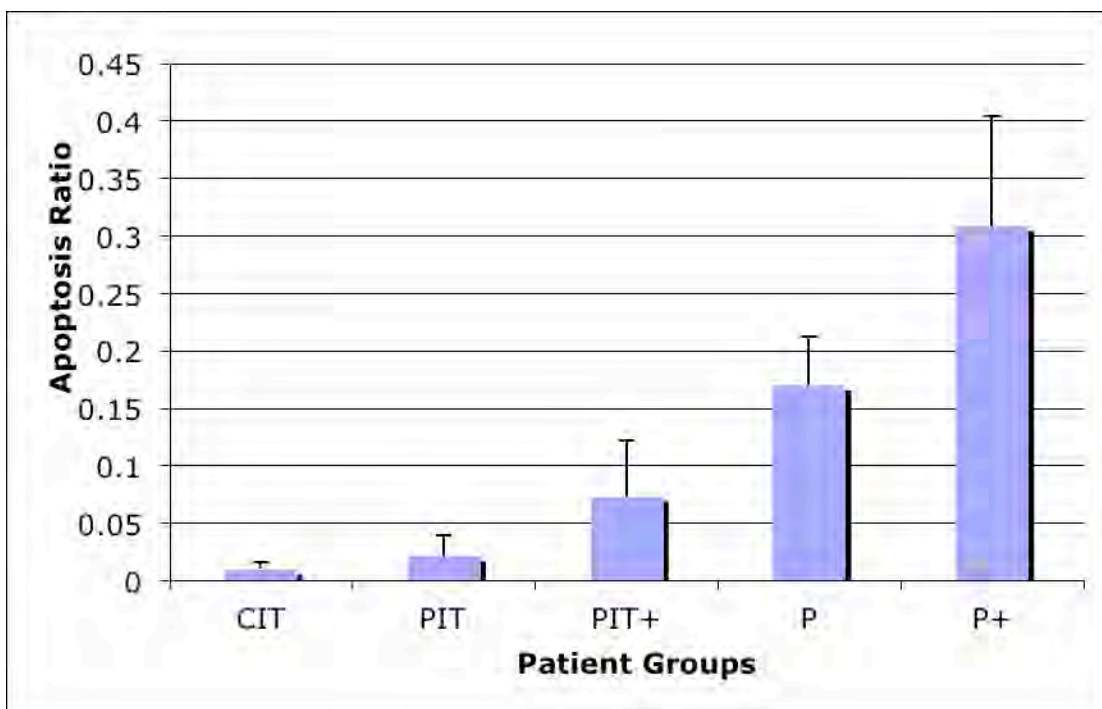


Figure 9.1: Apoptosis ratio in different patient groups.

Patient Group	Apoptosis Ratio	+/- SD
CIT	0.0094	0.0060
PIT	0.0213	0.0179
PIT+	0.0733	0.0488
P	0.1698	0.0422
P+	0.3086	0.0954

Table 9.2: Apoptosis ratios

9.1.4 Discussion:

Patient recruitment for this study was over slow, over an eighteen-month period, due to the serendipitous nature of the operating lists and the individual numbers of patients in each subgroup was low. The degree of apoptosis was greatest in polyps of patients with Samters triad and or allergy, and then in patients with polyps but no allergy, asthma or aspirin sensitivity. In the inferior turbinate group the apoptosis ratio was highest in the group with aspirin sensitivity, asthma and or allergy and lowest in the control. The standard deviations are wide but there is a definite trend. If one tried to quantify the degree of inflammation present in each of the tissue groups it is likely that they would be ordered in the same manor from the least inflammation in the control inferior turbinate (CIT) then in the PIT. A patient with nasal polyposis with allergy, asthma or aspirin sensitivity would be expected to have more inflammation than the PIT but less than a frank nasal polyp. Finally a polyp from a patient with Samter's triad would have the greatest degree of inflammation so the results seem to agree with the clinical and histopathological findings.

This study shows that having a history of asthma, allergy or aspirin sensitivity leads to more cell proliferation and apoptosis in nasal polyposis, and thus greater cell turnover. Both asthma and allergy increase the cell turnover, and the effect is additive, suggesting different mechanisms. Further studies will ascertain to what extent apoptosis and cell turnover occurs with asthma, allergy and aspirin sensitivity.

CHAPTER 10
GENERAL DISCUSSION PART II

10 General Discussion

As with many clinical trials there are issues with Ethical Approval, patient recruitment, funding and disease prevalence. We also relied on the serendipity of operating theatre lists containing the patient groups that we were investigating. This led to severe delays in gathering data for the proposed initial studies for a number of years. Given the additional complications of conducting research on a part-time basis whilst also handling a full clinical workload, and moving jobs every six months, it was thought to be pragmatic to refine the analytical techniques using material previously collected and immediately available. Although on skeletal muscle, this allowed me to develop proficiency with histochemical procedures and analytical approaches. In the first experimental chapter (Chapter 3) we measured capillarity in physiological adaptation. We refined the lectin staining technique to using *Ulex europeaus* agglutinin II instead of UEA-I as it seemed to give greater staining and we used a novel technique of capillary domains to calculate capillarity. We conclude that the capillary supply to human skeletal muscle is scaled according to fibre size and is relatively independent of fibre type.

The second experimental chapter looked at measuring capillary supply in pathological adaptation in muscle atrophy hypertrophy and we also conducted a meta-analysis of the available literature. We reaffirmed our previous experiments but this time in pathological muscle hypertrophy and atrophy with what seems to be a decoupling of angiogenesis from metabolic demand, so capillarity is mainly dependant on fibre size not fibre type, suggesting that the preponderance of angiogenesis-based diseases may be dependent on the tissue

involved.

When sufficient clinical samples were available we were able to begin refining the immunohistochemical techniques. In chapter 5 we developed a novel triple stain to examine remodelling using a 1:1:1 mixture of 3 lectins with Ki67 and DAPI, to assess the endothelium, degree of proliferation and nuclei all within the same section. This novel and robust method for analysing the capillary bed and the extent of cellular proliferation in unfixed samples of human respiratory mucosa enabled us to further assess vascular remodeling the nose.

The literature on the aetiology of nasal polyposis was lacking with many studies having poor patient selection or being inadequately designed. Our study was the first to robustly assess the condition, and specifically answer the question as to whether angiogenesis was the driving force behind nasal polyposis. For the first time we were able to categorically refute the common belief of angiogenesis-driven polyposis.

