Inflammation and Upper Airway Remodelling in Natural and Induced Models of Allergic Rhinitis

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Statement of contribution

My contribution to the work described in this thesis is stated below.

Under the guidance and supervision of Professor Stephen Durham:

I designed, undertook and established all study protocols. I was responsible for drafting, submitting, and editing all ethics applications and liaising with the ethics committee up to and beyond full ethics approval throughout the duration of the study.

I undertook or assisted with the recruitment of all study participants along with the study research nurse Ms. Andrea Goldstone.

I performed all bar two biopsy samples throughout the study. I was fully responsible for monitoring and documenting patient safety throughout and after the biopsy visits.

I performed all imaging data collection and all immunohistochemistry bar vascular and lymphatic markers where assistance was provided by Dr. Aarif Eifan. Tissue cutting and cell counting assistance was provided by Mr. Daffyd Lee and Dr. Mikila Jacobson (as blinded observer) respectively.

I was responsible for all statistical and data analysis. Data was double checked by Dr. Mikila Jacobson.

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Abstract

Introduction: Alterations in basement membrane thickness, collagen deposition, angiogenic and lymphangiogenic markers and changes in TGF-β pathways, are features of remodelling seen in asthma. Such changes are not well-established in persistent allergic rhinitis (PAR).

Aims: To establish whether upper airway remodelling is a feature of PAR using two models of allergic rhinitis: natural seasonal allergen exposure (NAE) and repetitive allergen challenge (RAC) model.

Methods: Total nasal symptoms scores, nasal biopsies, and Th1 and Th2 cytokines from nasal secretions were assessed in subjects with severe PAR (n = 46), healthy control subjects (n = 19), and nasal polyp (NP) participants (n=11) who acted as positive controls. Angiolymphangiogenesis was examined using immunohistochemistry staining against CD31 (vascular endothelial cells), vascular endothelial growth factor-A, and D2-40 (lymphatic endothelial cells). Collagen and extracellular matrix proteins, such as heat shock protein-47 (collagen synthesis), matrix metalloproteinase-9, and tissue inhibitor metalloproteinase-1, and alpha-smooth muscle actin (myofibroblasts) were evaluated as markers of upper airway remodelling using image analysis. Submucosal effector inflammatory cells as well as TGF-β, activin, ALK-4 and SMAD2 were measured using immunohistochemistry.

Results: Total nasal symptoms scores and quality of life were higher in PAR compared with healthy controls ($P< 0.0001$) in both NAE and RAC. Nasal secretion IL-4 ($P< 0.01$), IL-5, and IL-13 ($P< 0.001$) were significantly higher in PAR compared with healthy controls, with IL-5 increasing only in RAC. Submucosal eosinophils ($P = 0.06$) were increased in both NAE and RAC and neutrophils ($p< 0.01$) only in RAC. No differences were observed in angiogenesis, lymphangiogenesis, deposition of extracellular matrix, collagen markers, reticular basement membrane thickness, or glandular percentage area between PAR and healthy control subjects in either NAE or RAC models. TGF-β ($p=0.04$) and activin ($p=0.01$) were significantly raised in RAC with only TGF-β ($p=0.02$) increasing in NAE.

Conclusions: Although TGF-β pathway markers may be altered, remodelling is not a feature of moderate-severe persistent allergic rhinitis.

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“Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.”

Marie Curie

Dedication

To Piers, Henry, Beatrice, Daphne and Hugo

My sun, moon and the stars

Publications related to thesis

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Structural upper airway remodeling is not a feature of repetitive nasal allergen challenge in patients with seasonal allergic rhinitis

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Decreased expression of nasal tissue TGF-beta may contribute to allergic inflammation in allergic rhinitis but not remodelling

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Despite lack of remodelling, TGF-□ and activin pathways are activated in severe/ persistent allergic rhinitis

N Orban, A Eifan, M Jacobson, S Durham

List of abbreviations

A2	second allergen challenge biopsies
ADAM	a disintegrin and metalloprotease
APC	antigen presenting cell
CD	cluster of differentiation
CRSsNP	chronic rhinosinusitis *sans* (without) nasal polyps
CRSwNP	chronic rhinosinusitis with nasal polyps
DC	dendritic cell
ECP	eosinophil cationic protein
ECM	extracellular matrix
EDN	eosinophil derived neurotoxin
EPO	eosinophil peroxidase
GAG	glycosaminoglycans
GM-CSF	granulocyte-macrophage colony stimulating factor
GMP	granulocyte monocyte progenitor
HSP	heat shock protein
IL	interleukin
MBP	major basophilic protein
MMP	matrix metalloproteases
NAE	natural allergen exposure
NETS	neutrophil extracellular traps
PBMC	peripheral blood mononuclear cell
RAC	repetitive allergen challenge
RANTES	regulated on activation, T cell expressed and secreted
RBM	reticular basement membrane
SAM	synthetic absorptive matrix
Si	in-season biopsies
SMAD	“small” mothers against decapentaplegic

TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteases
TNSS	total nasal symptom score
TSLP	thymic stromal lymphopoietin

Chapter 1 : Introduction

Introduction to the thesis

The nose is the gateway to the lungs, acting both as a protector and by extension, a first responder. Knowledge of remodelling in asthma has led to fundamental changes in our understanding of the disease progression and its response to treatment. In the nose such in-depth understanding is lacking, partly due to the variable clinical picture of nasal disease and the interconnected role of allergic response to many nasal diseases. Allergic rhinitis is a gold standard model for analysing tissue responses that are classically type-2 inflammation driven but the existence and degree of remodelling is not fully understood. This thesis seeks to understand and define remodelling in allergic rhinitis employing a comprehensive set of histopathological markers and comparison with both chronic rhinosinusitis with nasal polyps and normal nasal tissue. It first validates an artificial in-vivo model of allergic rhinitis, and then describes the findings as they relate to natural allergic inflammation and to remodelling.

Allergic Rhinitis

Epidemiology

In the past, allergic rhinitis was often trivialised and by extension, under-reported, missed or an under-diagnosed condition. Allergic rhinitis accounts for over 50% of all cases of rhinitis (1) (2). Up to 40% of children and 10-30% of adults are affected, with 400 million sufferers world-wide (3). With recognition of the influence of AR on global health not only on patients' quality of life, but also tangible capital losses felt by employers and the state, resources for treatment and prevention have improved.

Lack of appreciation as to the wide reaching personal and societal effect of the disease may be the consequence of the very fast paced increase in the prevalence of allergic diseases in general and allergic rhinitis in particular. First noted in the early part of the 19th century (4), allergic rhinitis was rare enough to be presented as unusual at medical conferences. John Bostock, noting the seasonality of the disease, coined the term 'hay fever' (5). Charles Blackley was the first to note the link between airborne particles and the symptoms of hay fever. Both men were sufferers of allergic rhinitis (6)which at this time was primarily thought to affect the higher social classes (7). Surveys during the second half of the 20th century (1970 and 1981) saw primary physician consultations for allergic rhinitis triple, particularly in the 5-15 year age group independent of gender (8). More recent research suggests higher prevalence of allergic rhinitis in boys than girls who catch up in terms of increased incidence peri-puberty such that both genders suffer equally in adulthood (1) (9).

In any given age group, geographical prevalence estimates for allergic rhinitis vary by as much as tenfold(10) and epidemiological studies are sufficiently well established to provide excellent longitudinal data (11). Accessibility to allergy testing varies and can influence epidemiological studies (12). Over a period of fifteen years, the International study of Asthma and Allergies in Childhood (ISAAC) -the largest paediatric, cross-sectional epidemiological study to date- using standardized and validated questionnaires, conducted a study over three phases looking at the prevalence and severity of asthma, allergic rhinoconjunctivitis, and eczema in childhood (13). Phases 1 and 3 provided data for changes in prevalence within and between countries whilst phase 2 looked at possible aetiological variables such as socio-economic status, genetic factors, or the medical care received. Assessment was made in children aged 6-7 (66 centres in 37 countries) and aged 13-14 (106 centres in 56 countries) at two time points at least 5 years apart. In Phase 1 the median prevalence of allergic rhinoconjunctivitis was 6.9% in the younger and 13.6% in the older age group. Of the centres, 66% showed an increase in the prevalence of allergic rhinoconjunctivitis in the younger age group with the absolute median prevalence rising to 8.5% in Phase 3. In the older age group 45% of centres showed an increase with median prevalence rising to 14.6%. Decreases in prevalence were observed in 14%

of the younger and 25% of the older age group. With clear patterns for change, most centres demonstrated a change of at least one standard error for at least one disorder. Increases of prevalence were more common than decreases with over 20-fold difference in the prevalence of allergic rhinoconjunctivitis between individual countries (14). In contrast, worldwide asthma prevalence is stable (15) (16). Furthermore, low- and mid-income countries demonstrated plateauing or a fall in prevalence suggesting allergic rhinoconjunctivitis has peaked in these regions. The U.K. has one of the highest rates in Europe with Georgia and Lithuania having the lowest, fitting the trend of decreasing prevalence rates from West to East. Socio-economic status and industrialization is only one factor: Spanish Latin America has higher rates of prevalence than Spain itself, contradicting the idea that more affluent societies are more likely to develop allergy (17). Further updates on ISAAC discount ambient environmental particulate matter as a cause of variation in childhood prevalence of allergic rhinoconjunctivitis (18). The incidence of wheeze, asthma and eczema correlate well in an adult population study, the European Community Respiratory Health Survey (ECRHS) (19). To contrast, the ISAAC study prevalence for allergic rhinitis for the UK show a 12-month prevalence of 9.8% in 1997 rising to 10.1% in 2002 (20). Using similar and standardised methodology, the European Community Respiratory Health Survey found a mean UK prevalence of 21% of allergic rhinitis(21). This contrasts with prevalence of atopy at 50% in the general population (22), implying atopy is not synonymous with allergic disease. To date, this is still one of the biggest epidemiological studies in AR and asthma. More recently, utilisation of Google search terms has offered a novel epidemiological tracking tool (23).

These findings were further supported by the Swiss Study of childhood Allergy and Respiratory Symptoms with respect to Air Pollution and Climate (SCARPOL) study. This cross sectional study including four thousand children aged 6-15 showed that nocturnal or chronic cough is, but rhinitis is not, associated with long-term exposure to air pollutants (24). Using and testing the validity of the ISAAC questionnaires, the SCARPOL study demonstrated its high predictive value but low sensitivity amongst the general population (25).

Studies examining changes over time demonstrate variable evidence for the effect of geography and climate on prevalence of allergic rhinitis. A Swedish study on birth cohorts found a 3-4 fold increased risk of allergic rhinitis (26). Socioeconomic status showed a strong inverse correlation particularly among those borne during the 1950s with this effect present but decreasing over time. Industrialised nations bear a bigger burden of allergic diseases in general and allergic rhinitis in particular but this effect is also diminishing with time (1, 27). Even non-temperate climates have a low but significant disease burden (28).

Nonetheless, with over 400 million sufferers world-wide, allergic rhinitis is a global health issue (6).

Aetiology

Many epidemiological analyses have sought to answer the question of the rapid rise of allergic rhinitis in the last century. An amalgamation of pre- and post-natal factors exert their influence. Since the protective effect of large sibships was first reported (29), the effect of family size on rhinitis was expanded by Strachan (30). He coined the term 'hygiene hypothesis' which has since been applied to T cell skewing seen in response to environmental influences. Post-natal influences are unlikely to confined to childhood as the protective effect of having siblings is unrelated to birth order or the time lag between birth dates (31). Westernisation of society results in decreased parity and is thought to be in part responsible for the trend for increased prevalence of allergic rhinitis.

The relative significance of pre- or post- natal influences in not clear although good evidence exists as to the influence, at least in part, of both. Studies looking at pre-natal immunomodulation demonstrate significant association between cord blood immunoglobulin E and allergic sensitization in childhood, with levels of cord blood IgE decreasing with birth order (32). Post-natal alteration of T cell lineage commitment are supported by a review of 53 studies (33) which found that all studies looking at allergic rhinitis have an inverse relationship with family size.

The role of rural upbringing, via possible enhancement of a Th1 immuno-profile, may influence the decreased development of allergic rhinitis both pre- and post-natally. With increased infectious exposure (34), alteration in the dominant T cell profile away from Th2 picture may be exerted pre-natally. Offspring of mothers actively engaged in farm and stable work whilst pregnant were much less likely to be sensitized and to develop hay fever than children whose mothers had no such contact (35) (36). Evidence for post-natal immunomodulation is seen in infants and young children (37). Children exposed to farm milk and stables under the age of 1, or who ate fish had their risk prevalence of allergic rhinitis considerably reduced (3% vs 13%) (38). Furthermore, prolonged exposure to such influences bestowed a long-term protective effect until 5 years of age (35). Exposure to a multitude of infectious agents such as *Hepatitis A*, *Toxoplasma gondii* and *Mycobacterium* species or their products confers a preventative effect on the development of atopy (39). Presence and ingestion of endotoxin, whether around the house (40) or in unpasteurised milk may result in alteration of gut flora. In a study matching for non-atopic status, (41) comparison of Estonian and Swedish children (two countries with close geographical proximity but different lifestyles) showed fundamental differences in faecal bacteria developing soon after birth (42).

Other risk factors associated with predisposition to the development of allergic rhinitis are maternal smoking, particularly in the first year of the child's life, late entry into nursery/ schooling, early exposure to animal dander and birth during high pollen counts (43). Predilection to atopy has been

localised to a number of chromosomal loci (39). A German population-based birth-cohort study demonstrates an annual prevalence rate of allergic rhinitis of 6% at age three, rising to 24% aged thirteen. Similar figures, when one parent is affected by allergy, are 13% aged three, and nearly half the population studied at 44% aged thirteen. When both parents are allergic, the association with development of allergic rhinitis is significantly increased (adjusted OR 3.1; 95% CI [1.1-9.3]) (44).

Subjective measures of disease severity

The effect on the quality of life of sufferers of allergic rhinitis is on par with debilitating, chronic conditions such as diabetes and hypertension (45) (46). Various assessment tools have been used to quantify the personal burden of allergic rhinitis. Designed for investigating any disease, the generic medical quality of life questionnaires, such as the Medical Outcomes Study Short-Form with 36 items (SF-36) allows for comparison across disease entities.

Disease specific questionnaires carry the advantage of assessing the impact and influence of a specific disorder directly on individuals' psycho-social and physical functioning. Initially developed in 1991 by Juniper and Guyatt and specifically designed to assess the impact of rhinitis, the Rhinoconjunctivitis Quality of Life Questionnaire, RQLQ, is reliable and reproducible with good discriminatory properties. Acceptable correlation is achieved between the SF-36 and RQLQ when used exclusively for patients with rhinitic symptoms (47). These questionnaires have subsequently been modified e.g., The Rhinitis Quality of Life Questionnaire (for sufferers without ocular symptoms), the nocturnal, paediatric, mini (with 14 symptom parameters) and the standardized RQLQ, specifying the activities of daily living to be assessed (48). Assessment of validity and reliability of RQLQ in allergic rhinitis demonstrates good evaluative (responsiveness and longitudinal validity) and discriminative (reliability and cross sectional validity) properties of this tool with the SF-36 having good discriminative but poor evaluative features (49).

A variety of other assessment tools quantifying personal impact of other types of rhinological disease are in use (50). Within the clinical picture of chronic rhinosinusitis, the Sino-Nasal Outcome Measure, or SNOT-22, is highly suitable(51). Visual analogue scales (VAS) to assess disease severity are simple to use, easy to interpret and universally recognised (52). Their simplicity has led to their ubiquitous use. Validated (53) and used in primary care to assess severity of allergic rhinitis and strength of intervention (54), overall rhinitis severity VAS score correlate well with RQLQ (55).

Recently it has been demonstrated that visual analogue scores can assess severity of allergic rhinitis according to Allergic Rhinitis and its Impact on Asthma (ARIA) classification (56). In this study conducted amongst 3052 primary care patients. 41.8% had moderate/severe intermittent symptoms

and 51.2% moderate/severe persistent symptoms. More recent advances in electronic recording of symptoms have enhanced focus on patient empowerment in monitoring, reporting and treating symptoms (57) . The use of mobile technology correlates well with well-established methods of symptoms scoring (58).

Personal Burden

Traditionally focus of allergic rhinitis has been on treatment modalities. With the conception, development and translation into primary care parlance of the ARIA classification, better understanding of appropriate diagnosis, and targeted therapy has embraced better appreciation of the personal cost of allergic upper airway disease.

In adults, allergic rhinitis affects social functioning (59) as measured by SF-36. Population-based cross sectional surveys have shown that individuals with AR have higher scores than non-sufferers in five domains(60) as assessed by the RQLQ. Two million schooldays per annum are missed due to allergic rhinitis in the United States alone. In children, disturbed sleep leads to daytime fatigue and somnolence (61), which in turn negatively affects concentration. The consequent drop in academic performance may be tangible, seen as a drop in examination grades (62), or more abstract, manifesting itself as frustration and behaviour disturbance. The latter may also be seen when children are not able to partake in normal activities with their peers or in a family setting (63). The 'Allergies in America' survey, the largest and most comprehensive survey examining the paediatric population and their carers, showed 80% of sufferers felt tired frequently or sometimes during the allergy season (64) (65). Psychological effects of allergic rhinitis manifest as irritability in two-thirds of the population, with 36% reporting depression and 23% embarrassment frequently or sometimes during the season.

Socio-economic Impact

Socioeconomic impact of rhinitis can be direct, indirect, or hidden. Direct costs incurred in the form of outpatient hospital services, inpatient care and medication are rising. Previous health economic studies in United States showed a cost of $7.3 million in 2002 with the cost of rhinoconjunctivitis in children accounting for $2.3 million (66). Primary care visits accounted for 68% of this cost. These figures are likely to be modest estimates. Spending on allergic rhinitis in the United States was estimated at $6.1 billion in 2001, near doubling to $11.2 billion in 2005 (67). Over 50% of this sum was spent on outpatient or over the counter prescriptions. A total direct cost of $3.4 billion is split near evenly between medical prescriptions and outpatient visits (68). In a recent Swedish study, the combination of direct and indirect costs of allergic rhinitis was just under €1k per individual/ year (69).

Indirect costs in the form of impaired productivity at school and work are stark. In the United States alone, allergic rhinitis is responsible for 3.5 million lost work days (70) per year. The effect of symptomatic allergic rhinitis is further compounded by the sedating effect of antihistamines. Adults notice a decline in their work-place productivity. Patients' perception of their own efficiency at work is approximately 95% out of season dropping to 72% when symptomatic (60). Data from the National Health Interview Survey in the United States estimates the cost of such over the counter medication use to range $2.4 to $4.6 million (71). From an employer's perspective, cost of allergic rhinitis in the form absenteeism and presenteeism (decreased productivity whilst at work) accounts for 23% of all loss in productivity. The mean loss in productivity per employee per year of $593 is higher than any other condition including diabetes and coronary heart disease (72).

Comorbid associations lead to further, hidden costs of allergic rhinitis. Upper airways conditions such as rhinosinusitis, asthma, otitis media may exercise a symbiotically detrimental effect on overall symptomatology (66).

Clinical Phenotypes

Upper airway classification into well demarcated phenotypes may not reflect those found in asthma and linking to particular underlying pathology may prove more fluid (73). . Traditionally allergic rhinitis was classified according to the seasonal or perennial presence of aeroallergens. With the now ubiquitous use of the ARIA classification, categorisation is based on the duration of symptoms rather than the responsible aeroallergen. Persistent (present for four days or more, for four weeks or more continuously) and intermittent (present for less than four days and lasting less than four weeks at a time) are not interchangeable with perennial and seasonal disease respectively. A six nation European cross-sectional population based study found no association between the two systems, in that a large proportion of patients classified as 'seasonal' had year round symptoms, and those identified as having persistent disease were actually only intermittently symptomatic (74). The 'persistent' group regardless of seasonality of the allergic trigger experienced higher levels of discomfort, sought medical attention more often, had more medication prescribed and used it more often. This persistent group also reported higher mean scores for symptom frequency and severity.

Whilst noting the seasonality of allergen is helpful in directing immunomodulatory or symptom directed therapy, allergic rhinitis is clinically defined according to the duration and severity of the disease. Clearly defined seasons are not a climatic feature of every country. With multiple sensitivities, the ARIA classification is ideally suited to accurate categorisation of allergic rhinitis and is applicable world-wide.

Treatment

Up to date recommendations for treatment of allergic rhinitis are summarised in a recent ARIA update (75) (76). Simple allergen avoidance is advisable but often unrealistic. Interventions targeted at reducing allergen load may decrease the amount of aeroallergen but do not necessarily lead to significant clinical improvement (77). Non-pharmaceutical measures in the form of saline nasal douches are effective without side-effects. As such, they are well placed for use in pregnancy and paediatrics population (78) (79).

Pharmaceutical intervention should be guided by ARIA workshop stepwise approach to treatment. Intra-nasal corticosteroids are the single most effective treatment in allergic rhinitis with nearly 80% of adult sufferers responding to such topical treatment (80). Their efficacy is on par, or even superior to anti-histamines in combination with an anti-leukotriene (81). The safety profile of intra-nasal corticosteroids is sound and long-term use is advocated with data to support its use in adults and children (82, 83). Treatment should commence with intranasal corticosteroids, with additions of anti-histamines and oral leukotriene inhibitors in that order, should a step up in therapy be required (84). The addition of intranasal antihistamines has shown added benefit to intranasal corticosteroids alone and is superior to oral antihistamines. Glucocorticosteroids cause multifaceted effects in allergic diseases (85). Although some evidence suggests improvement in asthma with treatment of allergic rhinitis, guidelines are to treat the existing nasal condition, and not medicate established allergic rhinitis with a view to preventing asthma development or asthmatic-type symptoms (86) . Treatment benefit may be seen in other, non-airway related comorbid conditions. For instance, nasal topical corticosteroid treatment of allergic children reduces incidence of OME (Otitis Media with Effusion) by reducing nasal inflammation (87). The use of sublingual and subcutaneous therapy is now well established in AR with the benefit risk profile of each at an even balance (88).

Chronic Rhinosinusitis with and without Nasal Polyps

Introduction

Chronic rhinosinusitis with (CRSwNP) or without (CRSsNP) nasal polyps is a chronic inflammatory condition affecting the nasal mucosa with concomitant and obligatory involvement of sinus mucosa. Coexistence of the two conditions is now well-recognised and studies into epidemiological data, aetiological triggers and treatment modalities have embraced rhinitis and sinusitis as intrinsically linked. Diagnosis is dependent on clinical criteria and computer tomography (CT) or endoscopic findings. As one of the most common chronic conditions, CRS, presents to a wide variety of specialties, not all of whom have endoscopic facilities and endoscopic training.

Epidemiology

Heterogeneity of the disease as well as methods of diagnosis renders epidemiological studies difficult (89). Whilst 5-15% of the general population is estimated to be sufferers (90), consistent with the rate of physician diagnosed chronic rhinosinusitis (CRS) is 2%-14% (91) (92). Without access to endoscopic evaluation, the prevalence of CRS is likely to be overestimated (93). The Global Allergy and Asthma Network of Excellence (GA^2LEN) EPOS criteria compliant postal questionnaire study of CRS symptoms found a prevalence rate of 10.9% (6.9-27.1) across 19 European sites (94) with the mean age of onset of CRS is 42 (95).

Aetiology

The role of allergy in the aetiology of CRS is unclear. As nasal polyps are marked by Th2 cells and eosinophilia, initial epidemiological data aimed at establishing a link. Present evidence suggests that while allergy may compound CRS, treatment of either condition must not neglect management of the other if full benefit of treatment is to be gained (96). The prevalence of allergic rhinitis in nasal polyp patients varies from 10% (97) to as high as 64% (98). The prevalence of nasal polyps amongst allergic rhinitis patients is no different than within the general population (99) (100). Eosinophil counts in nasal polyps show correlation with specific and total IgE counts (101). Evidence of atopy is present in 50% to 84% of CRS patients (102) (103). Atopic CRS patients do not have infective episodes when exposed to the sensitised allergen (104). Allergy is likely to co-exist and possibly contribute to the aetiology of some phenotypic subsets of CRS without being a causative element *per se* (105).

Causes of both CRSwNP and CRSsNP are likely to be an amalgamation of factors causing dysregulation at the host-environment interface. Impaired protective effect of nasal epithelium may be initiated, propagated, and/ or influenced by pathogens present on nasal mucosal lining. 'Super-antigens' with ability to cross-stimulate the adaptive immune response and activate leukocytes responsible for the early-phase reaction are also likely to utilise impaired barrier mechanisms thereby initiating innate

immune responses (106). Presence of *Staphylococcus aureus* in epithelial cells of CRSwNP but not CRSsNP suggests particular impairment of adaptive and barrier mechanisms may initiate or drive polyp formation (107). Alteration in eicosanoid pathways, of particular relevance in aspirin sensitive CRS, are likely to at least partially influence disease progression. Enhancement of pro-inflammatory leukotrienes, with decrease in anti-inflammatory prostaglandin E2 will upset the delicate cytokine balance within CRS nasal mucosa (108).

A number of genetic factors are likely to contribute to the above. Genetically determined enhanced expression of inflammatory cell lineages or cytokines, together with environmental stimulation of epigenetic pro-inflammatory alterations assimilate in the form of Th2 driven and insufficient T regulatory responses

Clinical Phenotypes

Previously thought to differ only in severity and duration, CRSwNP and CRSsNP are now thought to be two different, if overlapping, disease entities co-existing under the umbrella of rhinosinusitis. CRSwNP demonstrates less fibrosis, more Th2 type T cell skewing, increased glandular formation, and eosinophilic, polyclonal IgE rich infiltrates with less TGF-β expression. CRSsNP in contrast has a more Th1/ Th17 cell profile, increased TGF-β and evidence of fibrosis (109). Clinical manifestation of this histological division is seen as expression of symptom severity. Nasal polyp patients actively undergoing therapy versus nasal polyp patients who have not sought, and do not receive medication, have significantly worse objective measures of the disease such as extent of polypoid disease and peak nasal inspiratory flow rate and worse subjective measures such as sense of smell (110).

Within the confines of diagnostic criteria of rhinosinusitis, there exists a broad, heterogeneous clinical group. Allergic and infective aetiologies all share the symptomatic manifestations of chronic rhinosinusitis with or without polypoid disease, albeit CRS lacks the atopy driven profile of allergic rhinitis.

Unified Airway Theory

The anatomical continuity between the nose and lung is reproduced in their histological picture (111). Recognition of this reciprocity of immunopathology has been long recognised. With the advent of the ARIA collaboration, definition, research aims, and summary recommendations are brought together under one roof guiding comprehensive medical management. Previous GA^{2}LEN reviews provide a structured investigative approach to tackling burden of asthma and allergy (112).

The presence of allergic rhinitis alters the symptomatology of asthma. Most asthmatics suffer from rhinitis (113). Allergic rhinitis in children (114) and in adults (115) is a risk factor for asthma independent of allergy(116). Although not all rhinitics suffer from asthma, those with more persistent clinical picture or on the more severe end of the clinical spectrum are more likely to be asthma sufferers. Rhinitics sensitive to both indoor and outdoor allergens (versus those only sensitive to either) are more likely to fall into this group. Thirty-eight percent of patients with allergic rhinitis have co-existing asthma. Conversely 78% of asthmatics report symptoms of allergic rhinitis (117) (118), with severe or persistent rhinitics more likely to have co-existent asthma (119). Existence of either condition may be compounded by the severity of the disease and in turn increase an individual's predisposition to the development of the other (120). Rhinitis, regardless of whether of allergic aetiology, increases susceptibility to asthma with an odds ratio of three (121, 122). Children with physician diagnosed rhinitis before the age of 1, were more likely to have respiratory symptoms and diagnosis of asthma aged 6 (123).

Asthma exacerbations, attacks and hospitalisations are more common in rhinitic asthmatics, with non-specific airway reactivity to cold air, than in asthmatics alone (124). Furthermore, bronchial reactivity and hyper-reactivity is more pronounced in persistent rhinitics than intermittent rhinitis sufferers. Such increased bronchial reactivity is triggered in hay fever sufferers during, and shortly after the season. It is possible that the perception of bronchial reactivity induced dyspnoea in this asthmatic rhinitic group is different from those without asthmatic symptoms.

Whether the link between rhinitis and asthma is causative or a continuum of the same disease is unclear. A model proposed by Togias would indicate the latter; the more severe the rhinitis, the worse the asthma (125). Most asthmatics have CT evidence of rhinitis. Histological evidence of an inflammatory reaction and possibly remodelling may be manifest in the nose and/or lung prior to clinical presentation. Eosinophils are found in both the nose and lung of asthmatics. Recognition of allergic rhinitis as a non-trivial complaint is essential to avoid high rates of underdiagnosis (126). Asthmatics demonstrate upper airway eosinophilic inflammation even in the absence of symptomatic rhinitis. Allergen challenge in one organ, results in eosinophilic inflammation in the other (87, 127). Presence of moderate rhinitis results in over fourfold increase in poor asthma control (similar to that of smoking) (87). Rhinitis with concomitant asthma confers increased access to healthcare, both in the primary care and hospital setting. How the coexistence of these two conditions affects work productivity and absenteeism is still contentious(128) (129). Nasal corticosteroids are more effective than inhaled corticosteroids in reducing AHR (130) (131). Clinically, allergic rhinitis is associated with increased airway hyperresponsiveness and reducing nasal inflammation improves AHR (132) (133).

Differences between the two conditions do exist. Although pollen-induced asthma exists, pollen allergy is not necessarily associated with asthma (134). Importantly, chest symptoms of rhinitics are not normally associated with airway obstruction(135).

Asthma and Allergic Lower Airway disease

Asthma is a chronic inflammatory condition of the lower airways associated with symptoms of airway obstruction which directly affect physical functioning (136). As with allergic rhinitis, asthma prevalence is rising in countries previously without a significant disease burden in response to industrialisation and a reduction in rural living. In contrast to world-wide trends for allergic rhinitis, the prevalence of asthma is however in decline in some areas. In both cases, quick and marked changes in disease rates are not explained by the slow effect of genetic variation and are more in keeping with unmasking of already present predisposition by rapidly shifting environmental factors. Influence of sibship size amongst a number of other environmental or individual factors (137) is less clear cut in asthma than allergic rhinitis (31, 138).

Persistent rhinitis, with accompanying loss in sense of smell, has a mean onset at 30 years of age. Two to five years later, asthma follows. Non-steroidal anti-inflammatory (NSAID) intolerance follows approximately five years later, with concurrent onset of nasal polyposis in the majority of patients. Such uniformity of aspirin-induced asthma bears true across countries (139).

Whilst within asthma, distinct clinical phenotypes exist, the immunopathological hallmark of the disease is a type 2 mediated inflammatory response. Although not all asthmatic subsets are driven by an atopic trigger, mechanistic pathways converge around a local IgE mediated reaction in the conducting airways. Resultant narrowing of the airways, referred to as airway hyperreactivity, comprises a clinical picture that varies in severity with environmental stimuli. It may be that such narrowing consists of two components, one of which serves to restrict the airways in a non-reversible, non-inflammatory fashion and is therefore resistant to acute anti-inflammatory treatment. The second may be responsible for acute, episodic and reversible airway narrowing that is amenable to anti-inflammatory therapeutic modulation. The hypothesis of remodelling being responsible for the former and acute Th2 driven exacerbation for the latter is gaining popularity.

Recent research explored the temporal relationship between airway remodelling and the clinical picture of airway hyper-responsiveness. Alterations in microanatomy, increased contractile forces due to smooth muscle proliferation, as well as enhanced mucus production do affect the asthma phenotype. Repeated allergen exposure causes structural changes collectively termed ‘remodelling’. This is likely to be compounded by a predisposition of the airways to faulty or incomplete repair

following relatively minor insults resulting in the clinical picture of asthma. There is evidence that airway responsiveness in the newborn period is predictive of increased asthma risk at 6 years of age (140). Non-invasive markers of remodelling can be found in asthmatic children (141). Furthermore, a cumulative effect exists between viral and allergic inflammation. For example, a child has a 20 fold increased chance of admission if exposed to rhinovirus and allergen (142).

Recognition of the role of remodelling in the pathophysiology of asthma is now opening up possible therapeutic avenues. Presence and role of remodelling within the context of allergic upper airway disease is much less defined.

Models of Allergic Rhinitis

Studies of asthma and allergic rhinitis depend on animal and human models. Animal models are primarily murine but may involve non-human primates. Mouse models have the advantage of ease of use and ready availability of different strains bred to express certain tissue responses to specific stimuli. Studies into asthma and AR mainly use BALB/c as they show a marked Th2 type response with high IgE-driven allergic inflammation. Mouse models of an allergic response rely on a period of sensitization followed by a period of allergen challenge. Sensitisation is characteristically induced systemically by administering an allergen such as ovalbumin suspended in an adjuvant such as aluminium hydroxide. Administration may be intraperitoneal or subcutaneous. During the subsequent period of repeated challenges, allergen may be administered directly, intranasally, intratracheally or be aerosolized and inhaled in a challenge chamber. Period and number of challenges may be variable (143). The possibility of altering specific gene expression, successive inbreeding, numerous different strains and methods of allergen challenge makes mouse models useful in studying allergic disease.

Murine models of remodelling and airway inflammation aiming to reproduce asthma or allergic rhinitis, rely on chronic repetitive allergen challenge lasting weeks to months (144). Without a sensitisation phase, mice may develop tolerance to chronic exposure (145). Moreover, the route of administration is critical with airway remodelling only witnessed via intranasal challenge of OVA in A/J strain mice (146). As such, murine models will aid but are unlikely to be able to faithfully replicate human upper airway diseases such as allergic rhinitis. Murine responses rely not only on method of administration, mouse strain but also only partially recapitulate human responses to chronic allergen challenge. Mice do not demonstrate marked influx of mast cells to the airway wall or epithelium (147), they fail to demonstrate significant increases in airway smooth muscle unless exposed to high doses of allergen (146), their inflammatory response is not restricted to conductive airways and they show vascular and parenchymal remodelling which is not seen in human asthma (148). Such inherent

differences imply the need for prudent interpretation of mouse models *vis a vis* human airway disease in general and human asthma or allergic rhinitis in particular (149) (143).

Such allergic models demonstrate acute airway/nasal allergic inflammation and develop airway hyperactivity post challenge with histological evidence of a Th2 mediated response. Murine and human models of allergic lower airway disease distinguish between the acute Th2 driven inflammatory response manifest as early phase reaction, and the AHR seen once early allergic inflammation has settled (150). The chronic asthmatic phenotype is not reproduced with baseline airway reactivity and chronic tissue changes seen in the absence of clinical symptoms. This intrinsic difference between murine models and human allergic airway disease means results should be interpreted in context and with caution when considering implications for detecting putative therapeutic targets in man. Expression of inflammatory and remodelling markers may differ between *in-vivo* and *in-vitro* models (151). Murine models may not fully elucidate the alteration in cellular and cytokine profile, or provide understanding that accurately mirrors what is found in man (152) (153) (154). For example, in murine models, IL-25 may be produced by mast cells and epithelial cells whilst in humans, eosinophils and basophils are the main sources of IL-25 (155). The well characterised murine immune system can enlighten our understanding by generation of over or under expression of signalling pathway constituents.

Human models provide the most accurate prism through which to view acute and chronic effects of allergic disease. In the case of allergic rhinitis, nasal sampling of tissue and/or secretions in different disease states is the gold standard. Ongoing natural environmental allergen exposure (seasonal for grass pollen, and perennial for house dust mite and pets) leads to chronic inflammatory changes in which mast cell and eosinophil/ T cell-dependent mechanisms become superimposed. The current experimental approaches used to model these changes for mechanistic and clinical studies each have major limitations.

In the 'single challenge' model, allergen is delivered to the nose in a simple one-off exposure. Though convenient, this model fails to reflect the chronicity of natural exposure. Moreover, the dose of allergen used is necessarily higher than that naturally encountered in order to ensure positive responses in most, if not all participants (156). The 'natural seasonal allergen exposure' model is physiological in that pollen-allergic subjects are studied during a period of natural exposure (i.e. in the pollen season). Such studies are hampered by the unpredictability of the pollen season. Additionally, the need to perform a baseline comparison outside the pollen season presents considerable logistical difficulties.

These problems may be partially overcome by use of an exposure chamber, such as the Vienna Challenge Chamber in which many subjects may be simultaneously exposed to grass pollen in a controlled environment. Mite allergic subjects similarly exposed to house dust mite (HDM) showed high correlation between allergen concentration and total nasal symptom score (TNSS) in (157). It is not clear how representative of natural exposure the chamber is, and there are very few available in view of their expense and need for high staffing and maintenance. A recent EAACI position paper sought to bring together up to date evidence for best practice and to optimise its use in future studies (158).

Limited existing data suggests that the cellular profile of nasal biopsies from patients subjected to repetitive challenge is different from that seen after single challenge with allergen. For example, 5-10 fold increases were reported in recruitment of key effector cells (eosinophils) and dendritic cells, consistent with 'priming' (159). In the lung, repetitive low dose allergen challenge results in non-specific bronchial responsiveness (160) and may provoke a late asthmatic reaction (161). Such models are currently used to investigate therapeutic interventions to control airway hyperresponsiveness (162) (163).

Nasal anatomy and physiology

Past the nares and the nasal vestibule, the mucosal lining of the nasal cavity like the lower airway, comprises pseudostratified columnar ciliated respiratory epithelium. Mucus is produced by goblet cells that lie interspersed amongst epithelial cells. Mucus within the nasal cavity acts as a mechanism of transport of inhaled particles, as a medium in which to dissolve odour-producing substances and as a non-specific barrier against infections. It contains a mixture of immunoglobulins, albumin, and bacteriolytic enzymes. Mucus is also produced by mucinous and serous glands in connective tissue deep to the epithelium. This layer is termed the lamina propria. Made up of collagen types III and IV as well as fibronectin, it is home to the network of veins which form thin-walled, cavernous sinusoids, also called cavernous bodies.

Blood supply to the nose is via the sphenopalatine and greater palatine arteries, both branches of the maxillary artery. Blood then flows to smaller arterioles via a network of superficial capillaries within lamina propria to reach the veins or via arteriovenous anastomoses. Both may be interspersed with venous sinusoids. Blood can be shunted between these two drainage routes regulating temperature control in the nose. Arterioles act as resistance vessels and post-capillary venules as capacitance vessels. While the arteriolar tone determines the amount of blood reaching the capillaries and venules, the muscular and collapsible venous sinusoids determine the drainage. Both are under neural as well as local mediator control. Therefore, nasal congestion is dependent on the intricate balance of

the local neurochemical environment. Small, superficial capillaries, not lined by endothelial cells, have fenestrations in their basement membrane. Capillary vascular pressure determines exudation through these fenestrations. Exudates are rich in albumin, immunoglobulins and contribute to the non-specific defence mechanism of the nose.

Inflammation

Introduction

The term 'atopy', initially coined by Coca and Cooke as a type of hypersensitivity, is the predisposition to production of specific IgE in response to a ubiquitous and under normal circumstances, benign protein at mucosal or epithelial surfaces (164). For clinical symptoms of atopy to manifest, sensitisation to an inhaled allergen begins at the nasal epithelium. The responsible allergen is recognised by local naïve antigen presenting cells (APCs) as such dendritic cells, macrophages and B cells. During intracellular antigen processing APCs lose their adhesiveness to the local tissues and begin to express surface markers that act as receptors for selectin ligands within peripheral lymph nodes. Here they meet and interact with naïve T cells within the T cell paracortex in an MHC class-II restricted fashion. Co-stimulators via CD28 on the T cell and CD80/ CD86 (B7-1/ B7-2) on the APC serve as a second signal for T cell activation. In the context of T helper cells, signal transduction is further facilitated via CD4. Newly stimulated T helper cells migrate towards the B cell lymphoid zone, where T and B cell interactions take place. Alteration of the local cytokine milieu both by the activated APCs and responding T cells, as well as the T/ B cell interaction, drives B cell class switching to antigen specific IgE. Such class switching is also present within the nasal mucosa (165). Locally generated antigen specific IgE is thereby available to sensitise mast cells and basophils via binding to their surface FcRIε receptors in order to sustain an ongoing cycle of allergic inflammation. Newly produced IgE coats local mast cells, an interaction dependent on specific FcRIε receptors on the mast cell surface. Such mast cells are now primed for further allergen interaction (166).

Once sensitised, IgE coated basophils and mast cells await activation by antibody-allergen interaction. Recognition of antigen epitopes by the variable portion of multiple surface bound antibody molecules allows for cross-linking of IgE. Such simultaneous activation results in degranulation of mast cells and release of preformed granule contents, de-novo manufacture of cell membrane lipid bilayer synthetic products and production of cytokines such as IL-4 and IL-5. Clinically, in a therapeutic setting, a drop in serum IgE levels corresponds to reduction in clinical symptom scores post Omalizumab therapy (167). Allergen challenge or natural pollen exposure results in increased eosinophil recruitment(168) in atopic individuals. Tissue eosinophilia is accompanied by increases in blood eosinophil levels. Together they are the hallmarks of allergy (169). Eosinophils are derived from progenitor cells under the influence of IL-3, IL-5 and granulocyte-macrophage colony stimulating factor. IL-5 plays a crucial role in eosinophil maturation, recruitment to site of allergic inflammation, and survival. Preformed highly basic granular proteins such as major basic protein (MBP) and eosinophil cationic protein (ECP) fight antibody bound parasitic infections in a healthy host.

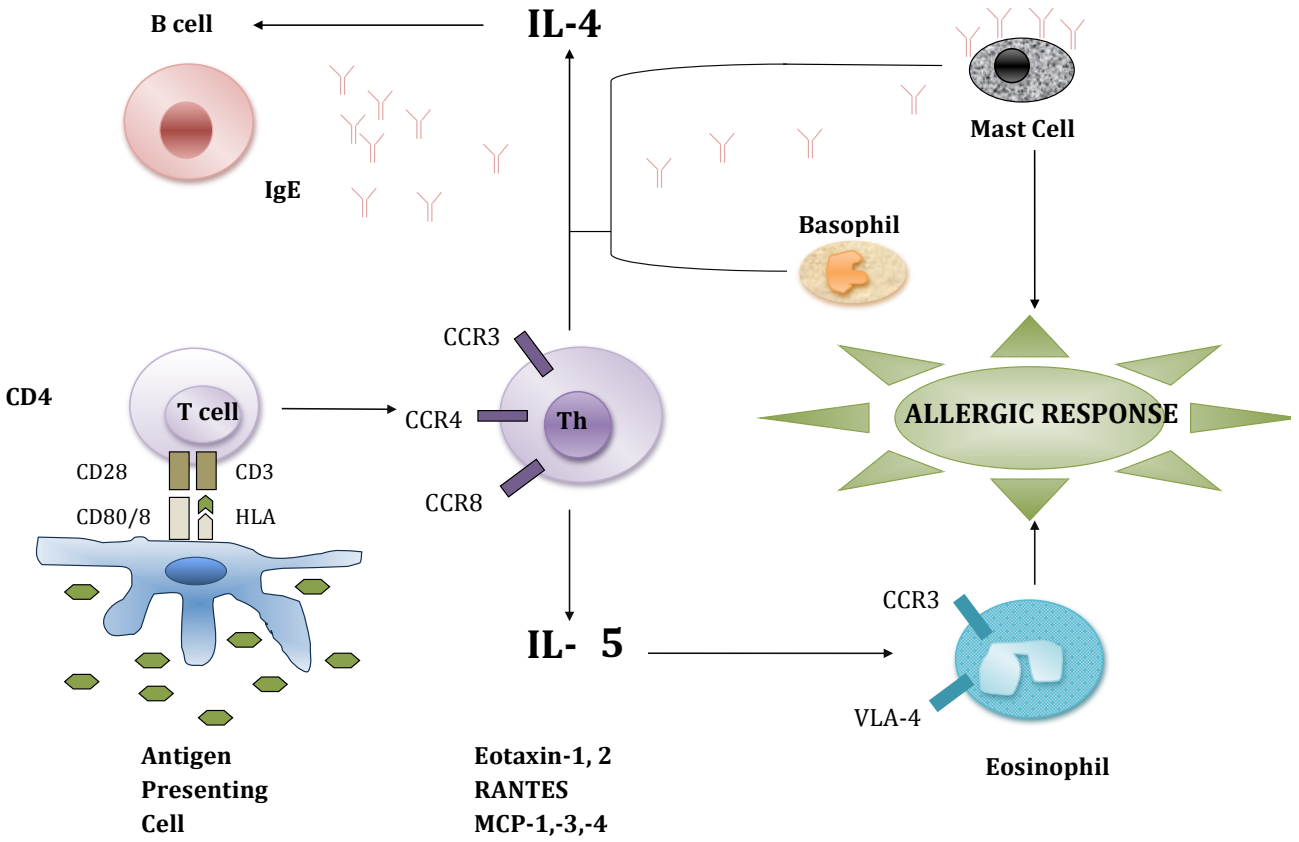


Figure 1.1: Central role of Th2 cells in allergic inflammation

Mast Cells

First described by Ehrlich in 1878 as Mastzellen or 'well fed cells' due to their highly granulocytic appearance (170) (171), mast cells form the firm foundations of immediate hypersensitivity in man. Of haemopoietic stem cell origin, mast cell progenitors are released from the bone marrow to enter the circulation but mature locally (172). Present at all peripheral sites of environmental interface, such as the skin, lung, and gastrointestinal tract, mast cells along with basophils and dendritic cells are ideally positioned to provide a first line of host defence (173). Absolute mast cell numbers along with increased mast cell migration, proliferation, survival and phenotypic alteration are all influenced by the cellular/ cytokine milieu (174). IgE receptor FcεRI is constitutively expressed on mast cells and further upregulated in an IgE rich environment. Consisting as either $\alpha\beta\gamma\gamma$ or $\alpha\gamma\gamma$ variants with a surface α, four transmembrane looped β, and mainly intracellular γ subdomains, immunoreceptor tyrosine-based activation motifs or ITAMs (one on β and on each γ) trigger downstream signalling. With activation by multi or covalent antigen via receptor bound IgE, aggregation of cell surface FcεRI activates mast cells with near immediate release of preformed cytoplasmic compounds such as histamine and cytokines, with further release of preformed, *de novo* synthesis lipid mediators such as prostaglandins and leukotrienes. Amongst these PGD_2 and LTC_4 coupled with histamine, enhance vascular dilatation and LTB_4 and TNF and IL-8 increase vascular permeability (175).

Mast cells increase in number at sites of allergic inflammation (176) (177) (178). Conversely, epithelial mast cells are absent in non-atopic states (179) . Epithelial migration of mast cells has, for example, been demonstrated in birch pollen sensitive patients during the season (180). Tryptase positive (+ve) but not dual tryptase and chymase +ve mast cells are elevated during the season in allergic rhinitics (181). In allergic rhinitic nasal secretions, tryptase levels are elevated over threefold above those observed in chronic rhinosinusitis or controls (182). Histamine rise coupled with levels of tryptase correlates with degree of allergic symptoms in allergen challenged nasal mucosa (183). Immunotherapy represses such seasonal increases in nasal mucosal cKit+ mast cells (184).

In the setting of lower airways disease, asthmatics have higher BAL tryptase and PGD_2 and demonstrate enhanced spontaneous histamine release from BAL mast cells than non-asthmatics (185). The ability of IgE in the skin (186) or lung (187) to produce the late phase reaction or late asthmatic reaction respectively is indicative of a role for mast cells during airway hyperreactivity. Within the context of remodelling, mast cell derived tumour necrosis factor TNF-α amplifies eosinophilic TGF-β1 secretion with mast cell tryptase encouraging proliferation of fibroblasts (188). Tryptase induces collagen matrix contraction and enhances myofibroblast differentiation (189). Both tryptase and chymase are capable of matrix metalloprotease activation thereby contributing to extracellular matrix alteration (190). In

murine model of asthma, mast cells contribute to increases in goblet cell numbers, and enhanced collagen production (191). Mast cells are capable of faulty restorative attempts culminating in tissue damage at non respiratory sites (192).

Tryptase but not tryptase/chymase positive mast cells demonstrate seasonal variations in allergic rhinitics (181). As ASM hypertrophy is a hallmark of lower airway remodelling and mast cells found at these sites are exclusively MC_{TC} , the key mast cell subtype responsible for driving ASM changes is missing in AR (193). In a murine model of chymase deficient mice (versus wild type), increases in airway smooth muscle, inflammation and hyperreactivity were seen post methacholine challenge, suggesting a possible protective effect of a mast cell derived protease (194). MC_T appear to be immune system dependent with MC_{TC} involved in angiogenesis and remodelling. Both may be activated by IgE dependent mechanisms but only the latter may respond to IgE independent kinetics (195). Eosinophils secrete Stem Cell Factor and Nerve Growth Factor, further endowing them with mast cell influencing abilities (196).

Mast cell FcγRI mediated and antigen independent IgE (at physiologically quiescent concentrations) is capable of sensitising or priming mast cell responses (197). IgE binding to FcγRI stabilises these receptors (198), which is clinically reflected as increased FcγRI expression on mast cells (199) and basophils in atopic individuals with high circulation IgE (200). Such 'arming' of mast cells may provide them with the ability to respond to multiple antigens, to do so at lower antigen concentrations and to release mediators at these concentrations, which similarly unarmed cells are unable to do thereby providing a positive feedback loop with ever increasing IgE concentrations in atopic individuals. Omelizumab, a humanised monoclonal IgE antibody, reduces circulating IgE thereby reversing this amplification loop (201). In asthmatic patients, presence of chymase positive mast cells and the increased ratio of chymase positive to total mast cells, correlates with severity of disease (202). Conversely, allergic rhinitics may demonstrate local IgE mediated reaction in the absence of systemic IgE responses (203).

Eosinophils

First described by Paul Ehrlich in 1879 to have intensely acidophilic staining, eosinophils are now recognised for their central role in both upper and lower airways disease. Derived from CD34+ pluripotent haemopoietic progenitor cells, they make up 3% of bone marrow and 1-3% of total peripheral blood leukocyte population. IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) both influence cellular commitment toward an eosinophil lineage, but it is IL-5 that is crucial for its development, activation, and survival. With a short peripheral half-life of ~18hrs, under

homeostatic conditions, eosinophils quickly migrate into the gastrointestinal tract and the thymus. When under stimuli, and via cytokine control, eosinophils home in on sites of inflammation where their action is exerted, and survival prolonged (204).

Eosinophils derived from allergic individuals undergo priming, firstly in the peripheral blood, secondly and more profoundly in the target organs. Such priming ensures eosinophils are fully activated when they reach their destination. IL-3, IL-5 and GM-CSF all influence the degree of migration, adhesiveness and degranulation. Cell membrane receptor CCR3 is crucial in signalling of both eotaxin (CCL11) and RANTES (CCL5) in both an IL-5 dependent and independent manner to enhance such cellular activation (205). Thymic stromal lymphopoietin (TSLP), both epithelial cell-derived and immune cell secreted (dendritic cells, CD4+ and CD8+ T cells, B cells, mast cells) signals through its TSLP receptor on eosinophils to enhance their activation and survival. Autocrine and paracrine activity of IL-25 secreted by both mast cells and activated Th2 cells acts to further enhance Th2 cytokine production thereby increasing IL-5 dependent eosinophilia. Endogenous cytokine production by damaged or necrotic cells further contributes to eosinophil activity. IL-33 produced by endothelial and epithelial cells, fibroblasts and adipocytes will enhance Th2 inflammatory cytokine production. All of the above coalesce to drive an effective and pronounced eosinophilic inflammation (206).

The expulsion of intracellular granules may occurs via classical (single) or compound (multiple) granule exocytosis, or via cytolysis (accompanied by cell lysis) or piecemeal degranulation, (where the cells itself remains intact) (207). Eosinophil granules contain eosinophil derived neurotoxin (EDN), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and major basic protein (MBP) with the latter making up around 50% of all granule content. In the context of lower airway disease, extracellular MBP and eosinophils are found in post-mortem samples of fatal asthma. MBP mediated bronchoconstriction via acetylcholine signalling on parasympathetic nerve fibres is thought to contribute to the pathology (208). Propagation of the inflammatory response by other leukocytes, with dysregulation of intracellular adhesions and epithelial ciliary activity further contributes to the allergic airway phenotype. Tissue eosinophilia is one of the hallmarks of allergic airway inflammation seen in allergic rhinitis and asthma. Cellular products of basic proteins and reactive oxygen intermediaries are fundamental in tissue injury subsequent to allergen inhalation in allergic upper airway diseases (188). Activated eosinophils and Th2 cytokine skewing is seen within nasal mucosa following allergen challenge, with IL-5 showing particular correlation with primed eosinophils (209). Both total (MBP) and activated (EG2+) eosinophils show seasonal increases in allergic rhinitics (181) with grass pollen immunotherapy inhibiting such an increase in MBP+ve cells (210). Immunotherapy conferred a significant decrease in seasonal symptoms which correlates with significant reduction of

submucosal eosinophils and IL-5 mRNA (211). Examining nasal turbinate tissue samples, atopic nasal polyp patients demonstrate significantly more total eosinophil counts as well as IL-5 and ECP (in nasal secretions), than either their non-atopic polyp, or allergic rhinitics without polyp counterparts (212).