There was still a large body of evidence that supported high levels of pro-angiogenic factors so we carried out a gene array. Gene microarray analysis shows upregulation of genes controlling the cell cycle and apoptosis, suggesting cell turnover is an important part of the pathogenesis of nasal polyps. This gene expression data was confirmed by TUNEL staining of cryosections, indicating an increased level of apoptosis in nasal polyp tissue, counterbalancing the increased cell proliferation. Inflammatory genes are also upregulated, however the data collected so far cannot distinguish between different types of inflammatory response, which means further research is necessary to elucidate the causal agents of nasal polyposis. A broad-

spectrum analysis of gene expression shows that there are greater differences between the inferior turbinate of the polyp patients and controls than between inferior turbinates and polyps of the same patients, implying that polyposis is primarily a disease of the whole nose rather than a local phenomenon. Both mRNA and protein expression of iNOS show a clear increase in nasal polyps, suggesting that iNOS has an important role in changing the whole nose disease into growing polyps.

We carried out proteomic studies using the luminex system but this did not clarify the situation despite using matched samples that were used in the gene array. They highlight the protein differences occurring in the polyps themselves. We have shown chemoattractants for eosinophils & macrophages (which are found in polyps), and significantly in iNOS, which is novel.

We have not shown significant results in the growth factors, specifically VEGF, seen by other researchers, although the trends seem to be in the same direction. We have also seen an increase in apoptosis, indicating that cell turnover in the polyps is high, and the impact of growth factors reported by other researchers may be less important than has been suggested.

Future directions would be to carry out similar genomic and proteomic analyses on further subgroups of patients; not only patients with nasal polyposis but also with asthma and allergic rhinitis, to further unravel the complex mechanisms that contribute to these common clinical

conditions. Nitric Oxide levels in the nose have been correlated with a number of nasal conditions and are currently used clinically in some tertiary hospital departments. Further investigation into our finding of high levels of iNOS is also very worthy of further research.

11 Abbreviations

APS	Ammonium persulphate
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CAF	Capillary around fibre
CPA	CAF per fibre area, normalized derivative of CAF
C:F	Capillary to fibre ratio
Ca(NO ₃) ₂	Calcium nitrate
CD	Capillary density
CF	Cystic fibrosis
CHAPS	[3-[(3-cholamidepropyl)-dimethylammonio] propane sulfonate
CIT	Control inferior turbinate
CRS	Chronic rhinosinusitis
CT	Computed tomography
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECM	Extra cellular matrix
EPC	Endothelial precursor cells
eNOS/NOS3	Endothelial nitric oxide synthases

FGF	Fibroblast growth factor
FBS	Fetal bovine serum
iNOS/ NOS2	Inducible nitric oxide synthase
IT	Inferior turbinate
KH ₂ PO ₄	Potassium dihydrogen orthophosphate
LCD	Local capillary density
LCFR	Local capillary to fibre ratio
MAP	Mitogen-activated protein
MCP-1	Macrophage chemoattractant
MMPs	Matrix metalloproteinases
Na ₂ HPO ₄	Di-sodium hydrogen orthophosphate
NaBO ₂	Sodium metaborate
NaDOC,	Sodium deoxycholate
NBT	Nitro blue tetrazolium
nNOS/ NOS1	Neuronal nitric oxide synthases
NO	Nitric Oxide
NOS	Nitric oxide synthases
NSAID	Non steroidal inflammatory drug
NSS	Normal sheep serum
P	Polyp
PAM	Predictive analysis of microarrays
PBS	Phosphate buffered saline

PCA	Primary component analysis
PCD	Primary ciliary dyskinesia
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PIC	Proteinase inhibitor cocktail
PIT	Ipsilateral inferior turbinate
PVD	Peripheral vascular disease
PVDF	Polyvinyl diflouride
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SAM	Standard analysis of microarrays
SDS	Sodium dodecyl sulphate
SELDI	Surface Enhanced Laser Desorption/ Ionization
TdT	Terminal deoxynucleotidyl tranferase
TBE	Tris-borate- EDTA
TEMED	Tetramethylethylenediamine
TIMPs	Issue inhibitors of metalloproteinases
TNF	Tumour necrosis factors
TRIZOL	Phenol & guanidine isothiocyanate reagent
TTBS	Tris-buffered saline

VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor

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Appendices

11.1 Appendix I: Published Papers

- i. Staining of nasal mucosa to examine remodelling
- ii. The causation and treatment of nasal polyposis.
- iii. No significant angiogenesis in nasal polyposis
- iv. Is human skeletal muscle capillary supply modelled according to fibre size or fibre type?
- v. Effect of asthma and allergy on nasal polyp cell turnover.
- vi. Scaling of capillary supply in human myopathies
- vii. Angiogenesis and cell proliferation in nasal polyposis.
- viii. The formation of nasal polyposis is independent of angiogenesis
- ix. Scaling of muscle capillary supply in humans: effect of strength training.

11.2 Appendix II: Ethical Committee Approval

- i. Dudley Local Research Ethics Committee

11.3 Appendix III: Successful grants

- i. British Lung foundation
- ii. Get-A-Head
- iii. MIO

11.4 Appendix IV: Luminex Data

APPENDIX I: Published Articles

Papers in peer-reviewed journals:

Ahmed SK, Williams J, Drake-Lee A, Egginton S
Staining of nasal mucosa to examine remodelling
Journal of Laryngology and Otology (2009) 123; 234-237.

Ahmed SK.
The causation and treatment of nasal polyposis.
The Journal of ENT Master Class (2008) 1; 77-81

Ahmed SK, Williams J, Drake-Lee A, Egginton S
No significant angiogenesis in nasal polyposis
American Journal of Rhinology (2008) 22(1); 24-28.

Ahmed SK, Egginton S, Jakeman PM, Mannion AF, Ross HF.
Is human skeletal muscle capillary supply modelled according to fibre size or fibre type? *Experimental Physiology* (1997) 82, 231-234

Abstracts:

Darlington DS, Ahmed SK, Williams JL, Drake-Lee A, Egginton S.
Effect of asthma and allergy on nasal polyp cell turnover.
The Otolaryngologist 2009; 2(3); 102

Ahmed SK, Williams J, Round J, Egginton S
Scaling of capillary supply in human myopathies
Microcirculation 2009; 16(5)

Ahmed SK, Williams J, Drake-Lee A, Egginton S
Angiogenesis and cell proliferation in nasal polyposis.
Clinical Otolaryngology 2008; 33(3); 303

Ahmed SK, Williams JL, Drake-Lee A, Egginton S.
The formation of nasal polyposis is independent of angiogenesis.
Microcirculation 2008; 15(7) 633-687.

Ahmed SK, Egginton S, Ross HF, Mannion AF, Jakeman PM.
Scaling of muscle capillary supply in humans: effect of strength training.
The Journal of Physiology (1995) 483, 134P.

Staining of nasal mucosa to examine remodelling

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Abstract

Background and objective: The process of embedding tissue in paraffin degrades many important molecules involved in respiratory epithelial remodelling. We therefore examined alternative methods.

Methods: Inferior turbinate and nasal polyp biopsies were either placed in formalin or immediately snap-frozen in the operating theatre. Novel protocols for staining remodelling markers were compared with current methods.