Murine and human evidence confirms the role of eosinophils in tissue fibrosis (213). IL-5 promoted and TGF-β driven, eosinophil enhanced collagen deposition is a feature of lower airways disease. In a human study of atopic skin, intradermal challenge increased eosinophil derived TGF-β driven myofibroblast transformation with increases in fibroblast expression of tenascin and procollagen-I (214). Via IL-5 dependent and independent activation, eosinophils can drive but not initiate thickening of reticular basement membrane (215) (216). In a murine repetitive allergen challenge model, administration of anti-IL-5 antibody before and after intra-tracheal challenge ameliorated tissue eosinophilia and significantly reduced subepithelial reticulin deposition (217). IL-5 deficient mice demonstrated less eosinophilia, epithelial proliferation and BAL fibronectin under repetitive allergen challenge protocols, but intra-tracheal IL-5 alone was insufficient to recapitulate the remodelling phenotype (218). IL-5 knock-out mice failed to demonstrate increases in both inflammatory and remodelling markers when exposed to repetitive allergen challenge. Increases in bronchial and BAL eosinophils, total collagen content, lung fibrosis, collagen III/ IV, peribronchial smooth muscle and epithelial mucus production were suppressed. TGF-β and the integrin responsible for its activation from its latent form $\alpha_v\beta_6$ also failed to rise (219). This finding was further supported by Tanaka *et al.* with IL-5 knock-out mice failing to demonstrate a rise in TGF-β and airway eosinophilia but IL-5 transgenic mice responding to repetitive allergen challenge by increased fibrosis (220). In human studies humanised monoclonal anti-IL-5 antibody therapy reduced bronchial tenascin, lumican and procollagen III in harmony with TGF-β1 mRNA expressing eosinophils (221).

Eosinophils will secrete pro-angiogenic VEGF in response to IL-5 and GM-CSF (222). Eosinophil derived IL-8 enhances MMP-9 and MMP-2 expression thus contributing to proteolytic release of ECM bound extracellular angiogenic molecules (223). Cellular localisation of IL-17a in the context of CRS with nasal polyps (using middle meatal polyp tissue versus sphenoidal mucosa) demonstrated eosinophils are the major source of this inflammatory cytokine. In this study, levels of IL17-A correlated with the degree of epithelial damage and basement membrane thickness (224).

Eosinophilic bronchitis leads to subepithelial RBM thickening similar to that seen in asthma but with no airway hyperresponsiveness which correlated with a lack of mast cells in airway smooth muscle (193) although in murine models dbl GATA mice demonstrate significantly less RBM thickening and airway smooth muscle proliferation than wild type mice following ova challenge (225). In murine

models of repetitive allergen challenge, airway hyperresponsiveness, collagen deposition and smooth muscle hypertrophy persisted for 8 weeks after the last dose, long after bronchial eosinophil and BAL IL-13 levels had returned to normal (226). This demonstrates the variable but vital role of eosinophils in the onset and propagation, but not necessarily the maintenance of remodelling in the airway.

Neutrophils

Neutrophils, also noticed by Paul Ehrlich in his pursuit of granulocytic leukocytes, have a short life span and are terminally differentiated making their *in vitro* study difficult (227). Despite their short life span of 6-8 hours, after which they undergo controlled apoptosis, taking care not to spill their granular cargo, neutrophils are the most abundant leukocyte in the peripheral blood. Neutrophils are under the influence of variable levels of IL-8. At low IL-8 concentrations, neutrophils shed their surface L-selectin whilst upregulating their integrins, higher levels encourage short lived oxidative bursts whilst yet higher IL-8 levels bring about complete neutrophil degranulation (228). Such neutrophil cell death is in the overall context of neutrophil activation, which terminates in extravasation release of cytoplasmic granules and NETS (neutrophil extracellular traps). Neutrophils produce lower amounts of cytokines than other granulocytes in absolute terms but their abundance at sites of inflammation ensures a significant contribution to the biochemical cellular environs (229).

In murine model of *Haemophilus influenzae* infection, viral load drives allergic airway disease typical of neutrophilic asthma (230). In human model of allergic rhinitis, neutrophils increased 24 hours post allergen (156). Attempts at linking neutrophilic polyp disease with clinical outcomes such as endoscopic or CT evaluations have failed to demonstrate a link (231). Treatment with LTB4 in human subjects subsequently challenged with respiratory viridae demonstrated a sentinel role for neutrophils in the orchestration of innate immune responses. Such treatment with LTB4 statistically enhanced MPO and α-defensin production, although this rise did not link in with clinical symptoms of common cold or rate of seroconversion (232). A study of community acquired viral upper respiratory tract infection demonstrated correlation of IL-8 and neutrophil counts with clinical severity of nasal symptoms including nasal congestion and rhinorrhoea (233). A study of SCID versus C57BL/6J mice demonstrated the central role of neutrophil migration in the clearance of nasal *Staphylococcus Aureus*. T cell orchestration with IL-17 secretion seems to dovetail into the need for neutrophil influx for effective clearance (234).

When compared to PBMCs, freshly isolated neutrophils released 20-fold greater amounts of activin. They did so quickly, one hour after stimulation by TNFα, with a period of quiescence following which activin mRNA increased at 12 hours lending support to the idea that initial activation releases

preformed stores, which is followed by *de novo* synthesis (235). Neutrophils are important sources of activin-A. Neutrophils and NETs are evidenced in a variety of disease states including ARDS, sepsis, systemic lupus erythematosus (SLE), rheumatoid arthritis and inflammatory bowel disease (236) (237). Whilst the causal and temporal relationship between neutrophils and activin is as yet undefined, it is likely the crosstalk is reliant on cytokines such as TNF-α, IFN-g, IL-8 and IL-17. Furthermore, neutrophil elastase plays a role in initiating and propagating parenchymal damage in lung conditions such as sarcoidosis and emphysema, thus playing a prelude to TGF-β driven ECM collagen disturbance (238).

Basophils

Basophils were first noted and named after their ability to stain with basophilic dyes. With low abundance in peripheral blood (basophils contribute to less than 1% of circulating lymphocytes), identification and study of this cell population has historically been challenging. Basophils share a common progenitor lineage with mast cells, developing from the haemopoietic stem cells GMP (granulocyte-monocyte progenitor), during bone marrow maturation, cell phenotypes diverge with some forming mast cell progenitors MCP, basophil MCPs, or basophil progenitors BaPs (239). The latter two groups are capable of forming mature basophils which, unlike mast cells which mature peripherally, will exit into the circulation in their mature form (240). There is some evidence to suggest extra-medullary haemopoiesis but the exact relevance of this in allergic inflammation is yet to be elucidated (241).

Lacking the surface receptors cKit and CCR3 which distinguish mast cells and eosinophils respectively, basophils share the high affinity FcεRI on their surface with the above granulocytes. With a life-span of around 60 hours, basophils need a constant cycle of replenishment from bone marrow progenitors (242). In the context of Th2 cytokine driven inflammation, IL-3 is a seminal promoter of basophil recruitment both into blood (243) and lymph nodes (244). In addition, TSLP has been demonstrated to engage innate immune system driven basophilic responses. Although capable of optimisation of IL-3 enhanced basophil function, TSLP-TSLP receptor engagement can drive basophilic responses in the absence of IL-3. Such bivalent initiators of basophil action propagate distinct cellular profiles. TSLP enhanced basophils are exquisitely potent secretors of IL-4 to a variety of cytokine sources including not only IL-3 but also IL-33 and IL-18 (245). Distinctively, this basophil phenotype lacks degranulating ability in response to IgE mediated FcεRI activation. Taken together, this demonstrates two distinctive pathways to basophil activity. One is IL-3 driven and IgE dependent, the other is a TSLP responsive, IgE independent mechanisms of basophil activation (246).

Although haemopoietically distinct in development basophils, like mast cells, can secrete histamine and these two cell types often coexist at sites of Th2 driven allergic inflammation (247). In response to cross-linking of FcεRI in an IgE dependent/ IL-3 associated manner they release histamine, with preformed eicosanoids and cytokine expression, particularly IL-4 and IL-13 (248). Noted to be the major source of IL-4 during parasitic infections (249), basophils are the main secretor of IL-4 and IL-13 in peripheral blood of sensitised asthmatics (250). Such Th2 driven cytokine release may also be presaged by house dust mite protease Der p1 in human *in vitro* studies. Furthermore heat inactivation of such proteases abrogates basophilic cytokine release (251). In the context of allergic rhinitis, basophils are present in nasal lavage fluid and contribute to late phase reaction (252). Co-localisation techniques evidence an increase in IL-4mRNA in 2D7 stained basophils post challenge (253). A genome wide meta-analysis study suggested correlation between allergic rhinitis and TSLP variants (254) whilst expression of TSLP is increased in nasal polyps with chronic rhinosinusitis (255).

Basophils numbers are higher in non-atopic asthmatics and rise further still in atopic asthmatics in clinically quiescent states. Further increase is demonstrated post allergen challenge (177). Contribution of basophils to nasal symptoms is underscored with Omelizumab therapy which sees basophil reduction before mast cell population decrease, with such basophil control correlating with decrease in post nasal allergen challenge symptom scores (256). Successful subcutaneous immunotherapy in grass pollen allergic rhinitics significantly suppresses seasonal rise in epithelial (but not submucosal) basophil numbers (210). Post bronchial allergen challenge in atopic asthmatics, eosinophils show maximal rise at 24 hours with basophils reaching their peak at 6 hours. Increases in mast cells and eosinophils are ten-fold those seen with basophils. Only a third of basophils demonstrate evidence of degranulation (177), linking in with the ratio of IgE dependent and TSLP driven/ IgE independent basophil populations. IL-33 may be able to synergise both these response pathways (257).

In the context of chronic rhinosinusitis in both children and adults, basophil (along with eosinophil) numbers rose reflecting a similar increase in subepithelial collagen deposition (258). Basophils are found in most sinugenic disease states implying a central role for this multipotent granulocyte (259) (260). Basophils may function as antigen presenting cells to facilitate allergen induced Th2 responses (261).

Macrophages

The capabilities of the monocyte-macrophage system extend across both adaptive and innate immune systems. Mostly quiescent, circulating monocytes are activated within tissues by a multitude of stimuli

and polarise to accommodate the needs of the localised immune response to perform inflammatory phagocytic roles. Macrophage phagocytotic compounds mature during endocytic development (versus neutrophils where this occurs at time of phagosome/ granulocyte fusion) (227). Phenotypic differentiation into two broad subtypes M1 and M2 generally follows the footsteps of the Th1/Th2 phenotype. Pro-inflammatory, 'classically activated' M1 cells respond to IFN-γ, bacterial LPS and TNF-α activating nitric-oxide synthase 2 whilst 'alternatively' activated M2 cells are activated by and may further secrete IL-4, IL-13, Il-10 and TGF-β and activate arginase 1 (262). As such, M2 cells possess increased endocytic clearance properties, heightened trophic factor synthesis and decreased pro-inflammatory cytokine production thereby facilitating resolution of inflammation. Varying chemokine expression and metabolism properties are seen between different macrophage types (263) (264).

The clarity with which macrophages subdivide into these characteristic subsets is likely to demonstrate temporal variation with both existing to differing extents at each point in the initial and resolution phases of inflammation. Although categorical division into a purely M2/ Th2 axis may overplay the degree of this paradigm, nonetheless macrophages play an important role in allergy and remodelling. Nasal macrophage numbers increase with local allergen challenge, seasonal allergen exposure and decrease in response to glucocorticoid therapy versus placebo control (265). Such a rise post allergen exposure is also evidenced in the lower airways with macrophages demonstrating activated status ready mounted with low affinity FCεRII (CD23) capable of binding antigen. The presence of macrophages can enhance a tolerogenic tissue profile (266) with some capable of antigen presentation to T cells (267). This expansion of the tissue macrophage population is due to both recruitment and *in-situ* proliferation and is a hallmark of Th2 inflammation driven by IL-4 (268).

CD163 and CD68 are both used to identify macrophages. CD163+ cells outnumber CD68+ cells in both Th1 and Th2 diseases with double staining immunohistochemistry demonstrating skewing of M1 versus M2 type macrophage phenotype respectively (269). Such polarisation does not conform to purity of division as both subtypes exist demonstrating a temporal change during the inflammatory and resolution process (270).

Pro-fibrotic, angiogenic environments co-exist in loci with IL-13 dependent, ARG-1 activation, in-keeping with an M2 type activation profile (262) (271). At present, evidence for skewing of macrophage profile in the nose is lacking, although in lower airways disease M2 polarisation is evidenced with pro-fibrotic, anti-inflammatory, Th2 driven cytokine profiles (272) (273). M2 macrophages are capable of such remodelling behaviour independent of a Th2 conduit (274).

T cells

Descended from pluripotent lymphatic progenitors, T cells exit the bone marrow and mature in the thymus. Following a 'Goldilocks' maturation whereby reaction to self-antigen has to be weakly positive (not too strong, not too weak but just right), T cells exit and circulate in the blood and primary lymphoid organs. Further maturation into a T helper or cytotoxic T cell phenotype as identified by surface markers CD4 and CD8 respectively, ensures such cells are in turn optimally placed to respond to extra or intracellular pathogens. T helper cells aid orchestration of adaptive immune responses with alteration and activation of B cell responses influencing the amount and class of immunoglobulin secreted. T cell responses are evidenced in allergic rhinitis with elevation of CD4+ CD25+ T cells following allergen challenge (156). Such change is accompanied by concomitant rise in eosinophils and neutrophils and doing so in the context of Th2 characterised cytokine response (209).

T-helper cells

The role of T cells in the pathogenesis of allergic lower airways disease was indicated on observation of a Th2 cytokine rich milieu in the late asthmatic reaction (275) and acute severe asthma (276). IL-2+/CD4+ Tcells are increased in both BAL and peripheral blood at 18hrs post segmental challenge (277). In murine model of AR, depletion of Th2 cells abrogated eosinophilic, IL-5 modulated lower airway inflammation post challenge (278). In human models of allergic rhinitis, T cells are the major source of IL-5 in nasal mucosa post allergen challenge (279). Th2 cell derived cytokines contribute to tissue eosinophilia during late phase reactions (280). T cells are a putative source of Activin A, as anti-CD3 stimulated CD4+ CD25- but not CD4+ and CD25+ regulatory T cells are capable of secreting Activin A, particularly under conditions that favour Th2 polarisation (281).

In vivo experimental models of inhaled allergen, low dose versus high dose allergen challenge induce TGF-β-dependent and IL-10 producing Tregs respectively (282) (283). Freshly isolated CD4+/ CD25+ T cells are anergic despite TCR stimulation unless in the presence of exogenous IL-2 indicating the importance of antigenic stimulation (284) (285).

Blocking of γδT cells with anti-TCRγ antibody prevents post inflammatory eosinophil and Th2 rise (as modelled by prolonged murine ovalbumin challenge) and reduces peri-bronchial collagen deposition as marker of remodelling via continuous murine HDM challenge (286).

T-regulatory cells

Finely tuned effector responses require control mechanisms via regulatory T cells or Tregs. Such cells adopt a double pronged approach of both central and peripheral avenues of controlling both desired

and unwanted immunological responses. Sites of central control during T and B cell maturation are the thymus and bone marrow respectively. Natural T regulatory cells are thymus derived and are characterised by the cell surface marker CD25 and transcription factor Foxp3. Both bystander suppression and infectious tolerance are viable methods of immune response control. Tregs are likely to utilise a multitude of avenues to affect such attempts at homeostasis. Constitutive expression of the high affinity IL-2 receptor, CD25, means Treg preferentially bind and utilise this cytokine, effectively robbing T effector cells from its stimulatory properties on T cell expansion, whilst endowing the growing Treg population with improved immune-modulatory characteristics. Cell to cell contact may result in apoptosis, whilst alteration of APC behaviour will indirectly affect T and B cell responses. T regs ameliorate the antigen presenting capability of dendritic cells and enhance secretion of suppressive factors in the target DC population (287). Dexamethasone or Vitamin D3 can induce differentiation of CD4 cells into IL-10 secreting Tr-1 cells (288).

With a plethora of immunomodulatory cytokine expression, T_{reg} responses are likely to mould prevailing immunoregulatory conditions, site of action and the aggressiveness of disease state. In their homeostatic role, lymphoid tissue resident Tregs will prevent DC orchestrated antigen presentation to resident naïve T effector cells. TGF-β, along with CTLA-4, is likely to play a role at this crucial step. When unable to control lymphoid resident effector activation, T_{regs} may engage further signalling molecules such as IL-10, and TGF-β. As such, Tregs are capable of abrogating tissue inflammation via local activation (289).

Remodelling

Introduction

Remodelling, meaning to model again or differently, within the context of asthma was born out of the need to better understand disease pathology and improve therapeutic outcomes. Despite the predominance of a Th2 inflammatory picture, standard treatment provided insufficient symptomatic control in many patients. Dissociation of inflammation from chronic, perhaps irreversible, histological changes goes some way to explain the persistence of airway reactivity and variable airflow seen in this disease. Whilst the temporal relationship between inflammation and remodelling is still under scrutiny, the symbiotic relationship between the two is now well recognised. With better appreciation of clinical, aetiological, and prognostic links between the nose and the lung, it is implicit that such a histological reciprocity is investigated and quantified.

Changes in tissue architecture are seen throughout the depth of respiratory mucosa. Epithelial damage, basement membrane thickening, alteration in goblet cells and submucous glands, neo vascularisation and lymphangiogenesis have all been identified within the context of asthma and lower airways inflammatory disease (290). Findings in the nose have yet to be comprehensively elucidated but are ongoing (291) (292).

Epithelium

The upper and lower airways collectively make up over 100 m^2 of surface area and come into contact with 10k litres of inhaled air. Epithelial linings provide a first physical barrier to noxious substances. Along with cilia enhanced mucus clearance, such epithelial blockades form an essential protective mechanism (293). The barrier function of airway epithelial cells is supplemented by unique characteristics working synchronously to trigger both the innate and adaptive immune system (294).

The uninterrupted nature of epithelial linings is achieved by a cell to cell connecting proteins and their receptors molecules such as occludins and cadherins (295). Further cellular communication pathways are facilitated by extracellular matrix protein coupling such as tight junctions, desmosomes, and connexins (296). Airway cells harvested from asthmatics and cultured in an air/ liquid interface, are shown to maintain their 'leaky' characteristic even after a number of generational passes, demonstrating that such deficiency in barrier function is cellular intrinsic and not a consequence of microenvironmental milieu *in vivo* (297). In the context of airway allergy, such leakiness between cells allows for potential entry of allergens and microbes capable of inducing a mast cell dependent early, and Th2 dependent late response (298). To combat the protective mechanism of airway epithelia,

many allergens possess either an intrinsically active protease or are coupled to one. This endows such compounds (299) with the collective ability to breach structural airway defenses by proteolytic cleavage of dendritic cell receptors (300) or direct destruction of cellular tight junctions (301).

Capable not only of stimulating the adaptive immune response, allergens work synchronously to effectively harness innate immunity. Toll like receptors (TLRs) recognise structural motifs shared by exogenous particles (dust or fragments of inhaled allergens/ infective organism) such as cell wall proteins or nucleic acid segments to activate epithelial cells, skewing T cell responses to particular phenotype (302). Importantly, TLR-4 located on airway stromal cells provides this link between adaptive and innate immune responses, and is pivotal in initiating an airway hyperreactivity in mice (303). Elucidation of inflammatory cytokine response (Il-33 and IL-25) and dendritic cell activation via recognition of allergen LPS by TLR-4, the adaptive immune response may elicit allergic inflammation independent of Th2 cell recruitment and exacerbate airway hyperresponsiveness (304). In asthmatic epithelia, secretion of TSLP, post TLR activation and downstream signalling enhances CD80/ OX80 and CD40 expression by resident naïve dendritic cells which, coupled with mast cell mediated cytokine release, works to enhance Th2 mediated responses (305) (306). Furthermore, TSLP, secreted by airway epithelial cells, is capable of B cell activation via STAT5 in a non-T cell dependent manner (307). Epithelial cells are able to affect such changes through their own cytokine release, with implicit dependence on autocrine secretion of EGF in response to non-specific tissue injury. Stress related EGF release engages with epithelial EGF-receptor to enhance cytokine release, with the long term result of healing by 'primary intention' (308). Pathways partly dependent on IL-5 mediate further inflammatory epithelial damage by eosinophils. GM-CSF secreted by epithelial cells and fibroblasts enhances epithelial eosinophilic inflammation by prolonging eosinophil cell survival (309).

It is thought that developmental links between mesenchyme and epithelium are maintained by a reciprocal relationship later on in life. This interaction has led to formally linking these two tissue types as a possible initiators and drivers of airway inflammation and remodelling. This so-called epithelial-mesenchymal trophic unit (EMTU) is activated on allergic stimulation(310). Epithelial-mesenchymal transition (differentiation of epithelial cell into fibroblasts and myofibroblasts) is regulated by SMAD dependent, TGF-driven signalling (311) (312). Furthermore, immature 'fibrocytes' may be recruited from CD34+ progenitors from the bone marrow (313) providing a variety of mechanisms through which fibroblast/ epithelial interactions lead to chronic histopathological changes in the airway.

Loss of epithelial integrity has long been thought of as pathognomonic of the allergic inflammatory lower airway disease (314) (315). The degree to which it signified disease severity (316) remains

contentious (317) (318). Such desquamation of airway epithelial cells is not unequivocally related to the presence of asthma or the severity of the disease, with some studies concluding such denudation of epithelia to be a processing artefact rather than feature of the disease (319). Despite this, evidence of epithelial stress exists in adults as evidenced by increased epithelial growth factor receptor in response to mechanical injury (320) and is also found in childhood asthma (321). Collectively this suggests that while loss of epithelial integrity is insufficient to drive airway remodelling in isolation, in combination with epithelial stress, with ongoing growth factor secretion, prolonged incomplete attempts at wound healing, such desquamation may result in chronic wound repair and remodelling. In the nose, epithelial shedding may be present in rhinitis albeit possibly without an allergic trigger or Th2 cytokine profile (322) with phenotypic variation of epithelial cells in experimental models of rhinosinusitis (323). Concrete evidence correlating epithelial cell loss with severity of rhinitis is presently lacking (324).

Glands

Secretion of mucus within the nasal vestibule and the lower airways forms a crucial part of primary host defence. Continuous clearance of trapped particles by the mucociliary transporter, posteriorly in the nose towards the naso/ oropharynx and superiorly by the lower airways where it is either expectorated or swallowed, depends on continuous ciliary movement by epithelial cells. Found throughout the upper airway and the nose, scattered within epithelial cells, goblet cells produce a number of mucin glycoproteins (325). MUC5AC, as the most abundant isoform in humans has its expression upregulated in asthma (293). Seromucinous glands within the lamina propria provide further substance to upper airway secretions.

Distribution of goblet cells and glands varies along the respiratory tract. Gland density is highest in the nose and lowest in the nasopharynx, trachea and bronchi with gland mass per gland being higher in the lower airways (326). Within the nasal cavity itself there exists a gradient of glandular density with inferior turbinate at the highest and the septum at the lowest axis of the spectrum. Presence of glands and mucus production is almost negligible within the sinuses. In the context of CRSsNP, maxillary sinus goblet cell density is reduced, but glandular density, possibly due to neogenesis, is higher compared to normal controls (327). Further support for differences in goblet cell and glandular density comes from histological studies examining either side of the uncinate process, which forms a section of the medial wall of the maxillary sinus dividing it from the nasal cavity proper. Total mucosal and basement membrane thickness, and area occupied by glands is greater on the medial side (nasal cavity), with increased epithelial thickness, more mast cells and epithelial goblet cells on its lateral portion- the maxillary sinus (328).

Cleaved from its membrane bound precursor by ADAM17, either through oxidative stress activation of dual oxidase (329) or via autocrine secretion in response to IL-4 and IL-13 (330), TNF-α release permits EGF dependent goblet cell metaplasia (331). Such Th2 dependent, IL-13 driven goblet cell alteration may be reproduced by adoptive transfer of Th2 cells in ovalbumin sensitised mice (332). When chronic, such goblet cell changes extend distally to the peripheral airways; a change not archetypal of milder disease (333). More structurally robust submucous glands in asthmatic airways, secreting more tenacious mucus further enhanced by eosinophilic basic protein result in thick viscous secretions (310). Such alteration in number and architecture of both goblet cells and submucous glands is found consistently in asthmatic airways, with weak relationship between degree of histological change and disease severity (334) (335).

Examining inferior turbinate biopsies, increased mucus production in perennial rhinitics (versus non-atopic controls) is due to non-hyperplastic increase in goblet cell function as opposed to increase in total goblet cell number *per se* (336).

Extracellular Matrix

The non-cellular component of tissues is known as the extracellular matrix (ECM). With wide-ranging function, providing support, nutrition, cell movement, diffusion of signalling macromolecules, the ECM is a vital part of tissue homeostasis. Composed of collagenous component, and the gelatinous non-fibrillar ground substance, the extracellular matrix is able to maintain tissue integrity. Allowing for sequestration of growth factors, extracellular ECM stores provide a rapid response by releasing such compounds on alteration of local cytokine and/ or chemokine milieu (337). Components of ECM are released from its constitutive cells via exocytosis. Playing a strong role in tissue responses to inflammation, the extra cellular matrix is fluidly responsive in its attempts to resolve injury.

The non-fibrous, non-collagenous component of the ECM known as ground substance, is primarily composed of glycosaminoglycans (GAGs), which in turn bind to cell surface and extracellular proteins forming proteoglycans. GAGs are composed of repeated disaccharide units of hexosamine and uronic acid and exist as linear polymers. They are polyanionic as their protein binding endows GAGs with a negative charge which attracts Na+ ions and thereby exerts an osmotic effect pulling water into interstitial tissues. This maintains tissue turgor helping it withstand compressive tissue injury. A number of polysaccharides belong to this family. Heparan sulphate binds to cell surface integrins, with keratin and chondroitin sulphate playing a role in extracellular homeostasis in certain tissue types. Although hyaluronic acid is a glycosaminoglycan that doesn't link with proteins, it is still able to affect a marked pro-osmotic effect. As such, it is found in abundance in load bearing joints (338).

Fibronectin secreted as a dormant glycoprotein, unfolds, dimerises and is activated on binding to ECM integrins. Once functional it will keep its attachment to cell surface integrins conjointly with ECM facilitating cell movement and cytoskeletal reorganisation.

Collagen

The main constituent of extracellular matrix protein content is collagen. Up to 29 different types of collagen are known to exist, in man making up 30% of all the body's total protein content (339). Collagen exists in a variety of tertiary structures, with each individual collagen molecule existing as a left-handed single α-helix with 18 amino acids per turn. The mature collagen molecule is tightly packaged as a right-handed turn triple helix (340), requiring a single residue stagger between each individual α strand and with every third residue a glycine. The resultant series of Gly-X-Y where X and Y may be any amino acid but often proline and hydroxyproline respectively, is the universal backbone of all collagens. The staggered overlay of fibrils gives a characteristic banding pattern of 70nm under the electron microscope (341).

Once assembled, the triple helix has a central core of glycine with bulkier sidearms positioned peripherally. Intra-molecular hydrogen bonds confer further stability on the mature protein. Telopeptides are small non-helical interruptions in this repeat present in non-fibrillar collagen. Such breaks in the structure expose areas within collagen to bond either to other collagen molecules or to ECM components. Collagen triple helixes exist as hetero- or homodimers with the former being more prevalent. Non- Gly-X-Y regions, termed NC, flank the main repeating section at either end. These are processed during mature collagen assembly, incorporating α-helices into aggregates called fibrils. C-propeptide is important in the initiation of helix formation, with the N-propeptide regulating fibril size (342).

Collagens are categorised by the type of tertiary structure post secretion and extracellular matrix assimilation. Classical collagen is fibrillar and network-forming, with other categories of fibril-associated (TACITs) or membrane-associated (MACITs) collagens with interrupted triple helices, and multiple helix domains and interruptions (MULTIPLEXINs).

Fibril forming collagen accounts for 90% of total collagen (343). Type I collagen is the most abundant of fibrillar collagen and accounts for 90% of organic mass of bone. Formed of two α1 and one α2 helix, it is integrated into functional collagen with either type III collagen (skin) or type V collagen (bone, tendons). In each case, it lends essential functional components to the tissues such as tensile or mechanical, load-bearing strength. Type III collagen is a homodimer of three α1 helices and is seen in

abundance with type I collagen in all tissues bar bone. Type V collagen is a heterodimer composed of 3 different alpha chains: α1, α 2, α 3. Type IV collagen is the primary constituent of basement membranes. The specific structure of type IV collagen includes short interruptions to Gly-X-Y repeat, a 7S N-terminal domain and a NC1 globular C-terminal domain. Cross linking of the former domain, and dimeric association of the latter, allow for a flexible but highly robust network. The glycoprotein tenascin produced during embryogenesis may be upregulated at times of wound healing or remodelling. At low densities, tenascin encourages cell mobility and influx but inhibits such migration at high densities (344).

Collagen producing gene transcription is activated following exogenous growth factor signals, such as TGF-β. Splicing of pre-mRNA to join protein coding exons occurs in the nucleus. Prior to leaving the nucleus, mRNA is capped at the 5' end and undergoes polyadenylation at the 3' end. Ribosome bound mRNA is translated in the cytoplasm into preprocollagen with a signal peptide at the N-terminus. This is recognised by a signal receptor on the endoplasmic reticulum (ER), such that the prepropeptide moves from the cytoplasm into the rough ER. This N-terminal signal protein is dissolved once the preprocollagen is in the endoplasmic reticulum. The product is now called the propeptide. Hydroxylation of prolines and lysines follows, after which hydroxylated residues on lysines but not prolines are further glycosylated. Hydroxylation is reliant on vitamin C as a cofactor. Deficiency of vitamin C as seen in scurvy, results in a loose triple helix. Single α-helical coils form disulphide bonds at the C-terminus with tight triple-helix formation 'zipping' from the C to the N terminus. Correct folding is dependent on cofactors such as heat shock protein 47 (HSP47). This tightly wound triple helix with loose non-intertwined portions at either end, is known as the procollagen. Polysaccharides are added onto the procollagen in the Golgi apparatus, after which the procollagen is secreted extracellularly. Protein endopeptidases cleave the linear N and C-terminal propeptides of the procollagen making tropocollagen. Formation of aldehyde groups by lysine oxidase prepares the tropocollagen for covalent bonding, thereby forming the final collagen fibril structure (343). Other non-covalent bonding, such as hydrophobic and electrostatic bonds are also present in some instances of collagen aggregation.

Heat Shock Protein

Heat shock proteins belong to a family of proteases known as serpin due to their *ser*ine *p*rotein *in*hibitory activity. Whilst HSPs do have a serpin loop characteristic of this enzyme family, they do not possess the inhibitory activity that characterises their peers. A 47 kDa variant of this peptide, heat shock protein (HSP) 47, is expressed at levels in harmony with collagen expression. HSP47 is constitutively present in all collagen producing tissues (345). Found within the endoplasmic reticulum

chaperone proteins such as HSP47 are increased with ER stress. This may be induced by accumulation of misfolded, misshaped protein aggregates which directly cause ER stress, or as in the case of HSP47, by heat shock alone (346). Whether caused by ER stress or heat shock, increase in production of such chaperone proteins (347) will ensure accurate folding and secretion of ER produced proteins, thereby ensuring ER 'quality control' (348). Whether aiding in proper disulphide bond formation between cysteine residues, or accurate N-glycosylation, molecular chaperones accurately fold and correct misshapen proteins thereby guaranteeing production of functional complexes (349).

Whilst other chaperone proteins have wide specificity, HSP47 is specific for procollagen. The function of HSP47 relates well to the variability of pH found in the ER-Golgi protein production system (350). HSP47 is associated with collagen at the neutral pH found in the ER but will dissociate at lower pH (<6.3) as seen in *cis*-Golgi or the ER-Golgi intermediate compartment (ERGIC). This coupling and uncoupling to procollagen depending on the pH is a quick response with rapid dissociation in the Golgi apparatus. KDEL receptors are thought to be responsible for recycling HSP47 back into the ER for re-association with a new procollagen molecule (351). Support for this theory was gleaned from experiments showing treatment with brefeldin A1- which blocks the secretory pathway between the ER and *cis*-Golgi- prevented the dissociation of HSP47 from procollagen. Furthermore, treatment with bafilomycin A1- which inhibits secretory pathways post Golgi- did not affect HSP47-procollagen interaction. Seen together with pH dissociation experiments, the authors concluded that HSP47 dissociates and is recycled within the *cis*-Golgi (352).

Trimerisation of single α chains commences with the formation of disulphide bonds at their C-terminal domains. As the three single chains are 'zipped' from C- to N-terminus, several folding proteins, oxyreductases and enzymes, work as assisting chaperones (353). Once tight triple helix of pro-collagen is formed, its outer surface obtains it's hydrophobic property (354). HSP-47 inhibits unfolding, prevents disordered aggregate formation of this still immature collagen precursor and facilitates transport of procollagen via the Golgi apparatus extracellularly.

In *hsp47-/-* cells, collagen is seen to form intracellular procollagen aggregates, a situation reversed by the addition of HSP47 to the cell line (355). In *hsp47-/-* mice, misshapen procollagen accumulation in the ER caused ER stress within the cell (356). Procollagen that was secreted was fragile with still-present N-terminal segments. Functionally useless triple helical procollagen accumulated in the ER is degraded by the autophagy-lysosome system. In contrast, single stranded propeptide is eliminated by the ubiquitin proteasome system (357).

HSP47 is produced by collagen producing cells only. It is not seen in neurocytes or myelocytes which do not make collagen. HSP47 production is directly linked to the amount of cell collagen production. Once exposed to heat shock, HSP47 transcription factor binds to its promoter region, HSE (heat shock element) to induce HSP47 protein production. Fibrotic conditions show irreversible excess collagen deposition, universally accompanied by increased HSP47 (358) (359).

Basement Membrane

Basement membrane performs both a structurally supportive as well as a communicative role, serving to facilitate movement of signal molecules between tissue planes and types. Its functionality as a selective filter to macromolecules is particularly important in the kidney, brain and placenta. Separation of endo/epithelial cells from the submucous compartment is relevant at organ sites potentially communicating with the external environment such as the gut, cornea or skin (360). Basement membranes are made up primarily of Type IV collagen which, along with collagens VI, VII and X structurally belong to the network forming collagens.

First noted by Kefalides to be a separate collagen (361), Type IV collagen is a trimeric strand made up of three out of a total of 6 available α collagen fibrils. By far the commonest composition is two α1 and one α2 chains around 100-300nm thick each. Occasionally α3/α4 combinations may be observed in the kidney with some α5 and α6 seen in the oesophagus and the lung (362). Trimeric fibrils making up the mature Type IV collagen fiber form linear supramolecular aggregates in a 'chicken wire' lattice like network to span the length of the basement membrane. Stability is conferred by disulphide bonds between C-terminal, so called, NC1 domains. Further cross linking and superhelix formation is stabilized by hydrophobic interaction resulting in a highly robust macroscopically linear structure (363).

The mature mesh-like structure is further supported by additional extracellular matrix proteins such as laminin, nidogen and perlecan (364). With passive movement of substances 50nm or less across the basement membrane, and the vital role of MMPs in continuous turnover, basement membrane synthesis and degradation is in constant flux (365). Although MMP2 (gelatinase A) is highly specific for collagen IV, it is likely that a number of other MMPs are involved in turnover as single MMP inhibitors did not alter metastatic ability, and therefore change survival, in studies involving tumours (366).

In the context of remodelling, studies focus on alteration in total collagen IV content, with or without the additional assessment of total basement membrane thickness. Such studies in rhinitis were prompted by evidence of such changes in lower airways disease. Basement membrane thickness is increased in perennial versus non-atopic individuals as shown by microscopic analysis of inferior

turbinate biopsies (367). Reticular basement membrane thickness is increased in asthmatic children to the same degree as asthmatic adults (368). This would suggest that structural changes influence AHR and do so early on in life. Furthermore, eosinophilic infiltration accompanies hyalinization and thickening of the lamina reticularis in children aged 1-3 suggesting that such changes are relevant to the initiation of asthma and not merely a consequence of long-standing disease (369).

Fibroblasts

Fibroblasts present in all extracellular matrix, are responsible for its content production. This is a relatively quiescent state as tissue injury, in the form of inflammation, trauma, metaplastic change, alters its phenomenology. When activated, fibroblasts transform acquiring a myofibroblast phenotype. Myofibroblasts play a crucial role in tissue healing. Aberrant, exaggerated or persistent myofibroblast activation leads to tissue fibrosis, which when irreversible, adversely affects organ function.

Originally identified over 40 years ago in skin wounds, myofibroblasts, with responsibility for dermal contraction (370), are now noted in a number of tissue and organ disease states. Under normal conditions, myofibroblasts will restore tissue integrity and mechanical stability following injury. When unchecked, they are responsible for pathological contraction, such as Dupuytren's (palmar fibromatosis) (371), lung or liver fibrosis (372), and contractions around solid prosthetics such as breast implants (373).

In order to fulfil its tissue healing potential, a vast array of cell types possess the ability to act as myofibroblast precursors. Smooth muscle cells, pericytes, fibrocytes, and epithelial cells during epithelial-mesenchymal transition, all may take on the myofibroblast phenotype following injury. Despite this heterogeneity of precursors, fibroblasts are still the most common cell type to undergo myofibroblast transformation. On full activation, myofibroblasts express α-SMA. Hintz et al. highlight the distinction between different stages in the fibroblast-myofibroblast transformation (374). Myofibroblasts have combined the phenotypic property of fibroblasts with extensive synthetically active endoplasmic reticulum, with the contractility of smooth muscles, hence the prefix of myo- (370). Normal undifferentiated fibroblasts, or 'proto-fibroblasts', are α-SMA negative and during early collagen deposition in wound healing will lay down early stress fibres and endow the extracellular matrix with only small contractile forces. Once transformed into 'differentiated myofibroblasts', these cells become α-SMA positive, and are able to exert large contractile forces responsible for wound contraction (375). α-SMA is ubiquitously used as an activated myofibroblast marker as this cell type is

negative for other smooth muscle cells markers –smoothelin, desmin, smooth muscle myosin heavy chain- whereas neo-expression of a-SMA is the exclusive ability of functional myofibroblasts (376).

A positive feedback loop exists between extracellular matrix stiffness, and myofibroblast activation. Small aggregates of fibroblasts, without stress fibres, form focal complexes or nascent adhesions as seen with early wounds such as fibrin clots. On further stiffening of the extracellular matrix, proto-myofibroblasts form α-SMA negative stress fibres that terminate in more mature focal adhesions (377). These focal adhesions provide information intracellularly about ECM dynamics and content. Matrix stiffness is normally expressed in units of Young's modulus which represents material tensile strength whilst abiding by Hook's law (force needed to extend a spring is proportional to the distance of that extension) (378). Full myofibroblast differentiation and the attainment of α-SMA positivity is seen at Young's modulus of ~20 kPa or above. At this tensile strength, focal adhesions mature and further enhance ECM stiffness (376).

Working alongside mechanical influences of the ECM, TGF-β stimulates myofibroblast differentiation. In response to TGF-β and TGF-β receptor activation, myofibroblasts express α-SMA, produce collagen, and enhance the contractility of the myofibroblast cytoskeleton (379) (380). Myofibroblasts themselves are a rich source of TGF- β, initially secreted as a large latent complex. Despite this strong feedback loop between myofibroblast activation and TGF- β levels, TGF-β alone is insufficient for activation. TGF-β is secreted prior to ECM stiffening, during early cellular responses to injury when inflammatory cells are abundant sources of TGF-β (381). Despite this, in the context of insufficient ECM tensile strength, myofibroblast activation is absent (382). Conversely, mechanical strain alone is not able to simulate fibroblast differentiation, as *in vitro* experiments of mechanically stressed cells do not produce α-SMA in the presence of TGF-β inhibitor (375). Timed orchestration of both responses is necessary to effect myofibroblast activation at the correct point in tissue healing. Transformation into collagen secreting myofibroblasts at the behest of growth factor TGF-β and cytokine IL-13 stimulus is likely to be a major driver in asthmatic airway remodelling (383) (384).

Transforming Growth Factor β Superfamily

Growth factors are a large group of pleotropic cell-signalling molecules involved in enhancing cell growth, differentiation, maturation, and a variety of regulatory cellular processes. Such functions may be carried out in an autocrine, paracrine or endocrine fashion. Growth factors share some structural homology as well as uniformity of stimulation of cellular growth and function, leading to this group of molecules being designated as a 'family'.

A type of growth factor, Transforming Growth factor-β is a superfamily with over 30 members. TGF-β superfamily is a group of disulfide-linked usually dimeric polypeptides encoded as carboxy-terminal segments of larger secreted pro-polypeptides (385). They encompass a wide variety of pleotropic proteins, including activins, inhibins, anti-Müllerian hormone (AMH), bone morphogenic protein (BMP), growth differentiation factors, amongst others. They collectively possess pivotal signalling and regulating purposes in embryonic development, health and disease states such as inflammation and remodelling. Left/right, ventral/ dorsal and anterior/ posterior orientation of organ development are also specified by the TGF-β superfamily (386). Such effects are mediated by membrane bound type I and type II serine/ threonine kinase receptors and utilise intracellular signalling molecules called SMADs. In addition to their involvement in signal transduction, SMADs may also be regulatory or inhibitory. Translocation of SMAD complexes into the nucleus controls gene transcription.

The TGF-superfamily is further subdivided into 2 subfamilies depending on the group of receptors and intracellular molecules used for downstream signalling: BMP/ DGF/ Müllerian inhibiting substance subfamily and TGF-β/ Activin/ Nodal subfamily (387). There are three TGF-βs, 5 activins and 8 or more BMPs in humans (388). Although the product of different gene loci, the three TGF-β isoforms demonstrate a high degree of homology with 74% between TGF-β1 and TGF-β2, 78% homology between TGF-β1 and TGF-β3 and 82% between TGF-β2 and TGF-β3 (389). De Larco *et al* were the first to observe TGF as a family of growth factors with the ability to endow *in-vitro* fibroblasts with a transformed phenotype.(390)

TGF-β

Synthesis and Activation

The three isomers of TGF-β are synthesised as large pre-propeptide precursors within the Golgi apparatus. Proper folding and dimerisation of the growth factor domain is dependent on its carboxyl terminal prodomain (386). TGF-β family prodomains demonstrate great diversity but are relatively conserved between species (385).The N-terminus signal peptide, linked to a pro and pre-prodomain is catalytically cleaved by a member of the family of endoproteases, furin. Furin cleavage produces the mature TGF-β molecule which then links in a non-covalent manner to its latency associated peptide (LAP) within the Golgi apparatus. This complex is now referred to as small latent TGF-β. This confers stability to the molecule, and further linkage with its binding proteins latent TGF- β binding protein (LTBP) makes TGF- β ready for secretion, now referred to as large latent TGF- β. LTBP binding of TGF- β enhances its secretion and confers stability to its folding when associated with LAP. The constitutionally inactive but mature complex of LAP-TGF-β-LTBP is secreted and then stored in the

extracellular matrix. TGF- β itself is made up of several (typically 2) antiparallel pleated β sheets perpendicular to a four turn α-helix, secured by disulphide bonds around a region of 6-9 cysteine residues called a cysteine knot which characterises this family of cytokines. Correct β sheet topology contributes to correct conformational arrangement of the cysteine knot and facilitates inter-monomer disulphide bonds (391). Hydrophobic interactions serve to further strengthen such inter-molecular bonds.

The structure of secreted pro-TGF-β has been subject to detailed study using multi- and single wavelength anomalous diffraction by Shi *et al* (391). They liken latent TGF-β to linked forearms, encircled by a prodomain straitjacket holding TGF-β in latency. TGF-β monomer topology is often referred to as an open hand with the central α helix forming the wrist, the two anti-parallel β sheets the fingers and the N-terminus the thumb (392). Such inactivity of TGF-β is conferred by the encircling pro-domain which blocks TGF-β from receptor interaction and also by holds TGF-β in a conformationally inactive form. Pro-domain β-strand arms extend from the straitjacket and around TGF-β monomers to meet and cross at a 'bow-tie' junction strengthened by disulphide bonds and hydrophobic residues. Latency lasso loops extend from and between the straitjacket α helices encircling the tip of each TGF-β monomer. Just below the 'bow-tie' junction are extra-cellular matrix integrin binding RGD motifs used to anchor latent pro-TGF-β to $\alpha_v\beta_6$ with considerable binding strength. LTBP binding to extracellular matrix fibrillins bestows further (393) conformational strength to the prodomain-TGF- β composite.

Activation of TGF-β from latent pro-TGF-β requires LTBP anchoring to the extracellular matrix at one end, integrin binding to the actin cytoskeleton at the other, and tensile forces exerted by myofibroblasts to unlock the prodomain (394). Disulphide bonds of LTBP hold the prodomain tight, with the β sheet of the arms most able to resist tensile forces (395). The resultant give of the straitjacket α helices unlocks and releases TGF-β molecule (Figure 1.2). Release of TGF-β results in a conformational change, enabling receptor interaction. Further roles for integrins in TGF-β activation may be by the provision of proximal docking sites for latent TGF-β and cleaving proteases (396), or direct enhancement of TGF- β receptor/ ligand interaction (397).

Tensile forces described above are activated with tissue response to inflammation or injury (374). Other mechanisms of TGF-β activation all relate to this premise of extracellular matrix disruption. Proteases active during ECM degradation cleave and release TGF-β concurrently. Serine proteases such as plasmin and metallo-proteases (MMP 2/9) play a role in proteolytic TGF-β activation (398) (399). There may be considerable protease redundancy as protease knock-out mice do not replicate

TGF-β knockout mouse phenotype (400). Interaction with thrombospondin, alteration in pH and release of reactive oxygen radicals, all present at times of tissue injury, are further supportive mechanisms of TGF-β activation. Heparan sulphates may be intermediaries and regulate LTBP-ECM interactions (401).

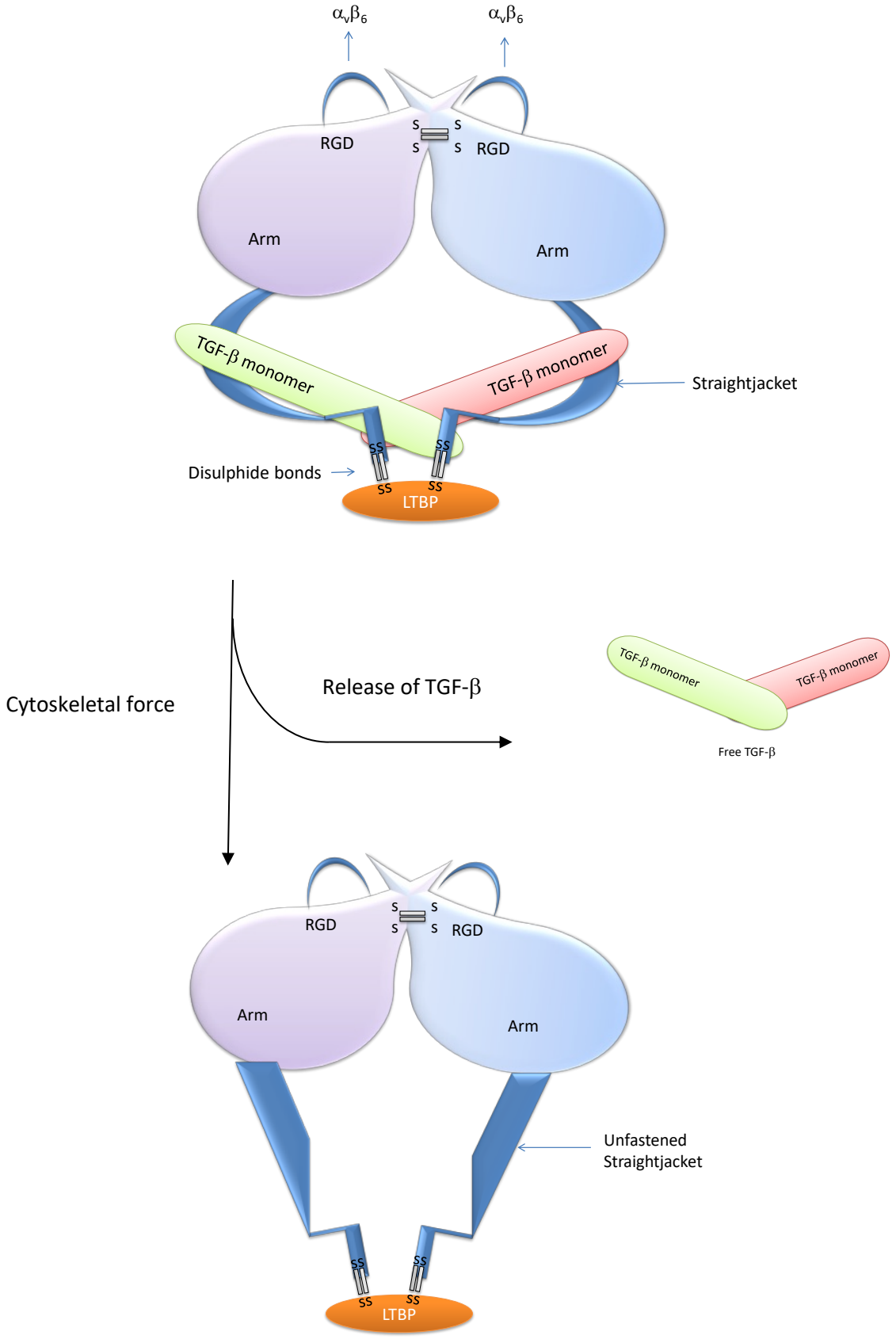


Figure 1.2: Schematic representation of latent and activated TGF-β (adapted from Shi et al Nature, 2011)

Receptor Interaction and Intra-cellular Signalling

The TGF-β group of proteins signal through two membrane bound receptors which are structurally similar serine/ threonine kinases. These exist as Type II and Type I receptors with each coupling as a homodimer. The resultant unoccupied heterodimer consisting of two Type II and two Type I receptors each is ready for binding with active TGF-β. Although structurally similar, Type I receptors have a conserved GS (glycerine/ serine rich) domain immediately upstream from their kinase domain. Certain Type II and Type I receptors have particular affinity for each other with particular heterodimeric confirmations dependent on receptor and ligand type (392). Binding of the Type II receptor ligand activates its kinase which phosphorylates the Type I receptor GS domain, which in turn phosphorylates and activates receptor complimentary intracellular SMAD proteins (Figure 1.3). Although some of the mammalian TGF-β group ligands may bind one or other of their surface receptors in isolation, such exclusivity of ligands to sole receptor binding is insufficient to endow them with activation and the capability for intracellular signalling (402). Co-receptors such as endoglin are needed to further collaborate in securing and modulating receptor/ ligand interaction (403). Such facilitators to TGF- β signalling are vital for proper signalling, as demonstrated by faulty endoglin gene mutations which result in fragile blood endothelial vessel cells leading to hereditary haemorrhagic telangiectasia and arterio-venous malformations (404) . Once activated, TGF-β receptors are able to remain so for hours (405).

The specificity of the intracellular response via SMAD recruitment and activation is determined through fine tuning conferred by the L45 loop within the Type I receptor kinase domain located downstream from the phosphorylated GS (Gly-Ser) region (388). L45 is a nine amino acid sequence vital in conferring fine tuning ability on the type of intracellular signal to be effected (406). SMAD binding, activation and signal specificity is further modulated by intracellular binding proteins such as SARA (SMAD anchor for receptor activation) and inhibitory SMADs (407). Signalling pathways may be SMAD dependent or SMAD independent and may exert effects via both transcriptional and non-genomic mechanisms (408).