Results: Our method, using a mixture of three lectins, stained a significantly greater proportion of samples, compared with using *Ulex europeus* lectin alone (84 vs 62 per cent; $p < 0.005$). Comparison of different proliferation markers showed that Ki67 was more suitable than proliferating cell nuclear antigen for frozen sections.

Conclusions: This study indicates that our robust, repeatable methods for examining whole mounts and for staining capillaries, cell proliferation and nuclei on the same section of nasal mucosa are superior to current methods. The use of fresh tissue that has not been paraffin-embedded would allow a greater suite of epitopes to be examined in the future.

Key words: Remodelling; Nasal Mucosa; Polyp; Turbinates; Histology

Introduction

Histological examination of nasal mucosal tissue is essential when studying the aetiology of conditions such as chronic sinusitis, polyposis, allergic rhinitis and asthma. Tissue remodelling is an important feature of these diseases, often being associated with increased severity or chronicity, although the mechanisms involved are unclear and require investigation.¹ Multiple labelling of different molecules on the same section is highly desirable, as it allows co-localisation of different factors involved in remodelling. However, most established protocols use paraffin-embedded human tissue.² This restricts the tissue markers available, as many antibodies will not recognise epitopes after paraffin-embedding. Thus, research into airway remodelling is currently limited to studying a relatively small set of proteins.

Using fresh tissue allows whole mount staining (using relatively large tissue samples); the sample can then be examined with confocal microscopy and analysed in three dimensions. This approach could yield a wider range of information, avoiding problems with heterogeneity of structure by examining a larger mass of tissue, and correcting for the large changes in tissue volume typically seen in the nasal mucosa due to the nasal cycle. This approach has been successfully applied to the examination of blood vessels with fluorescent lectins in animal models³ and in a proof-of-principle human study.⁴ However, the use of lectins can be problematic in large human studies, as no single lectin will stain a population with heterogeneous blood groups.⁵ Antibody staining, such as cluster of differentiation (CD) 31 or CD34, is poor at consistently staining contiguous structures such as blood vessels.

As different lectins bind to different sugar moieties, we reasoned that a mixture of the three different lectins reported to stain human blood vessels⁵ would successfully stain a far greater proportion of patient samples, and would therefore provide a more robust staining protocol for visualising capillary beds in three dimensions, allowing further investigation of vasculature changes during tissue remodelling in patients.

Materials and methods

Subjects

Patients were recruited from University Hospitals Birmingham NHS Trust, UK, with local ethical committee approval (numbers 0692 and 06/Q2702/15). Patients' informed consent was obtained. Polyp and ipsilateral inferior turbinate specimens, 1 cm back from the anterior edge, were obtained from patients undergoing routine polypectomy. Previous studies have found no difference in vascularity between the different areas of the lateral nasal wall.⁶ Inferior turbinate specimens from the same anatomical site were also taken from patients undergoing routine septoplasty or septorhinoplasty.

Immunohistochemical staining

Samples taken for confocal analysis were immediately fixed in 4 per cent buffered formalin and left for 24 hours. Samples were then washed in three changes of phosphate-buffered saline for a further 24 hours, before being stained whole with dilutions of either fluorescein-conjugated *Ulex europeus* lectin (Vector Labs, Peterborough, U.K.)

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or a fluorescein-conjugated lectin mixture (1:1:1 *Ulex europaeus*, *Griffonia simplicifolia* and *Euonymus europeus*; Vector Labs), in order to account for different affinities associated with different blood groups.⁵ Samples were then washed and stored in phosphate-buffered saline until viewed under a confocal microscope (DM IRE2; Leica, Milton Keynes). A total of 60 samples were stained with the single lectin protocol, and 50 different samples were stained with the triple lectin protocol.

Samples taken for immunostaining were immediately snap-frozen in liquid nitrogen cooled isopentane in the operating theatre, and 8 µm sections were subsequently cut on a cryostat. These were allowed to air-dry before being fixed in 4 per cent buffered formalin (for 1, 5, 10, 20 or 60 minutes) or ice-cold acetone (for 10, 30, 60 or 300 seconds). Antigen retrieval was performed in citrate buffer (10 mM citric acid adjusted to pH 6.0 with NaOH, 0.05 per cent Tween[®] 20) heated to 95°C for 5 minutes then allowed to cool to room temperature. Slides were blocked for 30 minutes at room temperature in wash buffer (either 5 per cent fetal bovine serum or 1.5 per cent weight/volume bovine serum albumin, 0.6 per cent volume/volume Triton[®] X-100 in phosphate-buffered saline, or 5 per cent fetal bovine serum in phosphate-buffered saline), then incubated for 2 hours with primary antibodies (Ki-67 and proliferating cell nuclear antigen; Dako, Santa Cruz, California U.S.A.) diluted in wash buffer to label proliferating nuclei. Sections were then washed in phosphate-buffered saline and incubated for 1 hour with secondary antibody (Tetramethyl Rhodamine Iso-Thiocyanate polyclonal rabbit anti-mouse; Dako) diluted in wash buffer. Sections were rinsed in phosphate-buffered saline and then incubated for 30 minutes with lectin to stain blood vessels. Sections were washed in phosphate-buffered saline, rinsed with dH₂O and mounted using Vectashield containing 4',6-diamidino-2-phenylindole, in order to label all nuclei and enable calculation of a proliferation index (Ki67 count/4',6-diamidino-2-phenylindole count), based on counts of four 500 µm × 500 µm sampling squares, using inclusion/exclusion lines. All counts were made either on the same section or on a serial section from the same sample.

Results and analysis

Samples from a total of 60 patients were stained using *Ulex europaeus* lectin, and compared to samples from 50 patients stained with a combination of *Ulex europaeus*, *Griffonia simplicifolia* and *Euonymus europeus*. Samples that showed stained blood vessels were designated as positive, and samples in which blood vessels were not visualised were restained. Repeated failure to stain capillaries was seen in 23 of the single-lectin samples and in eight of the triple-stained samples ($p < 0.005$; Pearson's chi-square test), representing a significant difference between single and triple lectin methods (see Table I and Figure 1).

TABLE I
COMPARISON OF SINGLE *ULEX EUROPEUS* LECTIN STAINING VS
TRIPLE LECTIN PROTOCOL

Result	Single	Triple
+ve (n)	37	42
-ve (n)	23	8
+ve (%)	62	84*

*Staining was interpreted as +ve = samples stained well after up to 3 attempts; -ve = samples repeatedly failed to stain

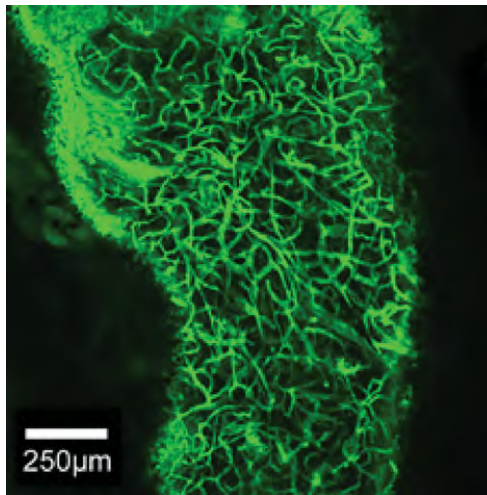


FIG. 1

Inferior turbinate mucosa stained with whole mount protocol using three lectins.

Both proliferating cell nuclear antigen and Ki67 staining gave the most consistent results when sections were attached to poly-L-lysine coated slides and fixed in 4 per cent buffered formalin for 20 minutes. Both stains gave best results with a wash buffer containing Triton X-100. Staining with Ki67 required antigen retrieval, and the optimal antibody concentration was 1:100 for both the primary and secondary antibodies. Staining with proliferating cell nuclear antigen required antibody concentrations of 1:50 for primary and 1:100 for secondary antibodies. A significant difference was seen in the level of proliferation marked by proliferating cell nuclear antigen and Ki67 staining ($p < 0.05$), with both the count per mm² and the proliferation index being significantly higher when measured using proliferating cell nuclear antigen staining compared with Ki67 (Table II).

Discussion

The objective of this study was to develop a robust multiple staining protocol using samples that had not been embedded in paraffin, so that a broader range of antibodies could be used. The use of lectins to stain blood vessels is well established in animal models, but it is less widely used in humans due to variability in staining between individuals with different blood groups.⁵ This study found a marked improvement when using a mixture of the three most commonly used human-specific lectins

TABLE II
COMPARISON OF PCNA AND KI67 AS MEASURES OF PROLIFERATION

Parameter	PCNA	Ki67
Proliferation (+ve counts/mm ²)	1127 ± 201	39 ± 9 [†]
Proliferation index*	34.3 ± 5.6	1.4 ± 0.3 [‡]

Data are expressed as mean ± standard error of the mean. *Proliferation index expressed as percentage of total nuclei; n = 12. [†] $p < 0.05$; Student's *t*-test. PCNA = proliferating cell nuclear antigen; +ve = positive

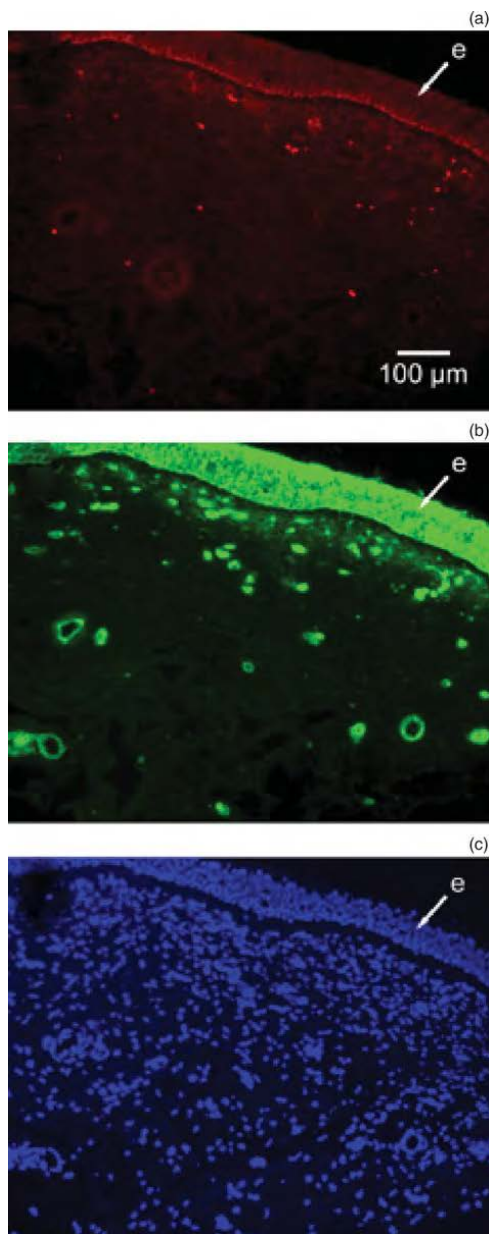


FIG. 2

Triple stain of a single section of inferior turbinate mucosa under different fluorescent illuminations. (a) Rhodamine-conjugated Ki67 stain; (b) fluorescein-conjugated lectin stain; (c) 4',6-diamidino-2-phenylindole stain for nuclei. e = epithelial layer.

(which bind to different sugar moieties present on the endothelium), compared with use of a single lectin. Lectins were also seen to bind to nasal mucosa epithelium (Figure 2), as previously described,^{7,8} however, as this layer

is avascular, it does not interfere with capillary visualisation. Lectins have several advantages over other immunohistochemical methods in the staining of blood vessels. They can be readily conjugated to several fluorophores, allowing multiple stains to be used on the same section. The one-step protocol reduces the signal degradation associated with other multiple labelling methods, as well as saving time. Lectins are relatively inexpensive, allowing whole-mount stains to be performed economically. Finally, using lectins avoids the problems commonly associated with the use of two primary antibodies raised in the same host species.

Proliferating cell nuclear antigen (also termed cyclin) is a protein that acts as a processivity factor for deoxyribonucleic acid polymerase delta in eukaryotic cells, and the staining of this antigen is well documented for paraffin-embedded sections. However, this study shows that staining for proliferating cell nuclear antigen is not appropriate for frozen sections, as the calculated proliferation index is unreasonably high. Normal mucosal tissue is highly unlikely to have 34 per cent of total nuclei undergoing mitosis at any one time (Table II). We surmise that this antibody stains proliferating cell nuclear antigen epitopes in fresh human cells that are not in their biologically active form, as we have also seen unrealistically high labelling indices in serum-starved Human Umbilical Vein Endothelial Cells monolayers where mitosis is arrested (J Williams *et al.*, unpublished data). Staining for Ki67 is far more successful after a brief formalin fixation with subsequent antigen retrieval. Results for proliferation indices for fresh tissue using this method broadly agree with those published for tissue prepared with paraffin-embedding.⁹

Conclusions

This study demonstrates a novel and robust method for analysing the capillary bed and the extent of cellular proliferation in unfixed samples of respiratory mucosa. This method will allow further investigation into the vascular response during tissue remodelling in human biopsies, and could thus help elucidate the aetiology and pathogenesis of tumours, chronic sinusitis, nasal polyposis, allergic rhinitis and asthma. The protocols used are detailed below.

Protocols

Lectin staining protocol. Place samples in standard hospital pathology formalin pots for 24 hours at 4°C. Stain with a 1:100 dilution, in phosphate-buffered saline, of a 1:1:1 stock of fluorescein-conjugated *Ulex europaeus*, *Griffonia simplicifolia* and *Euonymus europaeus* lectins for 24 hours at 4°C. Wash three times with phosphate-buffered saline for 1 hour each wash, before examining under confocal microscopy.