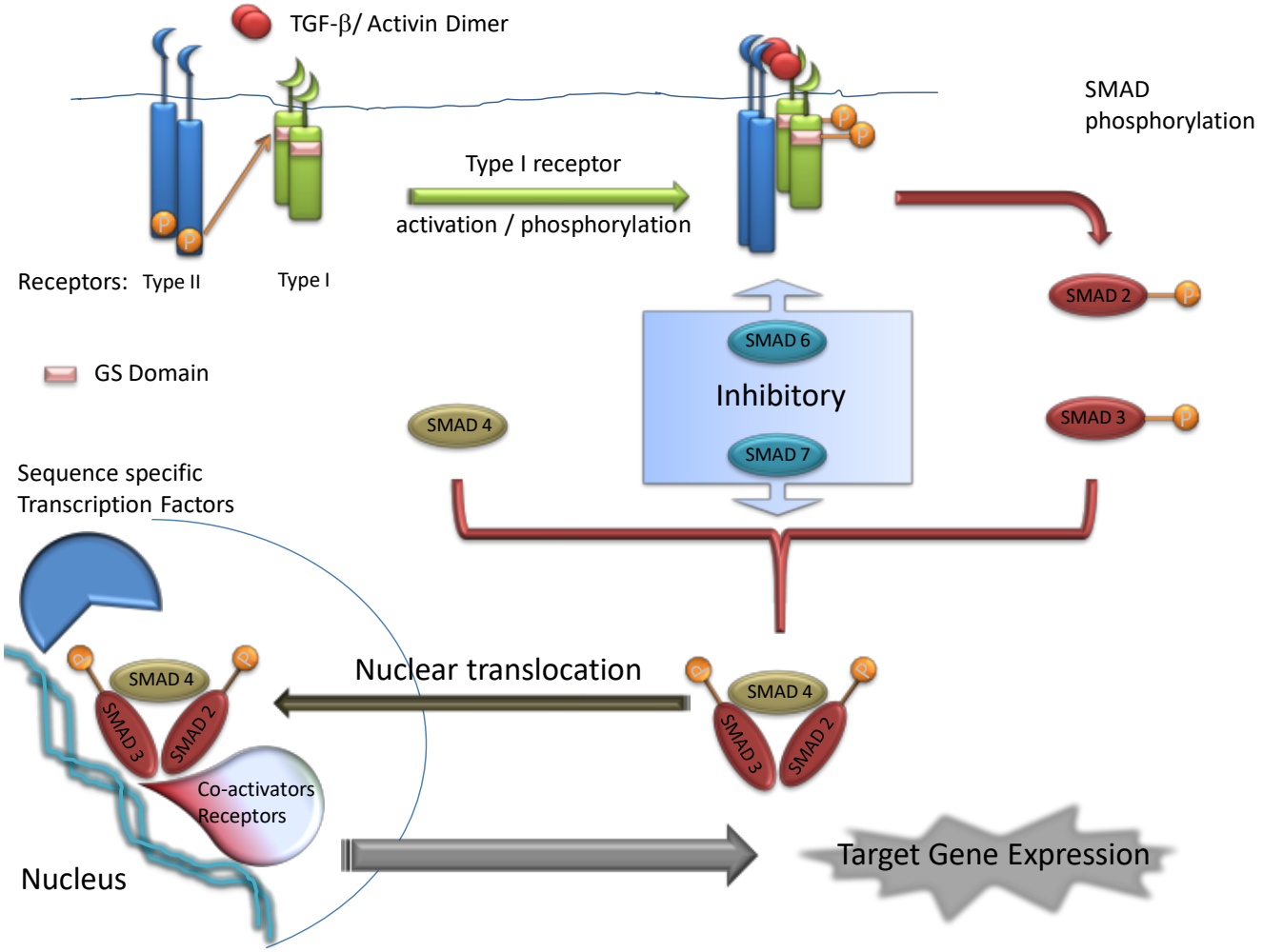


Figure 1.3: Schematic representation of TGF-β / activin signalling

SMADs

SMADs were identified during gene location studies in the *Drosophila Melanogaster* small fly species responsible for suppressing decapentaplegic gene in the embryo called MAD (Mothers Against Decapentaplegic) (409) and the SMA genes in the roundworm species *Caenorhabditis elegans* coding for small body size (410). Both MAD and SMA gene products were found to be vital in TGF-β signalling. SMAD is the amalgamation of the two acronyms.

TGF-β proteins rely on SMADs to affect their intracellular actions. SMADs exist as three functional variants: regulatory/ effector R-SMADs (1,2,3,5,8), common collaborative co-SMAD (4) and inhibitory SMADS (6,7) that counteract such effector functions. TGF- β 1/2/3 may activate SMAD2/3 if acting through ALK5 or SMAD1/5/8 if using ALK1 as its Type I receptor. R-SMADs and co-SMAD4 contain two well conserved MH1 and MH2 segments at the N-and C-terminus respectively. These two distal segments are separated by a less conserved polypeptide portion. The MH2 domain on R-SMADs contains a L3 amino acid loop responsible for directly interacting with the L45 on Type I receptor further securing this bond (411). Interfaces between phosphorylated Type I receptor and portions downstream from SMAD L3 loop confers versatility on intracellular SMAD mediated responses (412). It is the C-terminal SXS motif on the R-SMADs that undergoes phosphorylation by Type I receptors. R-SMAD phosphorylation results in a conformational change and dissociation from surface receptor complex. Further association with co-SMAD4 forms a trimeric complex which translocates to the nucleus and joins other transcription factors to effect transcriptional change(413). Although the co-SMAD/ R-SMAD complex shows weak binding affinity to DNA, it acts as a stabiliser and transcription enhancer for other transcriptional co-activators (387).

Inhibitory SMAD6/7 competitively engage Type I receptors preventing R-SMAD binding via their MH2 domain (414). Negative feedback is conferred by R-SMADs themselves that induce inhibitory SMAD expression as well as TGF- β signalling itself (415). Further inhibitory sequences are activated by a host of inflammatory mediators, amongst them IFN-γ, IL-1 and bacterial lipopolysaccharide (416, 417) . By marking receptor complexes for degradation, inhibitory SMADS further hamper R-SMAD mediated signalling (414).

Once R-SMADs have formed a heterodimer with co-SMAD4, translocation into the nucleus is followed by binding of the SMAD complex to a DNA promoter region, further binding to nuclear transcription factors and coactivators to exercise transcriptional control. The diversity of SMAD binding factors grants SMAD dependent signalling with a vast variety of effector functions (388). Heterodimerisation of R-SMAD with SMAD-4 is a prerequisite for displacement into the nucleus, although TGF- β may

exercise effector function in the absence of SMAD-4 (418). Conversely SMAD-4 deficient cell lines do not fully recapitulate TGF-β absent cellular phenotype (419). Ubiquitin marked proteosomic degradation of cytoplasmic SMAD by Smurf1 and Smurf 2 (Smad-ubiquitination-regulatory-factor) regulates SMAD bioavailability and modulates post-receptor signalling (420). Phosporylation of R-SMADs SXS motif on MH2 segments exposes importin-β binding sites on MH1 domains. Importin-β allows for nuclear translocation of R- SMADs by coupling to lyseine rich domain called Nuclear Localisation Sequence or NLS. R- SMADs may collocate with SMAD -4 but cytoplasmic complex formation is not a pre-requisite for nuclear transport (413). After achieving transcriptional relevance, R- SMADs are dephosphorylated and returned to the cytoplasm for recycling. Such phosphorylation is not a mechanism of control over SMAD-4 activity (421) (422). Following ligand binding, TGF-β receptors may remain active for hours, thereby sequestering pSMADs in the nucleus and prolonging their transcriptional influence (405).

SMAD signalling may be further modulated by enhancing inhibitory SMAD expression. Non-TGF-β specific tyrosine kinase receptors as well as inflammatory mediators such as interferon-γ signalling through STAT increase SMAD 7 production (414) and thereby inhibit R- SMAD activity. Conversely other ligands such as epidermal growth factor (EGF) may activate SMAD dependent signalling via tyrosine-kinase receptor interactions (423).

Non-Smad dependent signalling

Although tyrosine kinase receptor activation and SMAD mediated signalling of TGF-β family of proteins is now elucidated in some detail, it has also become clear that such signalling occurs in the absence of the above standard signalling pathway. Moreover, overlapping and reciprocal relationships between SMAD-dependent and SMAD-independent signalling serves to add further complexity to intracellular effector mechanisms of TGF-β.

Responses to TGF-β ligand binding take some time to manifest but responses elicited in minutes rather than hours indicate that non- SMAD dependent mechanisms are also triggered. The Erk, JNK, and p38 MAPK pathways have all been described as SMAD independent signalling mechanisms. Such pathways may affect transcription factors utilised during SMAD -reliant signal transduction thereby forming a mechanism where the two independent pathways may converge (419, 424).

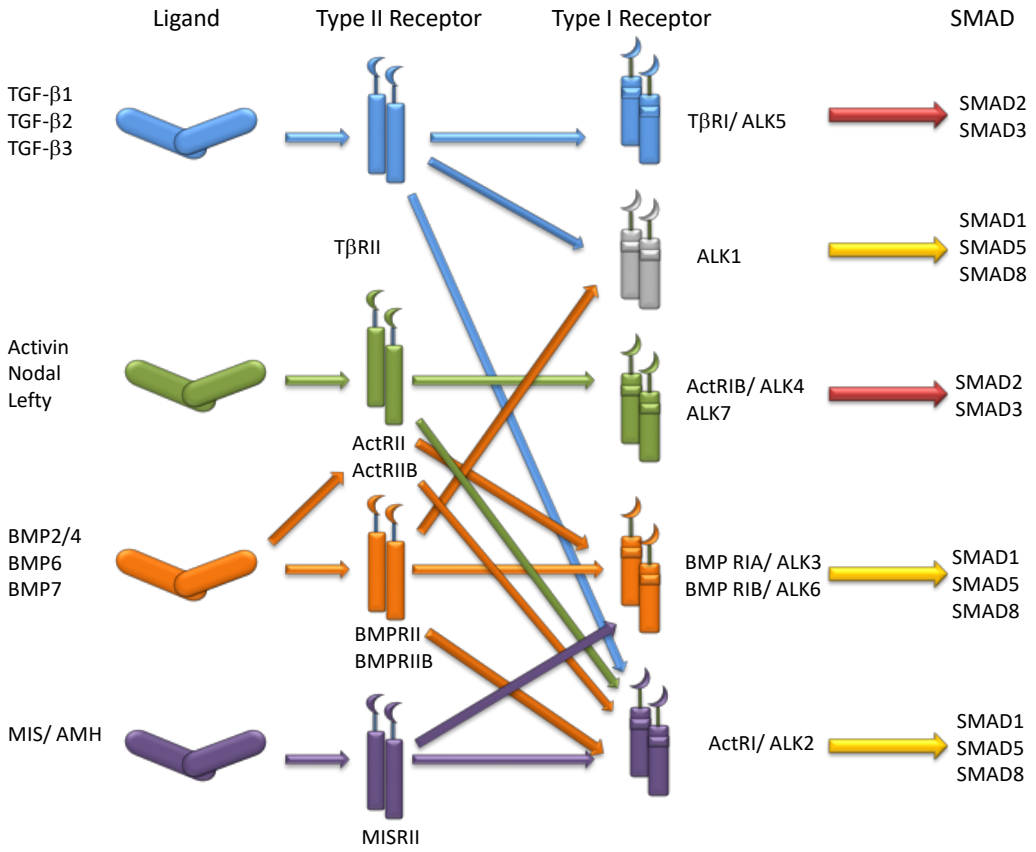


Figure 1.4: Combinatorial interactions of TGF-β superfamily ligands and their Type II/ Type I receptor interactions with complementary downstream SMAD signalling molecules. Adapted from Feng X, Derynck R, Annu Rev. Cell Dev. Biol, 2005

TGF-β in Disease

Allergic inflammatory insults induce reparative tissue responses. Functions of TGF-β are numerous and depend on tissue type, disease state and local cytokine milieu (425). TGF-β signals may be anti or pro-inflammatory and induce potent fibrogenic responses. TGF-β has been implicated as an active participant in processes leading to tissue remodelling which is a feature of chronic asthma. Initial focus was on its regulatory and anti-inflammatory properties as TGF-β knockout mice died of overwhelming autoimmune mediated infections (426). Within the scope of remodelling, the effects of TGF-β are many and varied, depending on the time post allergen challenge (427).

New synthesis as well as release from extracellular stores contributes to increases in TGF-β in response to tissue insult or injury. Some initial effects of TGF-β include chemotaxis of key inflammatory cells such as monocytes, mast cells and neutrophils (428) (429). Upregulation of inflammatory cytokines such as IL-6 and TNF-α follows (430). Such chemotactic effects are possibly enhanced by induction of tissue gelatinases (431). Alteration of extracellular matrix integrin expression further contributes to the orchestrating effects demonstrated by TGF-β. Importantly, TGF-β seems to influence tissue response to antigen. It does so by its influence over antigen presenting cells, such as dendritic cell differentiation (432, 433), as well as altering T cell function. Increased IL-2 production and T cell priming serve to alter the rate and nature of the T cell responses (434). There may be an initial delay in T cell apoptosis (435) but increased DC death post antigen presentation, with enhanced macrophage phagocytic activity demonstrates attempts at enhanced resolution of the disease process (436).

TGF- β as pro-inflammatory cytokine is chemoattractant to granulocytes and macrophages. It further enhances differentiation of T cells to a Th17 cell lineage (with resultant IL-17 secretion) (437) (438). With further proof of the multifaceted behaviour of TGF- β, there is evidence of diversification toward a Th9 phenotype as well (439) with roles in fibrosis and repair (440). In the context of remodelling, TGF-β slows epithelial cell turnover by promoting cell cycle arrest. In TGF-knockout mice, there is increased PCNA (proliferating nuclear cell antigen) expression with concomitant proliferation of epithelial cells(441). Effects of TGF-β on the extracellular matrix include increased tenascin, collagen I and collagen II deposition with upregulation of HSP-47. Myofibroblast transformation is potently enhanced by TGF-β (214). In a renal model of fibrosis, when epithelia of proximal tubular cells lines are injured, levels of TGF- β are increased with corresponding rise in number of cells in G_2/M phase of the cell cycle. This finding is replicated in a mouse model of acute kidney disease (442).

TGF-β drives SMA-α gene transcription via SMAD2/3 dependent mechanisms (443) with concomitant increases in collagen I and III gene expression (444). Conversely, blockade of ALK5 dependent SMAD

2/3 signalling via the kinase inhibitor SM16, decreases blood vessel fibrosis in the lungs (445). Bronchial epithelial cells control fibroblast function via TGF- β mediated activation. Fibroblasts exert further regulatory control over TGF-β secretion via $\alpha_v\beta_8$ mediated mechanisms. The inflammatory response to TGF- β is diverse due to the non-uniformity of fibroblast phenotype as well as fibroblast responsiveness in the upper and lower airways (311). In a TGF-β enriched environment, epithelial DCs are less potent at TLR mediated allergen driven responses (446).

Mice with mutated integrin binding motifs demonstrate a similar phenotype to TGF-β null mice with multi-organ failure underscoring the importance of integrin binding in TGF binding and activation (447). In humans, progressive diaphyseal dysplasia, a rare sclerosing dysplasia (Camurati-Englemann disease) is caused by mutations in TGF-β prodomain resulting in increased active TGF-β release. Characteristic features of the disease are painful long bones, muscular weakness and hyperostosis of long bone diaphysis (448).

In the lower airways, bronchial biopsy eosinophils demonstrate 75% positivity to TGF- β1 mRNA. Moreover, 65% of TGF- β β1 mRNA positive cells were eosinophils (449). Bronchial biopsies in COPD demonstrate decreased expression of SMAD 6 and 7 balancing epithelial cells that secrete TGF-β1 and possibly drive development of small airways disease (311). Alternatively, oxidative stress in turn activates MMP9 which may release latent TGF- β1(450).

In the upper airway, there may be a differential expression of TGF- β in CRSwNP and CRSsNP. TGF- β 1, receptor types I and II mRNA, and pSMAD2 positive cells are significantly lower in CRSwNP (polyp samples) when compared to normal controls (451). TGF- β 1 and collagen deposition predate symptoms in CRSsNP (452). Macrolide (Clarithromycin) therapy reduces cellular expression of TGF-β1 in CRS tissue *in vitro* but not *in-vivo* (151).

TGF-β plays a role in mast cell chemotaxis (453) and promotes expression of FoxP3 which in turn enhances T regulatory cell differentiation (454). Such CD4+ CD25+ Treg cells inhibit expression of IL-5, IL-13 and TGF-β in BALB/c mice (455). In a human study of the effect of allergen specific immunotherapy, Th2 and Treg associated IL-10 levels were higher, TGF-β and accompanying FoxP3 levels lower in seasonal allergic rhinitics both pre and post immunotherapy when compared to normal non-atopic controls (septoplasty candidates with all samples taken from inferior turbinates) (456).

TGF-β and Remodelling

The inflammatory cytokine profile of the nasal mucosa in CRSwNP and CRSsNP is reflected in sinus tissue underlining the collective involvement of the whole of the sinonasal epithelium in disease in these groups (457). CRSwNP patients' polyp tissue shows decreased FOXp3 expression and Treg cell counts when compared to CRSsNP turbinate biopsies (458). Furthermore TGF-β1 and its downstream signalling molecules are down-regulated in CRSwNP compared to CRSsNP. Such lack of regulatory cell expression in CRSwNP results in failed regulation of GATA-3 mediated Th2 cell and B cell activation (109).

When compared to the eosinophilic, Th2 type inflammation seen in Caucasian polyps, Chinese patients' polyps demonstrate a neutrophilic infiltrate, again with low TGF-β1 expression, with lower T-reg numbers, lower FOXP3 expression but a Th1/ Th17 skewing(311).

TGF-β1 effected imbalance between MMPs and their natural inhibitors (TIMP-1) is thought to be responsible for albumin deposition and the characteristic histological changes seen in Chinese CRSwNP: albumin deposition, pseudocyst formation, and oedematous stroma (459).

Activin

Now known to be a part of the TGF-β superfamily, when initially identified over 20 years ago, activin was classified as a hormone, with stimulatory properties on follicular stimulating hormone release from cultured gonadotrophin cells (460) (461). Activins and their natural antagonists, inhibins, form a subgroup of the TGF-β superfamily existing as homo- or heterodimers of two distinct subunits encoded by different gene loci. The α subunit is coded by one gene (inhibin-α), with four distinct β subunits coded separately (inhibin- βA, βB, βC, and βE). Inhibins are made up of one α and either a βA or βB subunit forming a heterodimer. Activins on the other hand dimerise using β subunits only to form activin A (βA/ βA), activin B (βB/ βB) or activin- AB (βA/ βB) (462). Tissue and cellular effects of the three activin dimers have been studied in some detail with activin-A subject to greatest scrutiny. Activin-A along with activin-B, are most biologically relevant. Although functions of activin-A and activin-B overlap, full redundancy is not achieved as knock-out mice without either activin species exhibit different phenotypes (463). Less studied activins-βC/ βE do not appear as vital for development and homeostasis as null mice without these activin subsets seem to bear no stigma of activin-A deficient phenotype (464). More recently, activin-C was shown to dimerise with βA subunit thereby competing with activin-A for receptor bonding(465). Moreover overexpression of activin-C does confer abnormal organ development in transgenic mice (466).

Like TGF-β, activin is produced intracellularly linked to its pre and pro-domains at its N-terminus with disulphide bonds conferring dimeric stability along the C-terminal mature protein domain (467). Enzymatic cleaving releases activin from its precursor, it is secreted from the cells where binding of its noncovalently bonded pro-domain weakly secures it to heparin sulphated proteoglycans in the extracellular matrix (468). Such ECM binding sites are present only on activin-A but not activin-B which may diffuse away from its parent cell with greater ease (469). Affinity between mature activin and its prodomain is much weaker than that of TGF-β (470) whereas the affinity between it and its constitutively active Type II receptor is strong. So although there is some sequestration of activin-A post cellular secretion, the pro-domain interaction is so easily displaced by receptor affinity that activin is considered to be secreted in an active form without any appreciable extracellular latent storage (471).

Bioavailability of activin is regulated not only by fine tuning its cellular secretion but also post-cellular inhibition. Both inhibins-A and –B share activins' β subunit and competitively engage constitutively active Type II receptors (472). Furthermore, follistatin and follistatin-like protein 3 modulate activin signalling by high-affinity binding resulting in endocytosis and degradation of the follistatin/ activin complex (473). Follistatin's affinity to bind activin-A is ten times that of activin-B (474). To a lesser extent activins may also be modulated by a variety of other molecules such as α2-macroglobulin and lipovitellin (475, 476).

Activins in Disease

Activin, a member of the TGF-β superfamily exists in four isoforms: A, B, C and E with dimerisation to form either homogenous or heterogenous variants. Activin A with two A subunits is most frequently expressed. It shares many similarities with TGF-β: it is also synthesized as a pre-proligand, may be expressed by a variety of cells, it is synthetised anew in response to inflammatory stimuli but is also present in its bound form extracellularly (477). A sheep model of inflammation demonstrates rapid rise in activin following LPS injection(478). Activin is raised in inflammatory diseases such as Crohn's disease and inflammatory arthritis. Like TGF-β, it is a potent inducer of fibroblast proliferation and enhances differentiation into myofibroblasts. Whilst human lung fibroblasts produce activin during times of tissue injury, they do so much more abundantly in the presence of TGF-β (479). Seasonal and perennial allergic rhinitis sufferers seem to have increased expression of TGF-β receptors which colocalise to mast cells(480). This finding correlates with gene expression studies. Such increase in receptor expression was not accompanied by altered SMAD-3, SMAD-6 or SMAD-7 gene expression(481). Activin A and activin B knockout mice have differing phenotypes and are additive when combined indicating non-compensation between these two monodimers (482).

Serum activin A levels are elevated in adult cystic fibrosis patients and correlate inversely with body mass index and lung function. In a murine model of cystic fibrosis, blockade of activin A via follistatin induced reduced mucus production, decreased neutrophil infiltration, and abrogated a number of inflammatory cytokines including IL-1, IL12p40 and IL-13 (483). Follistatin, which binds activin A with a 10 fold higher affinity than any other TGF-β family member, inhibits experimental models of allergic asthma and diminishesbleomycin induced lung injury in murine models (484) (485).

Matrix Metalloproteases

In their quest to identify the collagenase responsible for metamorphosis Gross and Lapiere identified the first metalloproteases back in 1962 (486). Introducing tadpole tails into a collagen matrix resulted in breakdown of the collagen triple helix hence the coined term matrix metalloprotease-1. Since this observation of tadpole tail involution during embryonic frog development, and the recognition of elevated levels of matrix metalloproteases (MMPs) in disease states such as rheumatoid arthritis (487) 23 human MMPs have been identified. Classification is determined by MMP substrate. Collagenases (MMP1/8/13), gelatinases (MMP2/9), matrilysin (MMP7/26), elastase (MMP-12), enamelysin (MMP20) and stromelysins (MMP3/10/11) are non-membrane bound MMPs. MMP14/ 15/ 16/ 17/ 24/ 25 are membrane bound. A number of tissue inhibitors of metalloproteases (TIMPs) have also been identified (TIMP1/2/3/4). Important during embryonic development, MMPs also play a major role post birth, in cell differentiation, migration, apoptosis, adhesion and during tissue turnover, in remodelling.

MMP Structure and Endopeptidase Activity

A conserved methionine residue termed 'Met-turn' beneath the zinc dependant active site, led Stocker and Bode to term this group of endopeptidases as metzincins (488). Comprised of four distinct subtypes (astacins, serralysins, disintegrin metalloproteases or ADAMs, and metalloproteases or MMPs), they share considerable homology most likely due to common evolutionary origins. All are involved in extracellular matrix reconfiguration within or without the context of remodelling. Metzincins are characterised by a pro-domain, a catalytic domain, and with the exception of MMP7/23/26, a hinge region and a haemopexin-like domain (489). Catalytic activity is bestowed by the zinc-metal containing three histidine residue sequence HExxHxxGxxH (where E is a glutamic acid residue acting as a nucleophile during proteolytic activity) within a hydrophobic cleft. The cleft is formed by 1,4 β turn within the highly conserved methionine sequence. MMP substrates are likely to be cleaved by the zinc motif bound water molecule previously activated by the glutamic residue within the three histone sequence (490).

The cysteine residue within the pro-domain serves to bind and stabilise the zinc motif on the catalytic domain site (Figure 1.5). Removal of the pro-domain from the catalytic domain and consequent exposure of the zinc dependent active site involves dual splitting both within the pro-domain as well as between the pro-domain and the catalytic domain. Upon cleavage, the zinc-dependent catalytic site is exposed enabling it to interact with the extracellular matrix substrate. Peptide structures with inherent proteolytic activity or the ability to enhance parent enzymatic function are termed *exosites* (491). The haemopexin domain attached by a hinge region to the main MMP catalytic segment belongs to such a group. Missing on matrilysins MMP7, -26, the haemopexin domain allows for anchoring, correct peptide-substrate interaction, molecular adhesion and substrate recognition of MMPs. Further MMP variations include the insertion of fibronectin type II domains in MMP2,-9 to mediate collagen/ gelatine substrate interaction (489). Excepting membrane bound MMPs (MT-MMPs: MMP14, -15, -16, -24), all other MMPs are secreted in their soluble format. MT-MMPs are linked to the plasma membrane via the haemopexin domain by a short anchoring segment which may be a glycosylphosphatidylinositol (GPI), transmembrane (TM) or an amino-terminal signal anchor. Soluble MMPs may still be membrane bound by extrinsic interaction with membrane bound molecules (492).

Regulation and Modes of Action

Although not constitutively expressed, MMPs can be detected in all disease states involving inflammation or injury. Remodelling processes ubiquitously involve expression of altered MMP and their inhibitors, TIMP. MMP activity may be regulated at multiple points during synthesis and activation: gene expression, post translational activation, cellular secretion and substrate activity (489). Release of MMPs' catalytic domain from their pro-domain may be by achieved by furin like pro-convertases, other membrane bound MMPs or enzymatic reactions via plasmin/ plasminogen activation cascade (493). Once secreted, specific inhibition via TIMPs, or non-specific inhibition via α2-macroglobulin further controls proteinase activity (494). With multiplicity of function and variable alteration, absolute MMP/TIMP levels as well as their comparative ratios, classification of action may be divided according to modality of action or the histological affect manifested.

MMPs are mainly noted for their ECM degradation ability. This may manifest as degradation of ECM constituents (495) (496) or the production of activated intermediaries as a result of proteinase activity. For example, the MMP9 cleaved α3-chain of Collagen IV called tumastatin is known to suppress angiogenesis and therefore inhibit tumour growth by binding to $\alpha V\beta 3$ integrin (497). Breakdown of intracellular structures as such tight junctions and basement membrane may confer altered phenotype onto epithelial cells themselves (498). Increased cellular motility is enhanced by the ECM degrading properties of MMPs, resulting in synergistic effects on epithelial remodelling or mesenchymal

transition. For example, ovarian cells with increased invasive properties show heightened claudin-3 and -4 expression, possibly mediated through enhanced MMP-2 levels (499). Enzymatic activity of MMPs can also serve to alter paracrine/ autocrine signalling by other extracellular molecules. Cleavage may result in activation or negative regulation by de-activation of such cytokines (500).

Studies into knockout mice devoid of a given MMP expression produces mice that survive to birth. MMPs each have a range of substrates which may signal enzymatic redundancy, compensation or other alternative enzymatic pathway activation to ameliorate any single MMP deficiency. Single knockouts do produce specific phenotypes with MMP7 (matrilysin)-null mice demonstrating drastically reduced re-epithelialisation in injured trachea (501). The same study demonstrated MMP7 is constitutively expressed and upregulated in cystic fibrosis epithelial cells. MMP9 deficient mice demonstrate aberrant and delayed growth plate ossification with abnormality of accompanying angiogenic activation during long bone development (502). Furthermore, increased collagen deposition and decreased collagen contracting ability by arterial smooth muscle cells in MMP9 null mice has been attributed to deficient arterial remodelling in this strain. Conversely, MMP7 deficient strains have shown suppression of tumour growth (503). MMP9-null mice demonstrate inhibited carcinogenesis in skin and pancreatic experimental models as well as decreased metastatic activity *in-vitro* (504).

Tissue Inhibitors of MMPs

Cytokines, growth factors, hormones and cell-to-cell interactions exert influence over transcriptional control of MMPs. Once in the extracellular space, endogenous peptides such as α2-macroglobulin and tissue inhibitors of metalloproteases (MMPs) will limit zymogen activation induced proteolysis by MMPs. α2-macroglobulin exerts such inhibitory activity in the fluid phase such as blood, whilst TIMPs do is in tissue.