Triple staining protocol. Snap-freeze samples in liquid nitrogen cooled isopentane and store at -80°C, before cutting 8 µm sections on a cryostat. Air-dry the sections, perform an antigen retrieval with citrate buffer (10 mM citric acid adjusted to pH 6.0 with NaOH, 0.05 per cent Tween 20) heated to 95°C for 5 minutes then allowed to cool to room temperature. Fix for 20 minutes in 4 per cent buffered formalin, then block for 30 minutes using a wash buffer consisting of 1.5 per cent weight/volume bovine serum albumin and 0.6 per cent volume/volume Triton X-100 in phosphate-buffered saline. Incubate for 2 hours with primary antibody (Ki-67, Dako) diluted 1:100 in wash buffer. Wash with three changes of phosphate-buffered saline for 5 minutes each, then incubate for

1 hour with secondary antibody (Tetramethyl Rhodamine Iso-Thiocyanate polyclonal rabbit anti-mouse; Dako) diluted 1:100 in wash buffer. Rinse with phosphate-buffered saline, then incubate for 30 minutes with 1:100 of stock 1:1:1 fluorescein-conjugated *Ulex europeus*, *Griffonia simplicifolia* and *Euonymus europeus* lectins. Wash with three changes of phosphate-buffered saline for 5 minutes each, rinse with dH₂O and mount with VectaShield containing 4',6-diamidino-2-phenylindole.

Acknowledgements

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IS HUMAN SKELETAL MUSCLE CAPILLARY SUPPLY MODELLED ACCORDING TO FIBRE SIZE OR FIBRE TYPE?

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(MANUSCRIPT RECEIVED 27 SEPTEMBER 1996, ACCEPTED 4 OCTOBER 1996)

SUMMARY

Analysis of muscle capillary supply usually relies on estimating either the numerical capillary to fibre ratio or capillary density. Both indices are scale dependent, i.e. they vary with fibre size. We have examined the use of an alternative approach based on the anatomical supply area of individual capillaries, which allows the calculation of a local capillary to fibre ratio or density based on area, rather than number of fibres. The results suggest that, in human skeletal muscle, capillary supply is primarily scaled according to fibre size, and is relatively independent of fibre type.

INTRODUCTION

An accurate quantitative analysis of capillary supply to skeletal muscle is an essential prerequisite for understanding the limits to peripheral oxygen transport. The capillary supply to skeletal muscle is influenced by its metabolic profile, oxidative capacity and fibre size (Hudlicka, Brown & Egginton, 1992). Quantitative differences in gross capillary supply are normally expressed as the ratio of capillary number to fibre number (capillary to fibre ratio, C:F) or to fibre area (capillary density, CD). Given that these indices are scale dependent, there is a need for a more discriminatory analysis that takes account of fibre size. By calculating the area of muscle tissue supplied by, or the domain of influence of, individual capillaries an unbiased measure of the local capillary to fibre ratio (LCFR) and local capillary density (LCD) may be derived (Egginton & Ross, 1989). This novel approach was used to investigate whether the local capillary supply was modelled according to the size or type of individual human skeletal muscle fibres.

METHODS

With the approval of the Local Ethics Committee and written informed consent, samples from the lateral portion of the quadriceps femoris muscle were obtained by percutaneous needle biopsy from fifteen active young subjects (10 male and 5 female): mean age, 23.3 ± 1.38 years (males) and 21.0 ± 1.73 years (females); height, 1.80 ± 0.02 m (males) and 1.66 ± 0.04 m (females); and body mass, 83.3 ± 3.70 kg (males) and 60.7 ± 1.56 kg (females). Serial sections ($10 \mu\text{m}$ thick) were prepared for quantitative analysis by histochemical staining of fibre types (myosin ATPase; Brooke & Kaiser, 1970) and capillaries (*Ulex europaeus* lectin, UEA-I; Holthofer, Virtanen, Kariniemi, Hormia, Linder & Miettineu, 1982).

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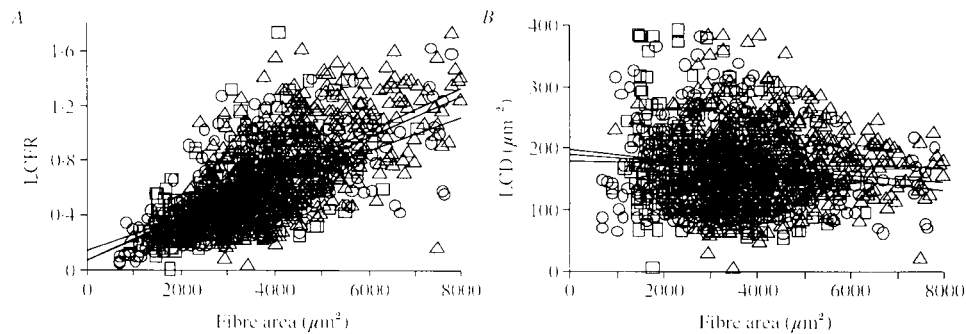


Fig. 1. Local capillary supply indices in the lateral quadriceps, split according to fibre type: type I (Δ), type IIa (\square) and type IIb (\circ). A, local capillary to fibre ratio (LCFR); B, local capillary density (LCD).

Muscle composition was determined by stereological analysis of fibre area and proportion of fibre types (Egginton, 1990). Digitized images of stained sections were used to determine the x, y co-ordinates for muscle fibres and associated capillaries. From these data the LCFR and LCD, indices of fibre-specific capillary supply, were calculated (Egginton & Ross, 1989). Briefly, this involves calculating the geometric supply area ('domain') of each capillary, defined as that portion of tissue cross-section closer to one capillary than any other, and the summed fraction of different domains overlapping each muscle fibre. Under conditions of maximal flow, assuming supply capacity to be similar for all capillaries, the domain size will be inversely proportional to the metabolic demand. Thus, LCFR represents supply capacity in 'capillary equivalents', which may be normalized for the effects of individual fibre size to give the LCD. For comparison, the equivalent number-based indices of local capillary supply, number of capillaries around each fibre (CAF) and its normalized derivative (i.e. CAF per fibre area, CPA), were also calculated. Data were analysed by ANOVA and results expressed as mean values \pm S.E.M.

RESULTS

Numerical fibre composition was found to be $47.9 \pm 4.2\%$ type I, $31.3 \pm 4.4\%$ type IIa and $21.6 \pm 5.1\%$ type IIb. In relation to muscle area the values were 51.8 ± 3.3 , 27.7 ± 4.4 and $15.7 \pm 4.1\%$, respectively. Mean fibre cross-sectional areas were 4538 ± 240 , 4005 ± 390 and $3957 \pm 814 \mu\text{m}^2$, respectively. Mean C:F was 1.3 ± 0.42 , while CD was $442 \pm 52 \text{ mm}^{-2}$. These values are comparable with those obtained previously for vastus lateralis biopsies from untrained individuals giving a C:F of 1.39–1.79 and a CD of 348–438 mm^{-2} (Ingjer, 1979), and a CD of 325 mm^{-2} (Andersen, 1975). A total of 1741 fibres ($n = 709$, 520 and 512 for type I, IIa and IIb, respectively) from three individuals were also matched across serial sections for fibre type, fibre area and capillarity. Capillary supply (domain) area showed a logarithmic-normal distribution. Domain area (μm^2) and $\log_{10}(\text{domain area})$ were 2831 ± 150 and 3.41 ± 0.024 , respectively ($n = 6$ biopsies and 944 domains). LCFR showed a positive correlation with fibre area ($\text{LCFR} = 0.075 + 0.0015 \times \text{area}$; $r^2 = 0.47$) that was independent of fibre type (Fig. 1A). Slopes for the regression with fibre type were very close (0.0012 – 0.0016 ; $r^2 = 0.40$ – 0.45), although mean values scale with oxidative capacity, i.e. type I > IIa > IIb. Normalizing the local capillary supply to give LCD produces a slope very close to zero ($\text{LCD} = 186.6 + 0.0042 \times \text{area}$; $r^2 = 0.009$), effectively removing the scaling effect of fibre size for both slope and mean values, which is consistent with the observed lack of any significant difference among fibre types (Fig. 1B).

Table 1. *Fibre type specificity of local capillary supply in the lateral quadriceps*

Fibre type	LCFR	LCD (μm^{-2})	CAF	CPA (μm^{-2})	Fibre area (μm^2)
Type I	0.723 \pm 0.084	174.1 \pm 10.71	1.323 \pm 0.168	329.7 \pm 34.9	4176 \pm 340
Type IIa	0.569 \pm 0.056	172.1 \pm 13.30	1.027 \pm 0.120	338.2 \pm 50.8	3399 \pm 366
Type IIb	0.532 \pm 0.047	169.8 \pm 8.34	0.912 \pm 0.076	311.6 \pm 23.7	3216 \pm 366

DISCUSSION

Many studies have sought to determine whether the capillary bed of skeletal muscle is limiting for aerobic capacity via its role in setting the level of functional hyperaemia. While capillarity is increased following a period of training, a number of variables may complicate the implicit relationship between supply and demand. For example, in a longitudinal training study Andersen & Henriksson (1977) showed that an expansion of the capillary bed was accompanied by fibre type transformation in vastus lateralis. Both the duration of training and size of fibres were also important factors, with C:F increasing significantly after 5 weeks, but CD only after 8 weeks due to the accompanying fibre hypertrophy. From biopsies taken before and following 24 weeks of endurance training, Ingjer (1979) found a significant increase in C:F, with the CAF for slow oxidative fibres increasing more than that for fast glycolytic fibres. Indeed, Saltin & Gollnick (1983) suggested that the supply area for capillaries would be 20–30% (type IIb) or 10–20% (type IIa) greater than that for type I fibres, which is similar to the differences shown by our measured values of local capillary supply (LCFR; Table 1). These and other studies are in agreement with the more extensive literature from animal experiments showing that local demand (in terms of fibre type) may influence capillary growth, and that CD often varies inversely with fibre size (Hudlicka *et al.* 1992). Such considerations may explain reported discrepancies in CD among human training studies.

Using a specific marker for capillaries and a discriminatory analysis of local capillary supply, we have examined over 1700 human muscle fibres. By comparison with the discontinuous distribution of values from any of the integer-based indices of capillary supply, which convey little information regarding muscle composition, the continuous indices can be used to describe the local capillary supply and have the potential to discriminate between more subtle changes in fibre composition and/or size. This may be of particular benefit where fibre size changes between samples, e.g. hypertrophy following high resistance exercise (Abernethy, Jurimae, Logan, Taylor & Thayer, 1994), or where an increase in CD does not reflect capillary growth, but rather the atrophy of muscle fibres (Hoppeler & Desplanches, 1992). In particular, while both fibre size and capillary supply are reduced with age, the apparent specificity of training-induced increases in capillary supply to type II fibres in elderly subjects (Proctor, Sinning, Wairo, Sieck & Lemon, 1995) deserves closer attention. Indeed, in a pilot study we showed a regression of LCFR against fibre area in trained muscle which was merely offset from that of controls ($\text{LCFR} = 0.798 + 0.0017 \times \text{area}$; $r^2 = 0.17$), with a similar slope and clustering for fibre types, although LCD showed a slight negative slope against fibre size ($\text{LCD} = 501.2 - 0.031 \times \text{area}$; $r^2 = 0.15$), suggesting that additional factors have some influence on local capillary supply (Ahmed, Egginton, Ross, Mannion & Jakeman, 1995). We therefore conclude that the capillary supply to human skeletal muscle is scaled according to

fibre size and is relatively independent of fibre type. The method of capillary domains offers a reproducible technique, with the potential to discriminate between subtle adjustments in local capillary supply at the level of individual human muscle fibres.

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ABSTRACTS

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Effect of asthma and allergy on nasal polyp cell turnover

Nasal polyps have a higher rate of cell proliferation and apoptosis than normal nasal tissue. Polyposis is often linked to asthma, nasal allergies and aspirin sensitivity, which also increase tissue remodelling. The aim of the study was to examine whether the conditions of asthma, allergy or aspirin sensitivity cause more active nasal polyposis and to what extent.

Polyp and inferior turbinate biopsies were taken from each side of patients with bilateral polyposis. Inferior turbinate samples were also taken from patients with no asthma or allergy undergoing routine septorhinoplasty, as an external control.

Polyp patients with a history of asthma and/or allergy, or Samter's triad were grouped accordingly. Frozen sections were TUNEL stained to label apoptotic cells, and parallel sections stained for Ki-67 to label proliferating nuclei.

Patients with a history of allergy and/or asthma had significantly higher apoptotic indices than patients with neither. There appeared to be an additive effect of asthma and allergy with more apoptosis occurring in patients with both or Samter's triad compared to patients with one condition only. Proliferation was higher in patients with asthma and/or allergy than in patients with neither.

This study shows that having a history of asthma, allergy or aspirin sensitivity leads to more cell proliferation and apoptosis in nasal polyposis, and thus greater cell turnover. Both asthma and allergy increase the cell turnover, and the effect is additive, suggesting different mechanisms. Further studies will ascertain to what extent apoptosis and cell turnover occurs with asthma, allergy and aspirin sensitivity.