To date, four TIMPs have been identified: TIMP-1, -2, 3, and -4. Acting as the natural inhibitors they form 1:1 noncovalent stoichiometric complexes with MMPs. Such binding renders the composite stable to heat or proteolytic degradation. Regulation of MMP activity is further modified by TIMP binding to pro-MMP molecules. TIMPs differ in solubility, constitutive expression and abundance per tissue type. TIMP-3 is matrix bound, whilst TIMP-1, -2, and -4 are soluble. TIMP-2 is constitutively expressed whilst TIMP-1, -3, and -4 is inductive on activation by external stimuli. TIMP-3 has a particular role in regulating inflammation by inhibiting tumour necrosis factor-α converting enzyme (TACE or ADAM-17) which would normally generate a soluble form of the membrane bound precursor of this inflammatory cytokine (505). TIMPs are 21-34kDa proteins with 12 cysteine residues folded

into two distinct domains- the N and C domains- by six disulphide bonds. Homology between TIMPs varies from 37% (TIMP-1 and TIMPs-2, -3, 4) to 50 % (TIMP-2 and TIMP-4). The N-domain includes the VIRAK sequence of 18-22 amino acids and is well preserved amongst all TIMPs. Crystallographic studies show a wedge shaped molecular structure with five stranded N-terminal β strands in a Greek key motif (the O-B fold), a β structure with two parallel and two antiparallel sheets and a small α helix C -domain, and a central helical region connecting the two (506). Inter-cysteine residue binding of MMPs in the N-domain are critical for TIMP inhibitory activity. Co-enzyme complex formation is via the C-terminal with structural flexibility intrinsic to TIMP molecules (507).

With different evolutionary origins, the N and C domains function independently. TIMPS exert a variety of cell proliferative, migratory, apoptotic and anti-angiogenic effects independent of MMP blocking activity. When first cloned, TIMP-1 was found to be identical to erythroid potentiating factor (508) with subsequent evidence of cell growth promotion in fibroblasts and a variety of other cell types (509). Receptor mediated intracellular signalling mechanisms vary between TIMP-1 and TIMP-2 but in both cases are independent of endopeptidase blockade activity as mutation or blockade of MMP-1 inhibitory action does not influence TIMP mediated cellular proliferation (510). Although both TIMP-1 and TIMP-2 are capable of inhibiting cell proliferation by arresting cell cycle at G_1 this activity appears linked to anti-apoptotic effects in TIMP-1 only (511). Such effects are both linked and independent of MMP activity. Linking of TIMP-1 to CD63, which in turn binds to b2 integrin, forms a trimeric complex constitutively turning on survival signals in some cancer cells (512). As such, high TIMP-1 levels in serum and tissue may be linked to worse clinical outcomes (513).

Although exerted via different pathways, all four TIMPs exert anti-angiogenic activities. As MMPs help to enhance epithelial cell migration and formation of new capillaries, their blockade provides MMP dependent inhibition of new vessel formation. In TIMP-2, non-MMP determined pathways are mediated via $\alpha 3\beta 1$ and its C-domain (514). TIMP-3 will bind directly to VEGF receptor-2 and angiotensin II type 2 receptors to exert its anti-angiogenic actions (515). Both TIMP-2 and TIMP-3 enhance quiescence in newly formed vessels by arresting further endothelial cell activation through inhibiting endothelial cell tube formation (516).

TIMP-1 -/- mice show alteration in steroidogenesis and altered reproductive cycle (517). They are less susceptible to corneal infections via decreased MMP inhibitory activity, possibly by enhancing innate immune responses through MMP7 (518). TIMP-2 -/- mice demonstrate wide ranging deficit in central nervous system developments (506), whilst TIMP-3 -/- mice show aberrant cardiovascular development including dilated cardiomyopathy (519).

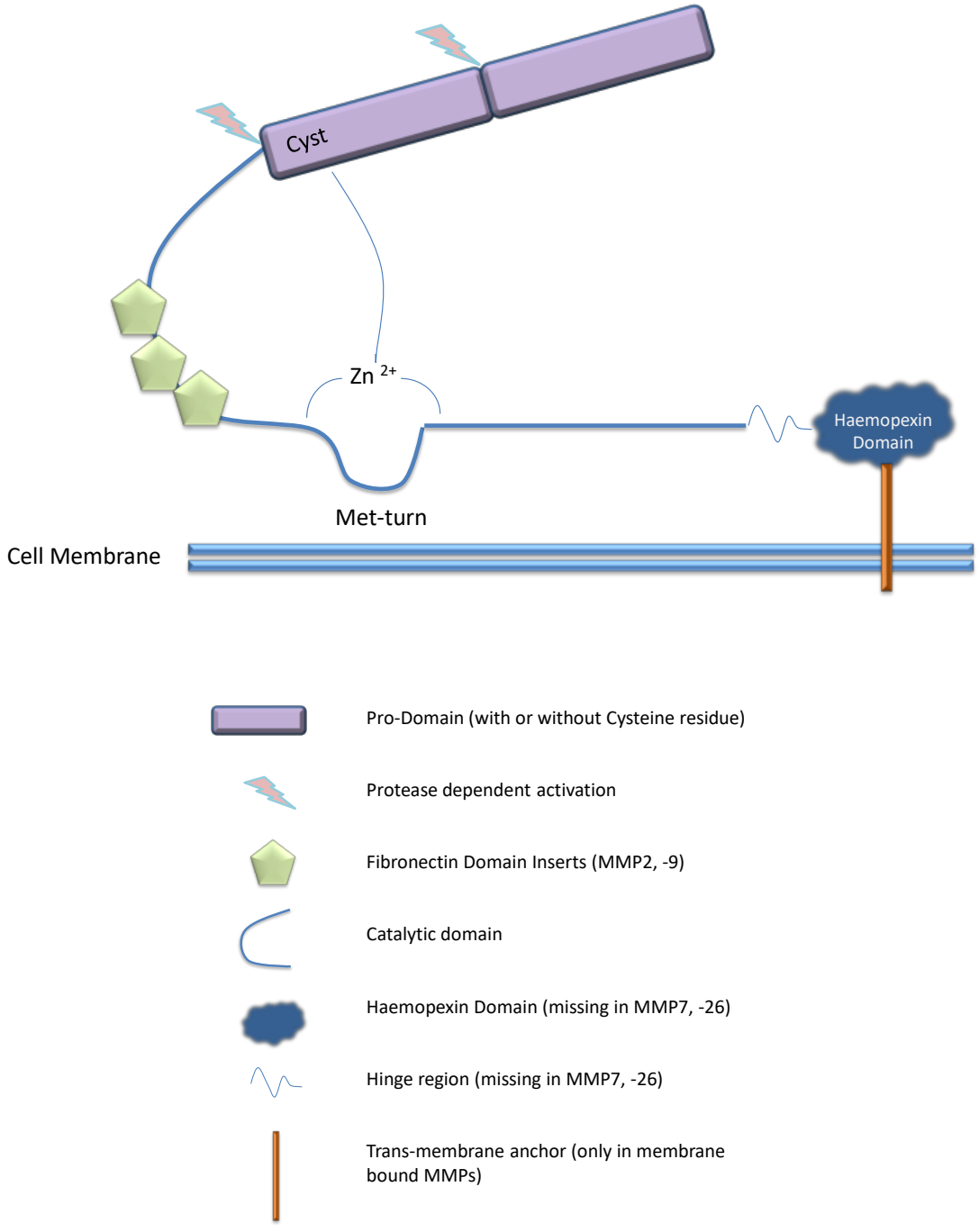


Figure 1.5: Schematic representation of MMP structure. Adapted from Page-McCaw et al, Nature 2007

MMPs and TIMPs in Disease

MMP7 activity within the confines of adaptive immunity may aid healing or enhance inflammation. Cleavage induced activation of E-cadherin, with which MMP7 colocalises, is responsible for easing epithelial inter-cellular adhesions allowing for migration of epithelial cells in wounded tracheal tissue. MMP7 whilst not expressed during embryonic development, is expressed exclusively and at high levels in normal mature epithelial mucosa (520). Defects in MMP7 expression result in absence of pro-cryptdin cleavage leading to a lack of cryptdin, an endogenous antibiotic peptide, and thus increases host susceptibility to intestinal bacteria (492). Respiratory mucosa is similarly affected by aberrant or absent MMP7 activity. Inflammatory cytokine induced neutrophil migration involves a twostep MMP7 dependent process. Syndecan-1, a member of the transmembrane heparin sulphate proteoglycan type syndecans, releases its ectodomain on cleavage by MMP7. Such splitting of syndecan-1 releases CXCL1, which in turn recruits' neutrophils into lung alveoli. In the absence of MMP7, neutrophils are prevented from entering alveolar spaces with a delay in cell mediated inflammatory responses (521).

MMP2 expression endows ovarian epithelial cells with increased malignant type invasiveness (499). Gelatinase A or MMP2 specific for collagen IV, cleaves this molecule at its $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin binding site (522).

Non-invasive markers in the form of sputum MMP9 and TIMP-1 are increased in children with moderate and intermittent asthma (523). A temporal relationship exists between MMP9 and osteitis in CRS as after an initial rise, MMP9 falls in keeping with the degree of severity of the osteitis (524). MMP9 mRNA and protein levels rise in murine models of acute lung injury following cardiopulmonary bypass in keeping with degree of inflammatory tissue insult. Attempts at mitigating ECM degradory properties of MMP9 are borne out by a similar and prolonged rise in TIMP-1 (525). In human models of AR, using house dust mite (HDM) allergen challenge, MMP9 shows an immediate rise at 30 minutes post challenge, correlating strongly with mast cell numbers. This rise wanes quickly, returning to baseline by 6 hours (526). TGF-β partly negotiates the balance between MMP9/ TIMP-1 (527, 528).

Angiogenesis & Lymphangiogenesis

Introduction

Development of a functioning vasculature is redundant for organisms such as *Caenorhabditis elegans* (roundworm) and *Drosophila melanogaster* (fruit fly), both small enough to utilise the diffusing properties of nutrients and gases. For more evolutionary complex organisms, such network of vessels is imperative. An area of considerable interest, the ancient Greek physician Galen supposed that once consumed, blood locally regenerated as opposed to circulated. Referencing descriptive analogy, Leonardo Da Vinci had likened the heart and circulatory system to a tree and its branches. In 1628, William Harvey noted the existence of arteries and veins and their respective role of carrying blood from and to the heart. In the mid 17th Century, Marcello Malpighi identified capillaries as linking the two types of vessels, and Caspar Aselius discovered the existence of lymphatics. Although blood vessels are present early on in evolution, lymphatics are only found in higher evolutionary species from amphibians onwards.

Angiogenesis

Within the growing embryo, vessels not only supply nutrition but act as conveyors of trophic and morphogenic signalling molecules. Early vessels share common angiogenic and haemopoietic progenitor cells. Genetic as opposed to haemodynamic forces influence differentiation as these young vessels are already destined for arterial or venous blood vessel differentiation at this early stage of development. Vasculogenesis will consist of an angiogenic followed by an arteriogenic phase. During angiogenesis, new vessels form, sprout, and develop from a central plexus. As these endothelial cells organise into a network, they are coated by pericytes and smooth muscle cells. Referred to as arteriogenesis, such external cellular reinforcement, hitherto only demarcated by endothelial cells, endows newly formed blood vessels with peripheral strength. Relative juxtaposition of these outer pericytes and smooth muscle cells will also determine vessel permeability.

Active in fetal life, present during post-natal development, neo-vascularisation is relatively quiescent during adult life. Exceptions are times of cyclical ovarian and placental growth. Balance between pro- and anti-angiogenic signals is upset at times of tumour growth, wound healing and repair, injury and inflammation.

Balance of pro and anti-angiogenic influences

The most studied growth factor capable of angiogenic properties is vascular endothelial growth factor, or VEGF. Previously known as vascular permeability factor, or VPF, it is the growth factor with the greatest affinity for endothelial cells. Vascular endothelial growth factors are a family with

multifaceted influences on both vascular and lymphatic blood endothelium. VEGF-A and VEGF-B act on blood vessels, whilst VEGF-C and VEGF-D exert their action on lymphatic endothelial cells (529). VEGF-A exists in a number of isoforms dependent on preferential splicing, VEGF-A_{165} is the most angiogenically potent (530). VEGF-A is produced by basophils, mast cells and also by airway epithelial cells via hypoxia driven TGF-β1 synthesis (531). VEGF features in vascular remodelling in asthma (532) (533) (534) (529) and plays a role in combination with inflammatory mediators in cancer development (535).

In humans, signalling of VEGFs is via three receptors, VEGFR-1, VEGFR-2, and VEGFR-3. Alternate splicing of VEGFR-1 mRNA gives rise to a soluble variant. VEGFR-1 is found on vascular tissue, VEGFR-3 on lymphatic endothelia and VEGFR-2 is present on both (532). Two further co-receptors Neuropilin-1 and Neuropilin-2 enhance intracellular signalling via VEGF receptors. Neuropilin-1 is found on arterial endothelial cells with Neuropilin-2 present on venous and lymphatic vessel walls (536) (537). VEGF-A_{165} signalling via VEGFR-2 is boosted by coreceptor action of neuropilin-1 by increasing post receptor intracellular phosphorylation (538).

Numerous other co-factors play a role in post-natal neovascularisation. Angiopoietins are structurally related endothelial growth factors exerting their affects via a tyrosine kinase receptor Tie-2. Angiopoietin-1 looks to control tissue oedema as normal expression stabilises newly formed vessels and minimises VEGF induced extravasation (539). Overexpression results in overgrowth, excess branching and proliferation of lymphatic vessels. Angiopoietin-1 is expressed in all tissues, with Tie-2 at its most abundant in the lung. Its negative active competitor angiopoietin-2 is not ubiquitously expressed but is present in the airway at times of remodelling (540). Hypoxic lung injury or inflammation will increase its expression causing pulmonary oedema, acute lung injury, tissue necrosis (541).

Other pro-angiogenic molecules exert their effect in a complex interplay between immunity and resolution of tissue injury. Angiogenin is one of the most effective tumour derived factors capable of enhancing new blood vessel formation. Implicated in a variety of non-malignant vasculoproliferative pathologies, like inflammatory bowel disease and diabetic retinopathy (542), angiogenin enhances cell adhesion molecule expression, encourages mitosis in vascular endothelial cells and suppresses action of polymorphonuclear leukocytes (543). Present in fibroblasts after lung injury, the heparin sulphate binding fibroblast growth factor FGF-β can stimulate angiogenesis *in vitro* and endothelial cell proliferation and migration *in vivo* (544). IL-8 acts as a multi-potent pro-angiogenic cytokine, exerting its effects partly via enhanced MMP expression in vascular endothelial cells (545). IL-17 produced by

Th17 cells enhances angiogenesis by inducing proangiogenic factors such as VEGF and encouraging endothelial cell migration (546).

Anti-angiogenic factors may do so competitively with VEGF at receptor level like thrombospontin (547), or by antagonising its effects via inhibition of endothelial cell proliferation and migration, for example, endostatin (548).

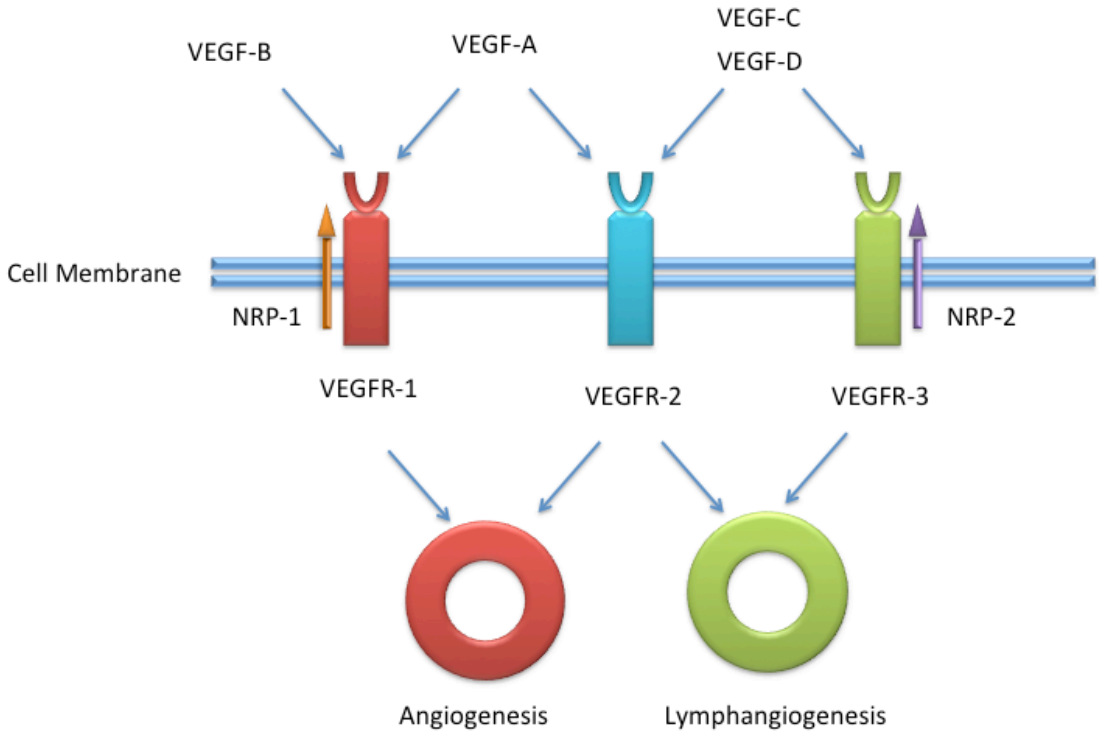


Figure 1.6: Summary of vascular endothelial growth factors and their receptors in inducing angiogenesis or lymphangiogenesis. (NRP: neuropilin , VEGF: vascular endothelial growth factor)

Lymphangiogenesis

Simpler organisms such molluscs have an open circulatory system where blood mixes with lymph (haemolymph) to provide nutrient and remove respiratory waste by simple osmosis. Increasing evolutionary and cardiovascular complexity sees blood retained within vessels. Although only mammals and some aquatic birds possess lymph nodes, lymph vessels are seen in animal of sufficient size, where fluids lost from blood vessels need a route of return (549). Proteins, leukocytes, activated antigen presenting cells move into superficial lymphatic capillaries found in all vascular tissues except the bone marrow and the central nervous system. Walls of lymphatic capillaries are held open by anchoring filaments that directly anchor into lymphatic endothelial cell surface membrane ensuring patency even in times of high interstitial pressure.

In amphibians and reptiles, small areas of contractility along lymphatic vessels, called lymph hearts ensure movement of lymph along the lumen. Whereas lymphatic capillaries are lined with a single endothelial cell, without a basement membrane or encircling supporting cells, the larger draining lymphatic vessels are lined by smooth muscle cells and contain valves. The rhythmic contraction along with compressive effects of surrounding skeletal muscle, ensure unidirectional lymphatic flow towards the thoracic duct and eventually back into the venous circulation at the left subclavian and left jugular vein (550).

Lymphangiogenesis *de novo* and lymphatic endothelial cell migration and survival is controlled by factors VEGF-C and VEGF-D. Both signal through the receptor VEGFR3 and its membrane bound semaphorin coreceptor neuropilin-2 (551) (552). Also expressed on neuronal cells, neuropilin-2 is necessary for correct signalling via VEGFR3 (553) (554). Neuropilin-2 deficient mice exhibit lymphatic hypoplasia (555). Formation of primary lymphatic plexuses from early embryonic veins is reliant on VEGF-C (556). In VEGF-C null mice, a total absence of lymphatic vessel development is seen. *Vegfc* +/- mice show severe hypoplasia of the lymphatic system (557). *Vegfr3* null mice die mid gestation due to fatal lack of functional vessel remodelling (558).

VEGF-C and VEGF-D may also signal through a second receptor VEGFR2, which could also be activated by VEGF-A. Although signalling via VEGF-A may encourage lymphatic growth of established vessels, working in part through VEGFR2, its sole actions are insufficient to enhance neo-lymphangiogenesis (559). Its pro-lymphangiogenic action may be mediated via its effect on inflammatory cells, which in turn, may increase VEGF-C and VEGF-D secretion (560) (561).

Further complexity is introduced into this system by extracellular matrix integrins which may activate VEGFR3 directly (562). Other members of the VEGF family such as platelet derived growth factor B, fibroblast growth factor 2 and other growth factors such as hepatocyte growth factor, will also enhance new vessel growth (549).

Angiogenesis & Lymphangiogenesis in disease

Innate immune response driven activation of VEGF-A synthesis may explain features of remodelling predating Th2 driven inflammation in airway of children prior to asthma development (563). Circulating bone marrow derived endothelial progenitor cells (EPCs) are higher in asthmatics than normal controls. Furthermore, these EPCs demonstrate ability to form neo-vessels and are proliferative *ex-vivo*. Such enhanced expression of EPCs is reproduced in an OVA sensitised, allergen challenged mouse model where these cells are actively recruited into the allergic airways (564).

VEGF will enhance nitrous oxide (NO) production by inducing endothelial NO-synthase and inducible NO-synthase (565). IL-13 inhibits, whilst TGF-β enhances, VEGF production (566). In transgenic VEGF-A_{165} mice, production by Th2 cells, innate immune responses, and IL-13 dependent and independent pathways are all responsible for VEGF-A levels. VEGF can regulate vessel permeability and is increased in asthmatic airways and also during exacerbations. Endotoxic stimulation as a consequence of viral infection will encourage sensitisation and enhance Th2 mediated inflammation (563). Conversely, there is evidence for decreased serum VEGF levels in allergic versus high serum levels in autoimmune diseases such as systemic lupus erythematosus (SLE) (567).

Absent or severely impaired lymphatic drainage causes stasis of protein rich vascular exudate clinically manifested as lymphoedema. If prolonged, tissue fibrosis, fatty degeneration of connective tissues, and weakened tissue immune responses ensue. Whilst primary lymphoedema is genetic in aetiology, secondary disease varies according to geography. Filariasis due to *Wuchereria bancrofti* or *Brugia malayi* is the most common cause in the tropics, whilst breast cancer associated treatment most frequent in industrialised countries (568).

Research around lymphangiogenesis in the last decade has particularly focused on its relationship to cancer metastasis and, by extension, clinical outcomes. Whilst new intra-tumour lymph vessel formation is seen in some solid cancer such as head and neck tumours (569), it may not provide the only route for possible metastatic spread. Interstitial pressure within the tumour mass is likely to collapse small lymphatic vessels (570). VEGF-C secreted around the periphery of the malignant mass

may be the lymphangiogenic effect needed to encourage vessel sprouting and intra-luminal migration of tumour cells (571) (572).

Inflammation initiated angiogenesis will lead to increased plasma exudation which needs to be counteracted by enhanced lymphangiogenesis if tissue oedema is to be prevented. *Mycoplasma pulmonis* infected mouse airways contained VEGF-C and VEGF-D producing inflammatory cells (dendritic cells, eosinophils, neutrophils and macrophages) with histological evidence of lymphangiogenesis. Amelioration of this VEGF-C and VEGF-D signal via VEGFR-3 receptor blockade prevented lymphangiogenesis, giving rise to oedematous mucosa but preventing lymphangitis. Underscoring the seminal role of VEGFR-3 in lymphangiogenesis, it also highlights its relevance in carrying inflammatory cells from sites of inflammation to local lymph nodes. This study further supports angiogenic signalling through VEGF-A with lymphangiogenic signal transduction via VEGF-C and VEGF-D. Adenoviral delivery of either group of growth factors enhanced their respective neo-vessel formation in isolation (560).

Amelioration of VEGF signalling, either through direct blockade or through depletion of VEGF secreting macrophages in a rabbit corneal model of inflammation, shuts down lymphangiogenesis (561). Dendritic cells expressing CCR7 and lymphatic vessels expressing their ligand, CCL21, may play a role in post-transplant inflammation and possibly rejection. These APCs expressing VEGFR-3 and VEGF-C on their surface will respond to lymphangiogenic signals as their blockade can prevent shuttling of dendritic cells between tissues and draining lymph nodes and thereby prevent delayed-type hypersensitivity reactions (573).

Hypotheses

Based on the above review and analysis of type 2 inflammation and remodelling, activation of the TGF family of proteins and features of remodelling of the epithelium, basement membrane zone, mucosal glands and extracellular matrix and associated blood and lymphatic vessels are characteristic features of allergic bronchial asthma. Whether or not type 2 inflammation in moderate severe persistent allergic rhinits is also accompanied by these features of remodelling provides the rationale for the following hypotheses:

- Allergic Type 2 inflammation is a feature of persistent allergic rhinitis that is demonstrable after repetitive nasal allergen challenge and during natural allergen exposure.

- Type 2 inflammation in allergic rhinitis is accompanied by histologic and immunohistologic evidence of nasal mucosal remodelling

- Nasal mucosal remodelling is associated with activation of the Transformation Growth factor beta family of cytokines.

- The repetitive nasal allergen challenge model is safe, well-tolerated and reproduces many of the features of natural allergen exposure.

Aims

The above hypotheses will be tested in participants with moderate-severe persistent allergic rhinitis as follows:

- Type 2 allergic inflammation will be assessed by immunohistology of nasal mucosa for inflammatory cells (lymphocytes, mast cells, eosinophils and neutrophils) combined with ELISA of nasal secretions for interleukin (IL)-L-4, IL-5 and IL-13.

- Upper airway remodeling will be evaluated by histology of nasal epithelial integrity, basement membrane thickness, nasal mucosal glands. Angiogenesis will be assessed by immunohistology of blood vessels (monoclonal antibodies specific for CD31 and VEGF-A) and lymphatic vessels (D2-40). Connective tissue will be assessed by collagen distribution and presence of extracellular matrix (ECM) proteins (HSP-47, MMP-9, TIMP-1 and alpha-smooth muscle actin).

- Relation of markers of remodeling to TGF family of cytokines will be determined by immunohistology using monoclonal antibodies directed against TGF- β and Activin

- The RAC model will be evaluated for safety and tolerability by patient questionnaires and clinical observation. Markers of Type 2 inflammation and remodeling in nasal mucosa will overall be compared with the natural exposure model, with nasal turbinate/polyp tissue from participants with chronic rhinosinusitis and nasal polyps used as a positive control.

Normal healthy participants will provide negative control nasal biopsies and samples from patients with chronic rhinosinusitis and nasal polyps will serve as positive controls for type 2 inflammation and remodelling.

Chapter 2 Methods

Ethical statement

This study was conducted in-keeping with the World Medical Association's 1964 Declaration of Helsinki. Ethics committee approval was granted by the Royal Brompton and Harefield NHS trust, via the COREC (Central Office for Research Ethics Committees), which subsequently became NRES (National Research Ethics Service). It is the latter body that granted site extension as needed. Informed consent was sought prior to enrolment in the study. The relevant consent form was signed in the presence of a witness and before commencement of the study. Informed consent was reconfirmed on the first and all subsequent visits in the presence of a witness.

Volunteer recruitment

Volunteers were recruited via advertising in print and on-line from the general public, Imperial College and Royal Brompton Hospital staff.