VEGF_{165b} or bevacizumab (a monoclonal antibody against VEGF-A). Hypoxic cells were cultured in a hypoxia chamber to which 5%CO₂/95% N₂ was connected. Cell viability (alive cells/alive + dead cellsX100) was determined by Trypan blue staining, and cytotoxicity by lactate dehydrogenase release. VEGF_{165b} increased trophoblast survival in normoxic (75 ± 2.3% VEGF_{165b}, 54 ± 8.1% control) and hypoxic (62 ± 1.8% VEGF_{165b}, 40 ± 3.8% control) conditions (One-way ANOVA, p = 0.0079, n = 16). In hypoxia, bevacizumab increased trophoblast cytotoxicity, and VEGF_{165b} addition to cells cultured in sodium butyrate (a cytotoxic agent) reduced trophoblast death (Unpaired t tests, p < 0.02 and p < 0.01 respectively, n = 14). VEGF_{165b} is a survival factor for 1st trimester trophoblasts. A lack of VEGF_{165b} may prevent adequate trophoblast invasion into the myometrium, promoting pre-eclampsia development.

PC77

SCALING OF CAPILLARY SUPPLY IN HUMAN MYOPATHIES

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In skeletal muscle ontogenetic growth and adaptive remodelling result in a capillary supply scaled according to fibre size [1,2]; whether this holds when remodelling is pathological is unknown. Biopsies were taken from control patients, and patients with idiopathic clinical muscle atrophy or fibre hypertrophy. Biopsies were snap-frozen and capillaries stained using *Ulex europaeus* lectin-1. Mean fibre area showed a 2-fold range among groups (~2300, 4500 and 6300 μm²). Capillary density (CD) of hypertrophic myopathies showed no significant difference from controls, but capillary to fibre ratio (C:F) increased in response to the greater fibre size (2.05 vs. 1.25), suggesting scaling effects similar to non-pathogenic stimuli. Atrophic myopathies showed no difference in C:F from controls, but an increase in CD (430 vs. 310 mm⁻²), suggesting maintenance of the vasculature independent of intramuscular scaling. These data are consistent with larger fibres being more susceptible to metabolic error signals, with reduced intracellular PO₂ acting as an angiogenic signal [3]. A meta-analysis of 44 human studies examined C:F, CD, fibre size, methodology, muscle type and experimental protocols as separate variables. Resistance training and chronic atrophies result in adaptive remodelling of the capillary bed, whereas acute atrophy shows vascular maintenance, and endurance training leads to a higher C:F than predicted by fibre size. [1] Egginton S. (1990). *Adv Comp Environ Physiol* 6:73-141; [2] Ahmed *et al.* (1997). *Exp Physiol* 82:231-4; [3] Deveci *et al.* (2001). *Am J Physiol* 281:H241-H252.

PC78

USING HUMAN ORTHOLOG RELATIONSHIPS AS A STRATEGY TO EXAMINE THE ORIGIN OF COLD-INDUCED ANGIOGENESIS IN A NON-MODEL SPECIES

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Some species increase their cardiovascular network to reduce the effects of reduced oxygen diffusion and increased blood viscosity associated with cold temperatures. Since many angiogenic and endothelial genes have been discovered in human, ortholog relationships between carp, zebrafish and human were used to predict the identity of genes responsible for cold induced angiogenesis. Carp sequences were collected and human-carp ortholog relationships derived, via zebrafish, using a new Conditional Stepped Reciprocal Best Hit (CSRBH) protocol. Then published articles, Gene Ontology and cDNA libraries were used to predict the identity of known or potential angiogenic genes. Finally, meta-analyses of cold carp microarray data identified carp genes up-regulated in response to cold temperatures. Using the CSRBH approach, 3,752 human-carp ortholog relationships (none redundant) were attained. 366 of these genes were predicted to have a potential role in angiogenesis. The total number of differentially expressed cDNAs in the carp data was 3603 and 1824 of these were up-regulated, representing 608 genes. The angiogenic prediction found 150 from the 608 genes to be angiogenic. Of these, 5 genes representing the orthologs NCL, RHOA, NFKBIA, GAPDH and MAPK1 are expressed in response to cold. Here we show that CSRBH ortholog relationships and meta-analyses can be combined in a none model species to predict genes of biological interest before a genome sequence is fully available.

PC79

VEGF_{165B} REDUCES HUMAN NEUROBLASTOMA GROWTH RATE *IN VIVO*

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Vascular endothelial growth factor (VEGF) has an essential role in angiogenesis and is up-regulated by a variety of tumours. An endogenous family of anti-angiogenic isoforms termed VEGF_{xxx}b has been identified in many normal, non-angiogenic tissues, and, in contrast with the angiogenic VEGF_{xxx} isoforms, is down-regulated in several

Angiogenesis and cell proliferation in nasal polyposis
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Objectives. Nasal polyposis has been proposed to be an angiogenic disorder. The aim of this study was to quantify the extent and type of angiogenesis in nasal polyposis.

Method. Polyp and inferior turbinate biopsies were taken from each side of patients with bilateral polyposis. External control samples were taken from patients undergoing routine septorhinoplasty. None of the patients had any history of asthma, allergy or aspirin sensitivity. One set of samples was snap frozen, cryosectioned, and stained using fluorescent lectins (stains capillaries), Ki67 (stains proliferating nuclei) and DAPI (stains all nuclei). Capillary density, capillary associated proliferation indices, epithelial proliferation indices and stromal proliferation indices were all measured. Parallel sections were also stained for apoptosis using TUNEL staining. A second set of samples were formalin fixed and immersion stained for capillaries, then analysed in multiple orientations using confocal microscopy. Capillary fractional area, surface area, surface to volume ratio and mean diameter were measured.

Results. There was no significant change from controls in capillary density, capillary-associated proliferation, capillary fractional area, surface area or surface to volume ratio. Increases in stromal proliferation and apoptosis were seen in polyp samples.

Conclusions. None of the commonly used measures of angiogenesis showed evidence of angiogenesis occurring in the polyp beyond that required for normal tissue growth. The architecture of the capillary bed is identical to the inferior turbinate tissue, hence angiogenesis is not a driving force in nasal polyposis. The previously reported presence of pro-angiogenic growth factors may be involved in stromal proliferation and cell turnover.

The role of nervus intermedius in side specific nasal responses

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Introduction. Nervus intermedius (NI) dysfunction is common in patients who have had vestibular schwannoma (VS) surgery.¹ Such patients have a unilateral parasympathetic-denervated nasal cavity. A number of side-specific nasal reflexes have been demonstrated in normals, including hand cold water immersion.² It is not understood whether these reflexes have parasympathetic or sympathetic efferent pathways.

Objectives. To evaluate the side specific nasal reflex to cold-water immersion in post-operative VS patients with NI dysfunction, in order to determine the nature of the efferent pathway of these reflexes.

Method. The study of Wilde² was repeated in 10 normal controls. Then a cohort observational study with internal controls was designed to evaluate the role of nasal parasympathetic innervation in patients who had unilateral VS surgery and NI dysfunction (Schirmer's test). Side specific response to cold-water immersion was tested by acoustic rhinometry.

Results. A consistent pattern of ipsilateral congestion and contralateral decongestion after the cold water immersion was seen in normals ($P < 0.001$ for both responses.) We found no consistent response in 18 VS patients on testing with stimulus ipsilateral to side of NI dysfunction and contralateral (the internal control).

Conclusions. We confirm the consistent side-specific nasal reflexes to cold water hand immersion in normals. This is disturbed in individuals following unilateral VS surgery with NI dysfunction. We have also

shown that the contralateral side-specific reflex is disturbed in these patients. These data suggest that the reflex is parasympathetic and crosses the midline.

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Is laryngopharyngeal reflux associated with changes in laryngeal mucosal flora?

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Objectives. To determine whether laryngeal reflux (LPR) affects the population size and distribution of laryngeal mucosal flora in humans; and whether LPR alters the integrity of the laryngeal mucosa. Further, to specifically assess whether *Helicobacter pylori* (*H. pylori*) is present in the laryngeal mucosa and if LPR is associated with this.

Methods. Laryngeal mucosal biopsies were taken from patients diagnosed LPR-positive or LPR-negative, none of whom had laryngeal disease. Presence of bacteria and epithelial integrity were determined by: fluorescent *in situ* hybridisation using oligonucleotide probes specific to all Eubacteria and *H. pylori*; real-time PCR specific for *H. Pylori* and all bacteria; and immunofluorescence using an antibody to occludin.

Results. *Helicobacter pylori* was discovered in all the biopsies tested – both in those taken from patients who did not have LPR and those that did – with no significant difference in its distribution between the two groups. *Helicobacter pylori* was found in both the epithelium and lamina propria of the laryngeal mucosa. Further, an increase in the number of total Eubacteria present in the laryngeal mucosa was found to be significantly associated with LPR (LPR mean 58.2 SE 19.0 bacteria/field of view, non-LPR mean 17.3 SE 4.7; $P = 0.02$).

Conclusions. The association between LPR and an increase in total Eubacteria in the laryngeal mucosa presents a possible aetiology for pathological symptoms associated with LPR. The fact that *H. pylori* was present in all of the laryngeal mucosal biopsies has significant implications for upper airway research.

Detection of lung tumours in patients with squamous cell carcinoma of the head and neck at the time of presentation

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Objectives. Screening for synchronous pulmonary tumours at initial presentation of squamous cell carcinoma of the head and neck is important as detection may alter management. Currently no consensus exists regarding best practice.¹ We present the largest series reported, and based on our data propose a pragmatic policy.

Methods. Retrospective review of the findings of pulmonary imaging performed on all patients presenting with HNSCC between January 1996 and January 2007.

PC27

THE FORMATION OF NASAL POLYPS IS INDEPENDENT OF ANGIOGENESIS

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Nasal polyposis is a common disease, the aetiology of which is currently unknown. Several mechanisms have been proposed, and recent studies have shown up-regulation of several pro-angiogenic factors [1,2]. The aim of this study was to assess and quantify the degree of angiogenesis in nasal polyps, and therefore whether angiogenesis is important in initiating polyposis. Biopsies of (1) polyp tissue and (2) nasal mucosa were taken from patients undergoing polypectomy, and compared with (3) nasal mucosal samples from control patients (n=5). Biopsies were either stained with fluorescent lectins for confocal microscopy or snap frozen and sectioned for histology for the examination of multiple measures of angiogenesis.

No significant differences in capillary density, capillary-associated proliferation, capillary surface density or capillary volume density were seen between the three study groups. The regression of surface density vs. volume density described the same linear relationship in all three groups, indicating no gross differences in architecture of the capillary bed. Polyp samples showed increased capillary diameter and interstitial proliferation. These results show no active angiogenesis or changes in capillary bed architecture in the polyp, although polyp capillaries appear more oedematous. As the capillary supply increases in line with the physiological needs of growing polyps, we conclude that angiogenesis is not a driving force in the aetiology of nasal polyposis, but is recruited in a feedback manner to support tissue expansion.

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Scaling of muscle capillary supply in humans: effect of strength training

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An accurate quantitative analysis of capillary supply to skeletal muscle is an essential prerequisite for understanding the limits to peripheral oxygen transport. Exercise-induced changes in the metabolic profile, oxidative capacity and fibre size are known to produce changes in muscle capillary supply, usually quantified as the ratio of capillary number to tissue area (capillary density, CD) or to fibre number (capillary to fibre ratio, C:F). Calculating the area of muscle tissue supplied by, or domain of influence of, an individual capillary provides a measure (by summing the fraction of all domains overlapping a fibre) of the local capillary to fibre ratio (LCFR) and local capillary density (LCD) (Egginton & Ross, 1989). The aim of this study was to utilize this approach to investigate whether the capillary supply to skeletal muscle was modelled according to fibre size or fibre type, in normal and post-training subjects.

With the approval of the local ethics committee and informed consent, muscle samples from the lateral portion of the quadriceps femoris muscle were obtained via percutaneous needle biopsy from twenty-one healthy, active young subjects (fifteen males and six females). Serial sections (10 μm) of muscle were prepared for histochemical analysis of fibre type (ATPase) and capillary visualization (Ulex Europaeus lectin, UEA-I). Digitized images of each stained section were used to determine the fibre area and the x, y co-ordinates for all fibres and associated capillaries. From these data the LCFR and LCD indices of capillary supply were calculated according to the procedures described previously (Egginton & Ross, 1989). Fibre type composition (mean \pm s.d.) by number for the subjects were: type I 47.4 ± 13.2 , type IIa 39.8 ± 12.2 and type IIb $13.0 \pm 9.2\%$; and by area occupied by type I $41.8 \pm 14.4\%$, type IIa 41.7 ± 14.4 and type IIb $11.0 \pm 7.7\%$. Mean fibre area was: type I 4224 ± 1006 , type IIa 5245 ± 1480 and type IIb $4293 \pm 1194 \mu\text{m}^2$. A total of 960 fibres were matched across serial sections for fibre type, fibre area and capillariness. Mean capillary supply was $310 \pm 71.6 \text{ mm}^{-2}$ and 1.77 ± 0.34 (CD and C:F, respectively). The LCFR analysis showed a significant positive correlation with fibre area ($r = 0.71$, $P < 0.0001$, ANOVA) that was independent of fibre type, while LCD showed no significant relationship to fibre area and no significant difference between LCD and fibre type. These results suggest that in humans capillary supply is scaled according to fibre size and is independent of fibre type.

The change in capillary supply with training showed an increase in both capillary supply and fibre size: 1.9 ± 0.49 vs. 0.62 ± 0.15 and 5073 ± 1459 vs. $1933 \pm 904 \mu\text{m}^2$ (C:F and mean area, respectively). LCFR increased, while LCD was unchanged after training (1.58 ± 0.65 vs. 0.54 ± 0.28 and 325 ± 129 vs. 306 ± 144 , respectively), suggesting that activity-induced changes in capillary supply are also determined primarily by changes in fibre size.

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