Exclusion Criteria

1. Previous treatment (within last 10 years) by immunotherapy with grass pollen allergen.
2. Any of the following underlying conditions known or suspected to be present: Cystic fibrosis, chronic obstructive pulmonary disease, moderate or severe dermatological conditions, malignancy, insulin-dependent diabetes, malabsorption or malnutrition, renal or hepatic insufficiency, chronic infection, drug dependency or alcoholism.
3. Any evidence of disease making implementation of the protocol or interpretation of the protocol results difficult or jeopardising the safety of the subject (e.g., clinically significant cardiovascular, serious immunopathologic, immunodeficiency, hepatic, neurologic, psychiatric, endocrine, or any other major systemic disease)
4. Pregnancy or risk of pregnancy (adequate contraception not being taken) or breastfeeding mothers.
5. Immunosuppressive treatment
6. Systemic steroid treatment in the 6 weeks preceding the start of the study
7. Topical steroid treatment in the 2 weeks preceding the start of the study
8. Underwent investigational drug use/ trials within last 6 months
9. Significant previous nasal trauma that has affected the alignment of the septum

10. History or family history of bleeding.

11. Current use of non-steroidal anti-inflammatory drugs (NSAIDS).

12. Smoking.

13. Starting new treatment for upper or lower airways disease at any point in the study.

Inclusion Criteria for all volunteers

- Male and female, 18-55 years of age
- Normal physical examination
- Willingness to fully comply with the protocol.
- Written informed consent obtained before entering the trial

Additional Inclusion Criteria for Seasonal Allergic Rhinitis Sufferers:

- A clinical history of grass pollen induced allergic rhinoconjunctivitis of two years or more requiring treatment during the grass pollen season
- Positive Skin Prick Test response (wheal diameter ≥ 5 mm) to *Phleum pratense.* Positive specific IgE against *Phleum pratense* (≥ 10 kU/ml)

Additional Inclusion Criteria for Perennial Allergic Rhinitis Sufferers

- A clinical history of house-dust mite induced allergic rhinoconjunctivitis of two years or more requiring treatment.
- Positive Skin Prick Test response (wheal diameter ≥ 5 mm) to house dust mite. Positive specific IgE against house dust mite (≥ 10 kU/ml).

Additional Inclusion Criteria for Nasal Polyposis Patients:

- Polyp disease that has failed medical therapy
- Polyp disease requiring polypectomy for routine medical treatment under general or local anaesthetic.

Additional Exclusion Criteria for Nasal Polyposis Patients:

- Unilateral nasal polypoid disease
- Suspicion of malignancy
- Suspicion of, or known, autoimmune/ vascular disorders

Study protocols

The study consists of two allergen challenge study designs: a natural allergen exposure (NAE), and a repetitive allergen challenge (RAC) protocol.

Participant groups in the NAE protocol include allergic rhinitics (to house dust mite (HDM) and Timothy grass), nasal polyposis patients and normal non-atopic controls. The RAC protocol includes Timothy grass allergic rhinitic patients and normal non-atopic controls only.

Natural Allergen Exposure Study Design

Patient recruitment

Subjects meeting the inclusion and exclusion criteria (see above) were considered for admission to the trial. In brief, we recruited participants with a history of seasonal allergic rhinoconjunctivitis (May-July) of at least 2 years duration and a positive skin prick test to Timothy grass. All participants were either mono-allergic to Timothy grass or allergic to Timothy grass and HDM only. Allergy to cats and dogs was permitted only if not a pet owner and not in contact with the animal responsible for atopy. For perennial rhinitis sufferers, we recruited perennial symptoms sufferers to perennial allergens such as house dust mite. Nasal polyp sufferers were identified from operative lists and samples were obtained intraoperatively. We excluded all potential participants who had any other systemic pathology which may compromise their health and/or the results of the study. Mild to moderate asthma was permitted. Recruitment was via local poster advertising, newspaper and other print media both from the public domain as well as in house Royal Brompton Hospital and Imperial College staff advertising.

Protocol

Visits for Seasonal Allergic Rhinitis Sufferers

Each subject was required to make 3 separate visits to the clinical research laboratory at the Royal Brompton Hospital (see figure 2.1). During the first visit, the potential participant underwent screening, received an explanation of the study and was offered the opportunity to consider their inclusion and ask questions. For eligible and willing participants, second and third visits were scheduled. The second visit was out of the hay fever season, with the third visit falling 4 weeks after the commencement of the season, running through the 3-week window just after the point when pollen counts are traditionally at their highest, i.e., end of May. At these 2 visits, nasal biopsy samples and nasal secretion samples were obtained.

Visits for Perennial Allergic Rhinitis Sufferers:

Following a screening and enrolment visit, eligible participants were called back for a single biopsy visit out of the hay fever season with all biopsies taking place November to January.

Nasal Polyp patients:

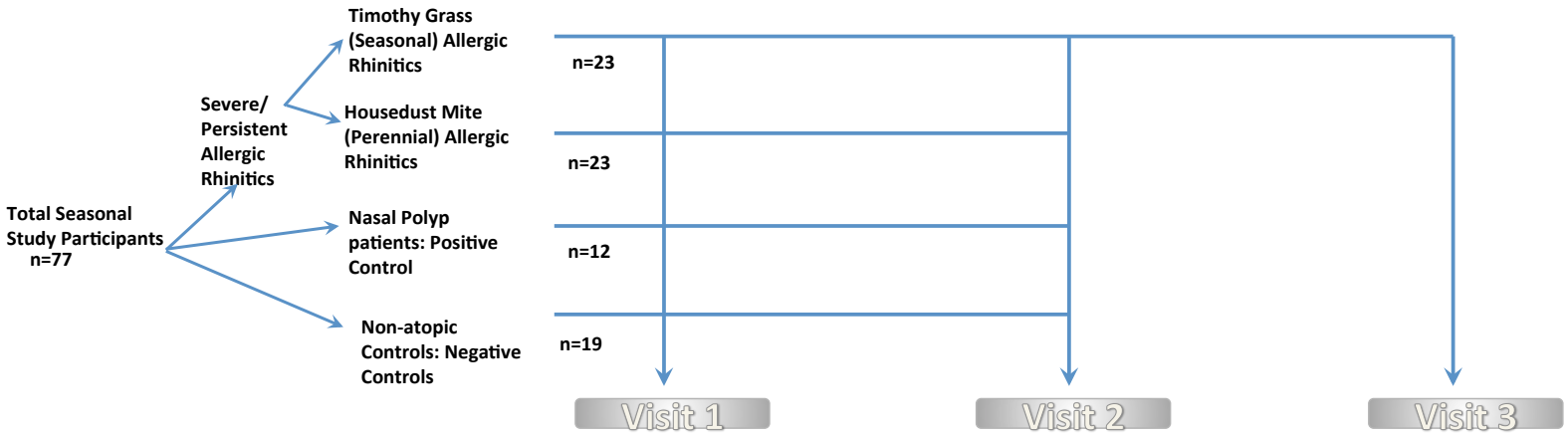
Nasal polyp participants were identified from routine ENT surgical lists at an allied sister hospital. Patients were initially contacted by phone to discuss the study and provisional enrolment. If suitable and willing to participate, a phone screening was carried out and this was revised on their admission when skin prick testing was carried out. We recruited patients undergoing surgery as part of their normal surgical care for their polyp disease. Recruitment of polyp participants was carried out at the same time as the other nasal biopsies. A small piece of tissue from their polyp and turbinate was used for our study. These were tissues that would have normally been discarded as part of routine surgery.

Normal Controls:

Following a screening visit, eligible participants were called back for a single biopsy visit out of the season.

All participants enrolled in the study completed the relevant protocol.

Table 2.1: Natural Allergen Exposure Study design



	Screening	Out of season	In season
History & Examination	√		
Skin Prick testing	√		
IgE/ RAST	√		
Quality of Life: TNSS/ VAS/ RQOL		√	√
Nasal secretions for cytokines		√	√
Nasal Biopsies		√	√

Repetitive Allergen Challenge Protocol

The Repetitive Allergen Challenge (RAC) protocol included ten allergic rhinitis participants and 16 normal controls.

Non-Atopic Healthy Volunteers

Non-allergic, non-atopic normal control participants attended the clinic four times out of the hay fever season. Each participant underwent a diluent challenge first, followed by an allergen challenge at least 4 weeks later. Twenty-four hours after each challenge, participants had a nasal biopsy. Only one side of the nose was utilised at each biopsy visit, although both sides were challenged at each challenge visit.

Allergic Rhinitics

Allergic rhinitis participants attended the clinic 15 times over the course of 8 weeks. A screening visit established eligibility and willingness to take part. If enrolled, a participant had 7 visits for the diluent phase and 7 visits for the allergen challenge phase of the study. For each phase, 5 visits were for the challenge (diluent or allergen) and 2 were for nasal biopsy. The biopsies took place 24 hours after the first and the last challenge in both phases (Table 2.1).

We used participant questionnaires during both phases to monitor the severity of nasal symptoms. Each participant completed the questionnaires at regular intervals for 24 hours prior to, and for 24 hours after each challenge. Blood samples and nasal secretions were taken to determine markers of the allergic response immediately prior to the nasal biopsies.

Randomisation of Biopsy Location

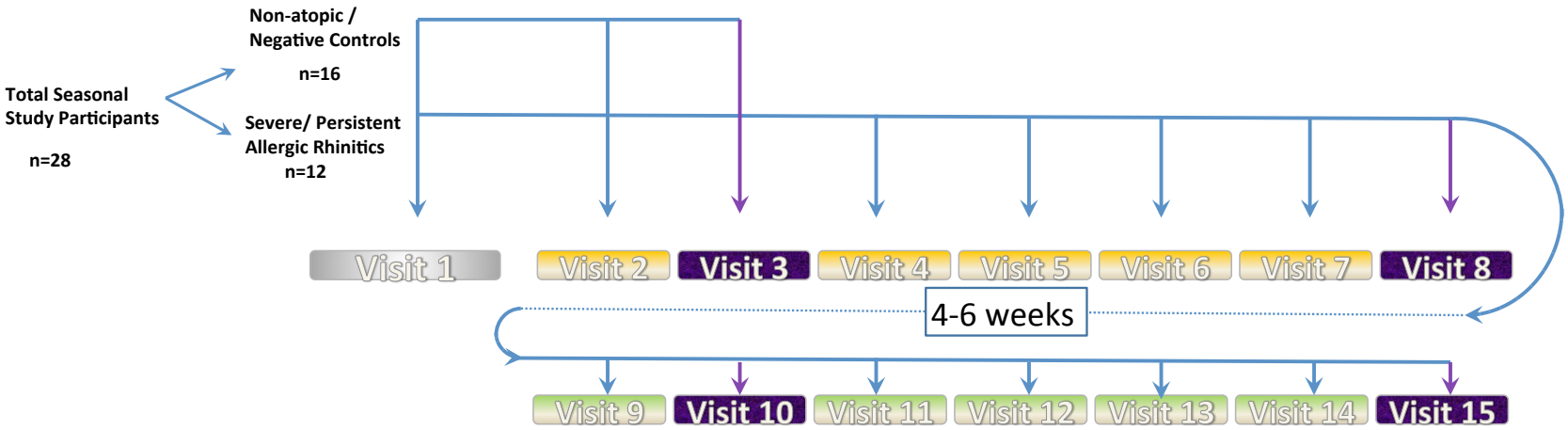
Each side of the nose was randomly designated as either side 1 or side 2. This was determined on the first diluent challenge visit by 'flip of a coin'.

Table 2.2:Diluent Challenge Phase: biopsy visits (visits 2 and 7) are in highlighted in red.

Visit 1	**Visit 2**	**Visit 3**	**Visit 4**	**Visit 5**	**Visit6**	**Visit 7**
Diluent challenge 1	Biopsy 1 (at 24 hrs)	Diluent challenge 2	Diluent challenge 3	Diluent challenge 4	Diluent challenge 5	Biopsy 2 (at 24 hrs)
Side 1	Side 1	Side 2	Side 2	Side 2	Side 2	Side 2

Table 2.3: Allergen Challenge Phase: biopsy visits (visits 2 and 7) are in highlighted in red

Visit 1	**Visit 2**	**Visit 3**	**Visit 4**	**Visit 5**	**Visit6**	**Visit 7**
Allergen challenge 1	Biopsy 1 (at 24 hrs)	Allergen challenge 2	Allergen challenge 3	Allergen challenge 4	Allergen challenge 5	Biopsy 2 (at 24 hrs)
Side 1	Side 1	Side 2	Side 2	Side 2	Side 2	Side 2



	Screening	Challenge	Biopsy	Challenge	Challenge	Challenge	Challenge	Biopsy
History & Examination	√							
Skin Prick testing	√							
IgE/ RAST	√							
Diluent/ Allergen Challenge		√		√	√	√	√	
Clinical response to challenge: sneeze, secretion		√		√	√	√	√	
Quality of Life: TNSS/ VAS/ RQOL		√		√	√	√	√	
Nasal secretions for cytokines			√					√
Nasal Biopsies			√					√
Clinical outcome post biopsy: pain, recovery			√					√

Table 2.4: Repetitive Allergen Challenge Study design

Clinical methods

Skin prick tests

Skin prick testing (SPT) was performed using a sterile stainless-steel lancet to introduce the allergen on the volar aspect of the forearm. Allergen extracts (Soluprick ALK Abello, Denmark) to common inhaled allergens were tested (Timothy grass *Phleum Pratense* extract, mixed grass pollen, birch pollen, mixed tree, cat hair, dog hair, horse, house dust mite (*Dermatophagoides pteronyssinus*), mixed weeds, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Alternaria alternata*, Soluprick at 10 HEP strength in 2ml). Histamine (Soluprick ALK Abello at 10mg/ml) acted as positive control and normal saline diluent as negative control.

Nasal allergen challenge

Nasal allergen challenge was performed during the RAC protocol only. Previous studies have indicated 320BU is sufficient to provoke an immediate and a delayed allergic response (574). Timothy grass extract (100, 000BU in 5ml) was diluted to twentyfold to provide 10,000BU in 10mls. The use of an atomiser applicator (Bidose/ Dolphin, Valois) dispensing 0.23mls per puff delivers 230BU per puff. After randomly assigning the first side of nasal biopsy, and blowing the nose to clear any secretions, the designated side was challenged with two puffs in quick succession in the first instance and with a single puff thereafter until clinical response threshold was surpassed. Clinical response of sneezing and anterior rhinorrhoea was recorded, and positive clinical threshold was reached with 5 sneezes and 30g of nasal secretions, which were captured in tissue and weighed. Immediately post response, and up to 24 hours post challenge, nasal symptoms were recorded using a visual analogue scale VAS (for itching, runniness, congestion, sneezing) and total nasal symptom scoring (TNSS) quantifying each of the above four symptoms.

Nasal diluent challenge

Nasal diluent challenge was performed during the RAC protocol only. Using the Bidose nasal challenge atomiser, 2 puffs of diluent were administered to the designated side. Number of sneezes and nasal secretions were recorded immediately post challenge. As above, clinical response using VAS and TNSS was recorded for 24 hours post challenge.

Symptom scoring of clinical response

Three methods of symptom scoring were used during the study: a visual analogue scale (VAS), a nasal symptom score (NSS), and the mini rhinitis quality of life questionnaire (miniRQLQ). The VAS required a mark to be placed along a 0-100 mm scale to indicate severity of four nasal symptoms: congestion

(blockage), anterior rhinorrhoea (runniness), itching and sneezing. The NSS assigned a number 0-3 to indicate the overall severity of these identical four symptoms. The miniRQLQ ascribed a score of 0-6 across 14 items in five domains: activity limitation (3 items), practical problems (2 items), nose symptoms (3 items), eye symptoms (3 items) and other symptoms (3 items). In all instances higher values indicated worsening symptom severity.

Symptoms scoring during Natural Allergen Challenge protocol

Nasal Symptom Scores (NSS), VAS of 0-100mm, and mini rhinitis quality of life (RQOL) questionnaires (575), establishing severity of nasal symptoms for the week preceding participants' visit, were completed at each biopsy visit.

Symptoms scoring during Repetitive Allergen Challenge protocol

Nasal symptom scores and visual analogue scale scores were collected for 24 hours after each (allergen and diluent) challenge visit. Post biopsy questionnaires examining six aspects of recovery were completed after each of the four biopsy visits. These six fields were: degree of pain at time of biopsy, degree of pain for 24 hours following the biopsy, nasal blockage, anterior rhinorrhoea, time until peak of discomfort, and time to total recovery in days.

Collection of nasal secretions

Prior to the collection of nasal secretions and biopsy the selected nasal cavity was first irrigated using 10mls of normal saline drawn up into a 10ml syringe. An 'olive' at the end of the syringe was used to occlude the nares. An 'olive', shaped like its namesake, is a custom made solid, stainless steel device, cored out such that it allows the end of a syringe at one side and occludes the nares at the other. These were built in-house at the Royal Brompton Hospital and were made in three different sizes allowing for differences in body habitus and nose size. With the head in a slight chin-tuck position and the neck flexed (576), whilst occluding the contralateral side by pressing on the alar, participants were asked to breathe through the mouth to elevate the soft palate and prevent the irrigation fluid from escaping the nasopharynx. Ten millilitres of normal saline were gently flushed and withdrawn three times with any residual fluid allowed to drip out. Although, not formally documented, loss of irrigation fluid was minimal. Participants were asked not to blow the nose. Nasal lavage fluid was discarded and nasosorption carried out using synthetic absorptive material (SAM). (577, 578)

Wearing non-latex gloves Accuwik filter papers (579) (Accuwik Ultra Membrane paper, Pall Corporation) were cut into uniform 30mm by 7mm strips. Under direct vision, two strips of Accuwik filter paper were placed, with their absorbent surfaces in direct contact with the medial surface of the

inferior turbinate and the lateral surface of the nasal septum, such that the two filter papers were lying back-to-back. After two minutes, using crocodile forceps, the filter papers were removed and placed into 1 ml solution of Beadlyte (Beadlyte, Upstate Cell Signalling Solutions, New York) and centrifuged for 30 seconds. The Accuwick filter papers were wrung out and removed, with the Beadlyte filled Eppendorf tubes frozen and subsequently stored at -80 °C. Nasal cytokines were analysed using MSD (MesoScale Discovery Th1/Th2 10plex, 96 well, Mesoscale Rockville, Maryland) multi-array human cytokine assay analyser.

Nasal biopsy

The side for nasal biopsy was pre-selected at random. Local anaesthesia was achieved using 'Co-phenlycaine' (5% lidocaine, 0.5% phenylephrine) administered via a pump aerosol. The biopsy site was then prepared with a vasoconstrictor (1-1.5ml of 10% cocaine) soaked onto 3 small cotton wool balls, each placed adjacent to the inferior turbinate. Ten minutes later, the cotton balls were removed, and 2.5 mm nasal biopsies taken from the inferior turbinate using cup-and-ring (Fokkens) biopsy forceps under direct vision. Haemostasis was achieved by 0.3 ml of 1/1000 adrenaline on a cotton swab applied to site of biopsy for 10 minutes, followed by cautery using a silver nitrate stick. Subjects were observed for 30 min before discharge. A supply of paracetamol was provided for any minor discomfort after loss of local anaesthetic effect. An information sheet with post-procedure epistaxis instructions and 24 hr contact telephone number was provided for advice in the event of complications.

Laboratory methods

Sample Processing

One biopsy was processed for immunohistochemistry by mounting on a card in OCT mounting media (ThermoShandon, Altrincham, UK) followed by snap-freezing in isopentane (VWR, Leicester, UK) pre-cooled in liquid nitrogen. A second biopsy was collected in phosphate buffered saline (Sigma Aldrich, Dorset, UK) and then prefixed in 4% paraformaldehyde for 10 minutes, followed by 2 washes in 15% sucrose (1st wash for 1 hour, 2nd wash overnight). All samples were then mounted in OCT and snap-frozen in isopentane pre-cooled in liquid nitrogen. Air-bubble free OCT mounting media was essential to ensure minimisation of artefactual damage. All samples were labelled both on the card and on its corresponding Bijoux container. All specimens were stored at -80^{o}C pending cutting for immunohistochemistry studies. 4μm biopsy sections were cut using a Bright Cryostat and placed three sections per Superfrost Plus microscope slide (VWR, Leicester, UK). Slides were dried overnight, then wrapped in aluminium foil, labelled and stored at -80 ^{o}C. These two different fixative methods were

used to optimise IHC techniques. For all antibodies, paraformaldehyde fixed sections were used unless otherwise specified.

Principles of immunohistochemistry

Immunohistochemistry is a technique which allows us to detect specific target antigens in tissue sections using antibodies which are directly or indirectly linked to a chromogen or fluorophore.

Three-step indirect method

Immunohistochemistry relies on the potentially strong and multiple binding capabilities of antibodies to reveal target antigens either through enzyme mediated chromogen deposition or fluorescence labelling. A primary antibody (normally raised in a different species to the tissue source) commonly mouse or rabbit, binds the antigen of interest. A secondary antibody directed against the species of the primary antibody is applied in excess. Thirdly, an enzyme conjugated antibody is added linking the resultant antibody/ antigen complex to an enzyme capable of releasing a coloured precipitate from its respective chromogen. The signal is amplified by the sandwich nature of this indirect antigen capture method, more than one antibody molecule can link to respective antigens at each step.

Soluble enzyme immune complex method

Three step antibody amplification systems allow for enhanced detection of tissue antigens. Systems are named according to the enzymatic reaction used. The alkaline phosphatase, anti-alkaline phosphate APAAP system was used throughout. Surplus secondary antibody was added, each antibody molecule had several Fab binding sites capable of binding both the primary antibody as well several tertiary antibody/ enzyme immune complexes. Further amplification was achieved by the presence of two enzyme molecules on each APAAP complex. The principle of detection was for the alkaline phosphatase enzyme to catalyse the conversion of its chromogen, Fast Red, to a red precipitate at the site of the antigen of interest. AP acts on its substrate naphthol-phosphate esters by hydrolysis. In alkaline conditions, end products of this reaction are phosphates and phenolic compounds, with the latter coupling to colourless diazonium salts (chromogen) to form coloured insoluble azo dyes. Fast Red was used throughout this study, resulting in a bright red final product. Another common chromogen system used with APAAP is the BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/ NBT (nitro blue tetrazolium chloride) compound. When BCIP is the substrate, NBT acts as an oxidant to form insoluble NBT formazan which is dark blue.

Avidin-biotin methods

Avidin biotin detection methods exploit the high affinity of the protein avidin for the vitamin biotin. Avidin is a 66-69kDa tetra or dimeric biotin binding protein with dissociation constant of Kd~ 10^{-15}M, one of the strongest non-covalent bonds. With up to four sites for binding, two will bind biotin which is also known as co-enzyme R or water-soluble vitamin B_7. ABC-AP kits (Vector Laboratories, Peterborough, UK) are designed to detect antibodies from a particular species allowing them to be used in conjunction with any primary antibody of that species. The secondary antibody is biotinylated and binds to the tertiary layer, the avidin/biotinylated enzyme (alkaline phosphatase) complex. The avidin-biotin complex alkaline phosphatase (ABC-AP) system was used unless otherwise stated.

Staining protocol

Normal human serum at 10% (HS) was used for any slide washes intended to reduce non-specific binding. All other washes were performed using phosphate buffered saline (PBS) unless stated otherwise. All extracellular markers were incubated for 1 hour, with all intracellular markers left overnight. Following retrieval from -80°C, slides were unwrapped in small batches to prevent condensation that may otherwise wet and destroy sections. Slides were kept at room temperature for 10 minutes before each staining run. All incubations were performed at room temperature unless stated otherwise. Clinical details were left blinded until all cell counts were complete.

Antibody dilutions were prepared in 10% HS unless otherwise specified. Optimal dilutions were determined in optimisation staining runs. Incubation with 20% Horse serum for 30 minutes prior to primary antibody application ameliorated non-specific binding. Primary antibodies against extracellular antigens were applied to tissue sections and incubated for 1 hour at room temperature whilst antibodies against intracellular antigens were left to incubate overnight at 4°C. Blocking of endogenous alkaline phosphatase activity was achieved by use of Levamisole (Vector Laboratories, SP-5000), as part of ABC-AP system (both Vector Laboratories, Peterborough, UK). Depending on the species in which the primary antibody was raised, an ABC-AP mouse or rabbit VectaStain kit was used. Substrate chromogen was Fast Red in all instances. Harris haematoxylin (Sigma-Aldrich, St. Louis, MO) was used to counterstain. All runs included a positive (eosinophil) and negative control (mouse or primary antibody species raised against IgG1 isotype control [eBioscience, 14-4714). Slides were kept in water troughs until ready for mounting to prevent drying out. Slides were coverslipped with Glycergel mounting media (Dako C0563). Fast Red is alcohol soluble hence use of aqueous mounting media.

Enumeration of inflammatory cells

Detection of monoclonal antibodies

Inflammatory cells were detected using the APAAP technique outlined above. The mouse anti-human monoclonal antibodies used were: BMK13 (MBP major basic protein) at 1:100 dilution (for eosinophils, Santa Cruz SC18241), AA1 at 1:80 (for mast cell tryptase, DAKO M7052), Neutrophil elastase at 1:100 (for neutrophils, Dako M0752), 2D7 at 1:50 (for basophils, Biolegend 346201), CD68 at 1:50 (for macrophages, Dako, M0876), CD3 at 1:100 (for T cells, Dako M7254), CD4 at 1:20 (for T helper cells, Dako M0716), and CD8 at 1:10 (for cytotoxic T cells, Dako M7103). After defrosting in small batches, taking care the slides did not get wet, the primary mouse antibody diluted in PBS was incubated for one hour in a humidity chamber. Two 5-minute washes in PBS were then followed by incubation with a secondary antibody, rabbit anti-mouse (Dako, Z0259) diluted to 1:30 in 10% normal human serum for 30 minutes. This again was followed by two 5 minutes washes in PBS. The tertiary mouse APAAP conjugated antibody (Dako, D651) 1:30 in 10% HS in PBS, was added and incubated for a further 30 minutes. A third cycle of 2 washes of 5 minutes followed. Fast Red (Sigma F4523) was applied for 20 minutes and slides were then washed in tap water to terminate the reaction. Sections were counterstained with Harris Haematoxylin for 5 seconds and serial washes until clear. Slides were coverslipped with Glycergel (Dako, C563).

Enumeration of remodelling markers

Detection of antibodies

Remodelling markers were identified by the following mouse monoclonal antibodies: heat shock protein-47 at 1:800 dilution (Stressgen, SPA 470), smooth muscle actin at 1:800 (Dako, M0851), MMP7 at 1:20 (AbCam, Ab 3205), MMP9 at 1:50 (AbCam, Ab51203), TIMP1 at 1:50 (AbCam, Ab1827-250), CD31 at 1:30 (for blood vessels, Dako, M0823), D2-40 at 1:150 (for lymphatic vessels, Dako M3619), VEGF at 1:75(AbCam, Ab 1316), TGF-β at 1:400 (R&D, MAB 1835), , ALK-4 at 1:100 (GeneTex, GTX 10594). All primary antibodies were diluted in 5% HS/ 0.1% saponin/ PBS solution, and incubated overnight. Washes in 0.1% saponin/ PBS were followed by incubation with biotinylated rabbit anti-mouse secondary antibody diluted to 1:30 (in 0.1% saponin/ PBS) and incubated at room temperature for 30 minutes. Further washes in 0.1% saponin/ PBS were then followed by Vectastain ABC-AP complex according to manufacturer's instructions, incubated for 30 minutes before washing. Activin was detected by a goat primary polyclonal antibody at 1:80 (R&D, MAB 3381), with horse anti-goat biotinylated secondary antibody, and goat Vectastain-ABC-AP kit. pSMAD at 1:200 (Santa Cruz, CS-11769) was a rabbit primary antibody detected by goat anti-rabbit biotinylated secondary antibody and the appropriate rabbit Vectastain-ABC-AP kit. Fast Red was used as chromogen for all the

remodelling markers, and Harris Haematoxylin was the counterstain. Positive cell counts were expressed per mm^2. TIMP-1 specific staining of submucosal glands was subjectively quantified using a scale 0-5. On this scale: 0=no staining, 1=just visible staining, 2=weak staining, 3=moderate staining, 4=very obvious and good staining, 5= strong staining throughout section.

Enumeration of nasal cytokines

Nasal cytokines were quantified using a Meso Scale Discovery device (MSD, Rockville, MD) with a human TH1/TH2 10-Plex Base Kit. MSD technology uses electrochemiluminescence to quantify nasal cytokines via a multiplex plate capable of measuring ten cytokines per sample well. All runs were as per manufacturer's instructions. Reagents were brought to room temperature. Known quanta of each of the ten cytokines, via 7 lots of four-fold serial dilutions, were used to establish calibration curves for each analyte. This standard curve was included on each analyte filled plate to ensure accuracy of detection. First, 25µl of human cytokine assay diluent was added to each well with a gentle tap to release any bubbles from the bottom. The plates were sealed with adhesive seal and incubated for 30 minutes on an agitator at 800rpm. Calibrator fluid or samples were then pipetted onto the plate, which was sealed and incubated for a further 2 hours with vigorous shaking at 800rpm. Plates were then washed three times with PBS / 0.05% Tween-20. Detection antibody solution at 25µl was added to each well. Plates were again sealed and incubated for 2 hours under agitation at 800rpm.

Samples were analysed with SECTOR Imager 2400 (MSD, Rockville, MD) which counts units of light measurements via a charge-coupled device (CCD) camera. The lowest threshold, or noise floor is 30 counts for a 10 spot 96 well plate with the highest signal value being 1-2 million counts. The camera system measurements were linear over a 6 log-dynamic range between these two extremes. The generation of a standard curve utilized a least squares fitting algorithm. The software used a sigmoidal dose response (4-parameter logistic model) with 1/y2 weighting function as per manufacturer's instructions. Each assay was performed in duplicate and the results averaged using a total of 50µl of each sample.

Reverse pipetting was used throughout to avoid introduction of air bubbles. All reagents used during this technique were MSD (MSD, Rockville, MD) unless otherwise specified. All incubations were carried out at room temperature.

Reticular basement membrane

Reticular basement membrane thickness (RBM) was determined using Puchtler's Picro-Sirius Red staining (580) which stains collagen III (and I) red visible by light microscopy. Slides were first immersed

in Celestin Blue (Sigma-Aldrich, St. Louis, MO) for 15 seconds. Ion salts in the dye penetrate and fix into the sections which are then rinsed gently under running tap water. Slides are then plunged gently into Harris Haematoxylin (Sigma-Aldrich, St. Louis, MO) for 15 seconds where the haematoxylin dye binds to the section fixed ion salts and thereby resists acid (581). Slides were rinsed under tap water until water from the wells ran pale blue. Sections were then incubated for 1 hour in Picro-Sirius Red after which they underwent two changes in acetic acid water with 8 dips in each change. A brief rinse in tap water was followed by serial dehydration (30 seconds in 70%, 90%, two times 100% IMS (Industrial Methylated Spirit) with gentle agitation). Finally, following three changes of Histoclear, 5 minutes each, slides were mounted with DPX (VWR International).

Measurements of basement membrane thickness were performed at 20μm intervals at right angle to the basement membrane with at least 20 measurements for each section (582). A computer-generated right angle was used to cross check observer determined line of measurement with final measurement expressed as an average per section.

Epithelial and submucous glands

The glycoprotein, mucin which is the primary product of glands in the airway, was stained using Periodic Acid-Schiff stain. Periodic acid oxidises glycogen by breaking carbon-to-carbon bonds not engaged in glycosidic linkage. Oxidisation needs to be controlled by the presence of free hydroxyl groups with the result of two aldehyde groups at either end of the cleaved monosaccharide ring. Addition of Schiff reagent alters the unstable colourless dialdehyde group into a magenta colour by returning the quinoid chromophoric grouping. Haematoxylin was used as a counterstain.

In brief, slides were first oxidised in 0.5% periodic acid solution (Sigma-Aldrich, St. Louis, MO) for 5 minutes. A rinse with distilled water was followed by 15 minutes in Schiff reagent (Sigma-Aldrich, St. Louis, MO). Slides were the washed for 5 minutes in lukewarm tap water. This was followed by Harris haematoxylin counterstain for 15 seconds and then gentle tap water washes until clear. Slides were then dehydrated through alcohol, cleared using Histoclear and mounted with DPX.

Epithelial integrity and mucus content

A reliable histological imaging modality used to assess epithelial thickness is optical coherence tomography, which relies on interferometry and low coherence optical sources, not dissimilar to ultrasound (583). Our choice of technique was guided by reliability, reproducibility and ease, with epithelial mucus content, mucous staining intensity and epithelial integrity quantified using periodic acid-Schiff (PAS) stained slides. First, the whole section was captured at x200 magnification using Nikon

80*i* light microscope coupled to colour camera (QImaging, Media Cybernetics, Inc). Images were saved and stored on a coupled hard drive. Saved images could be magnified on screen to identify histological structures. PAS staining captured epithelial and submucosal glands (serous, mucous and sero-mucinous). This total gland related area was expressed as a percentage of total submucosal area and included: total area of the gland itself, total area of gland content, and the sum of the two to give total glandular area. Epithelial integrity was assessed by light microscopy using a BH-2 Olympus microscope. A scale of 0-5 was used to subjectively quantify epithelial shedding with grading system of: 0= no epithelial cells, basal lamina only; 1=single epithelial cell layer; 2=severely damaged but more than two cell layered epithelium; 3=moderately damaged but mostly intact epithelium; 4=mildly damaged with most of epithelium intact; 5=intact epithelium. A similar scale of 0-5 was used to subjectively quantify intra-glandular epithelial mucus staining. On this scale: 0=no staining, 1=just visible staining; 2=weak staining; 3=moderate staining; 4=very obvious and good staining; 5= strong staining throughout section.

Cell counting

Observers counted all sections blind to clinical status. Cells were counted throughout the epithelium and divided by the total epithelial surface area. Submucosal cells were counted to a depth of 200μm from the basement membrane. In all instances, cells were counted at x200 magnification. Epithelial sections were counted using computer assisted image analysis (Image Pro-Plus 7.0 system, Media Cybernetics, Inc) with Nikon 80*i* light microscope coupled to a colour camera (QImaging, Media Cybernetics, Inc). Submucosal cells were counted by light microscope using a square eyepiece graticule (0.202mm^2 at 200x magnification), one grid depth, (Olympic Corp., Lake Success, NY, USA). Final cell counts are expressed as cells/mm^2.

Statistical analysis

Cell counts are expressed as scatter plots with median and interquartile range (whiskers). Small scale discreet value measurements are shown with median and interquartile ranges (box) and minimum and maximum values (whisker) plots. Paired within subject data was analysed using Wilcoxon signed rank test. Mann-Whitney U test was used for all unpaired data. ANOVA test was used for multivariate analyses. Correlation of cell counts used Spearman rank order method. Area Under the Curve method was used to analyse clinical response to allergen and diluent challenge. All data was analysed using Graph Pad Prism software (GraphPad Prism Software Inc, San Diego, CA). For all data, significance level was taken as $p<0.05$.

Validation of Method for cell counts in the nasal epithelium and submucosa.

To validate accuracy of investigator cell counts versus experienced and independent cell counter, sections stained for eosinophils following allergen challenge were used to establish intra and inter-observer reliability at different x20 and x40 magnifications. High (submucosal) and low (epithelial) cell counts were performed and analysed for correlation and plotted on a Bland-Altman plot.

Epithelial and submucosal cell counts show high correlation with intra- and inter-observer counts and at x20 versus x40 magnification. Bland-Altman plots demonstrate bias of 4.2 intra- and 13.3 inter-observer variability at low cell counts, and 0.9 intra- and 13.3 and inter-observer variability at high submucosal counts. Effect of field magnification on bias was negligible on low epithelial counts (bias=0.7) but increased at high submucosal counts (bias=21.4). Cell counts of 50 and under show very little difference between observers. Deviation of observations from the mean difference increases at higher cell counts but does not demonstrate a trend for either higher or lower counting with symmetrical spread around the mean.

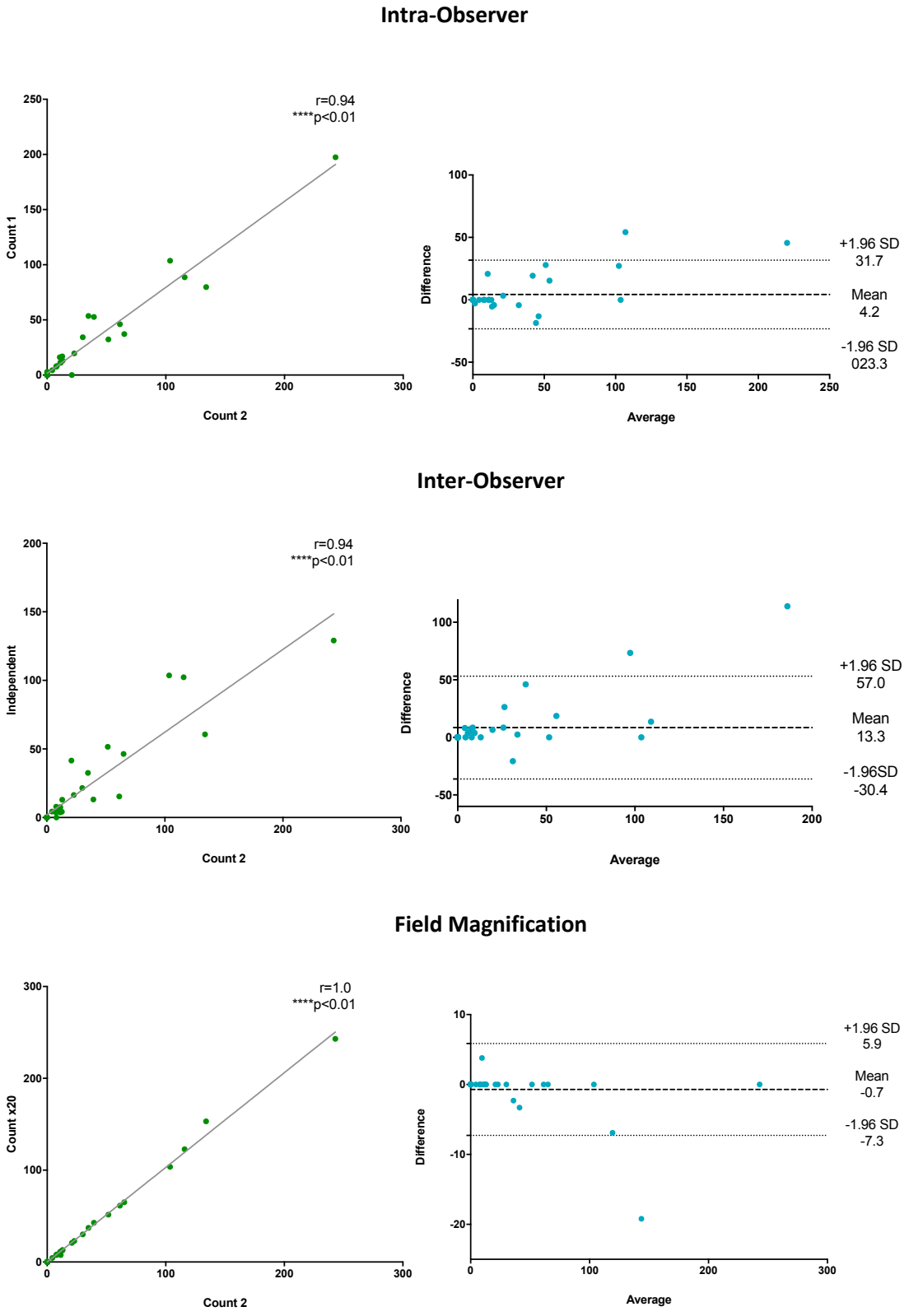
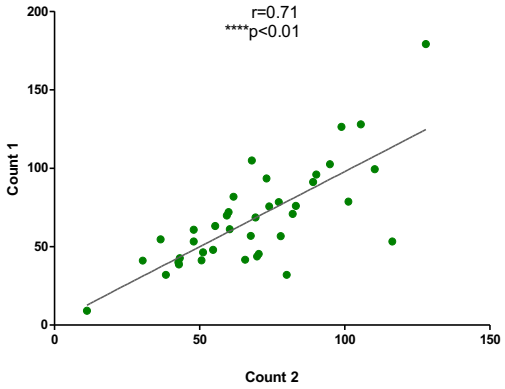
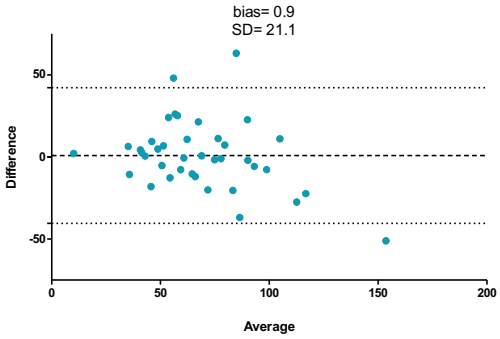


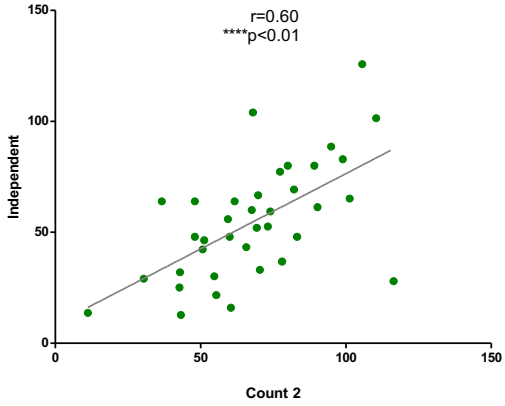
Figure 2.1: Epithelial Cell Count Reliability and Reproducibility graphs. Left column shows correlation, with Spearman rank coefficient. Right column shows Bland-Altman plots with bias and 1xSD.

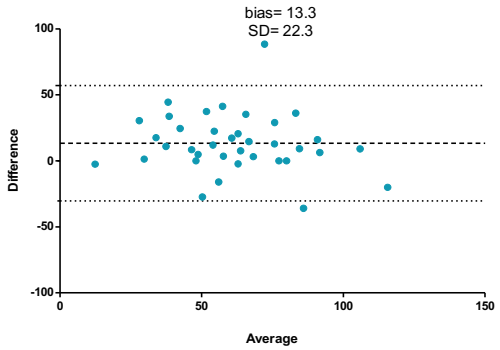
Intra-Observer



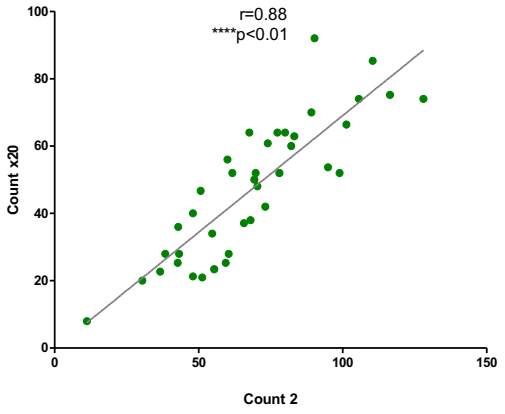


Inter-Observer





Field Magnification



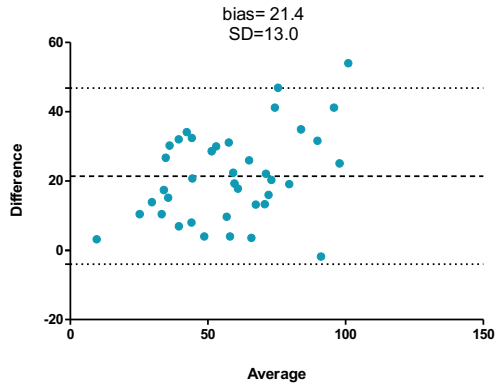


Figure 2.2: Submucosal Cell Counts Reliability and Reproducibility graphs. Left column shows correlation, with Spearman rank coefficient. Right column shows Bland-Altman plots with bias and 1xSD

Chapter 3 : Clinical characteristics of participants during natural allergen exposure and following repetitive allergen challenge

Introduction

Natural allergen exposure, for both seasonal and perennial allergic rhinitis, forms the gold standard for investigative study however the seasonal variability of pollen counts means standardisation is difficult. Depending on the affected organ(s) allergen provocation challenge with known allergen concentrations is the primary method of replicating the season. Allergen challenge may vary in site: conjunctival, nasal or bronchial. Environmental exposure chamber will offer an alternative but is expensive and only available to a small number of centres. A cheap, reliable and reproducible allergen challenge model able to demonstrate early and late phase reactions to known allergen means opportunity to perform multiple challenges.

Studies into artificial reproduction of the pollen season have included variable dosing and biopsy timings, with relative paucity of such findings in human models of the disease. This study is unique in quantifying clinical and immunological characteristics of both seasonal and perennial allergic rhinitics. All participants were characterised by ARIA classification as severe/ persistent regardless of allergen and were matched in all other demographic aspects. Seasonal rhinitics were studied in and out of season. Normal controls were non-rhinitic, non-atopic individuals and positive controls were polyp samples of chronic rhinosinusitis patients with nasal polyps (CRSwNP).

This chapter describes the clinical changes observed amongst the study group of severe/ persistent rhinitics during the natural seasonal limb of this study and the repetitive allergen challenge limb. Patient demographics for each study group are described. Clinical symptom scoring via the total nasal symptom score (TNSS), visual analogue score (VAS) and quality of life (QoL) was carried out for both the natural and induced models of allergic rhinitis and presented in figures 3.3-3.6. In the induced model, symptom scoring was subdivided into the early and the late phase response and plotted against normal non-atopic controls with diluent and allergen challenges.

During natural models of allergic rhinitis, seasonal allergic rhinitis participants were sampled immediately after the highest pollen count peak with those sensitive to perennial allergens biopsied whilst symptomatic out of the pollen season. Clinical responses show statistically significant changes in TNSS, VAS and QoL. The repetitive allergen challenge model clinically demonstrated priming of the early phase reaction at day 5 and of the late phase reaction at day 11. Non-atopic, non-rhinitic

participants represented a negative control population for the effects of pollen, and in the absence of sensitisation, control for the effects of diurnal variation on the diluent day biopsies of allergic rhinitics.

In the repetitive allergen challenge (RAC) arm of the study, incremental doses of allergen were used to define individual thresholds

Results

Participants enrolled in the 'natural model' arm of the study were biopsied in June and July over the period of two years (Figure 3.1). Pollen counts were obtained from the London Met Office, North Islington Site. Peaks in pollen counts varied from mid-June to late July. To allow an allergic phenotype to develop prior to biopsy, participants were seen after this initial 2-week period, but when pollen counts were still high.

Our groups' collective previous experience indicates that 50 grains/m^3 is the requisite threshold for allergic rhinitic symptoms to develop. Historically peak pollen counts usually occur during the first two weeks of June. In this study, during the first year of biopsies, pollen counts increased early, the last week of May, and reached their peak in the first week of June. Counts then dropped until mid to late June but were still higher than 30 grains /m^3 and for most of the time, higher than the 50 grains /m^3 needed for marked allergic symptoms to develop and peaked again during the first half of July. This was due to a period of frequent rain giving rise to an extended growing season. Biopsies were taken after the first peak and during the second, and stopped when pollen counts dropped to below 30 grains/m^3.

The highest weekly pollen count in Year 1 was 124 grains/m^3 compared to a high of 170 grains/m^3 in Year 2, in addition counts remained high for longer in Year 2 following the peak. Year 2 biopsies were performed immediately after the peak, from the middle of June onwards and stopped at the end of July when pollen counts dropped to below 20 grains/m^3.

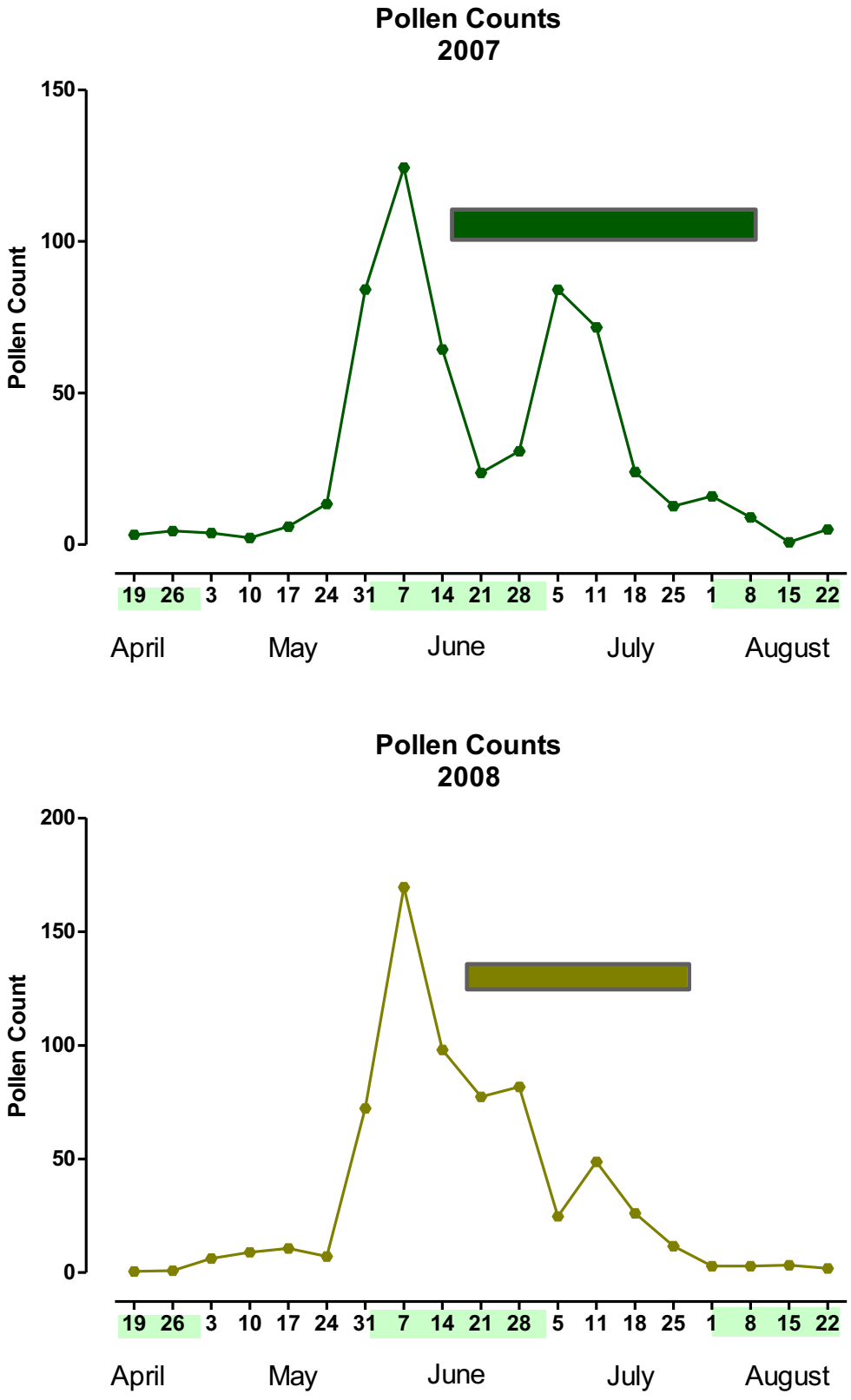


Figure 3.1: Pollen counts for 2007 (top) and 2008 (bottom) with weekly averages. Pollen counts were obtained from the London Met Office, North Islington site. Timing of biopsies is represented by solid bars

Patient demographics demonstrate that in both arms of the study, the two groups of non-atopic individuals and allergic rhinitics, were well matched for age and gender.

Non-atopic normal controls showed low total IgE levels with near zero titres of specific IgE to house dust mite and timothy grass. Seasonal allergic rhinitics in the natural allergen exposure arm of the study (pale red column) demonstrated high specific IgE to grass and very low IgE to house dust mite. Indeed, only two participants demonstrated above zero titres to house dust mite. These findings were reproduced on skin prick testing with marked responses to Timothy grass but very low (6 mm^2) to house dust mite. The perennial participants in the same natural allergen exposure arm (middle red column) showed increased specific IgE to both house dust mite and grass with very high total IgE level of 538 UI/ml. Skin prick test results mirrored IgE levels, with high average weal size of 42 mm^2 and 34 mm^2 to Timothy grass and house dust mite respectively. The combined group of seasonal and perennial allergic rhinitics in the natural allergen exposure arm of the study (dark red column under allergic rhinitis) had a high weal size for both grass (44 mm^2) and house dust mite (20 mm^2) with high specific IgE levels of 45 IU/ml and 25 IU/ml respectively.

The repetitive allergen challenge arm of the study (purple columns) included non-atopic controls (left purple column) with no positive skin prick test weals or specific IgE to both Timothy grass and house dust mite. The severe/ persistent allergic rhinitics (right purple column) included participants with no skin response or specific IgE to house dust mite, and high skin response (54 mm^2) and specific IgE (17 IU/ml) to Timothy grass.

We aimed to exclude smokers from all studies, but this was not entirely possible, and each study group had either 1 or 2 participants who were active smokers. Eleven percent of normal control participants in the natural allergen exposure arm, and none in the repetitive allergen challenge arm were asthmatic. Of the allergic rhinitics, 7% in the natural exposure arm and none in the repetitive allergen challenge arm (middle grouped columns) were asthmatic. Non atopic controls had low rates of atopy with 2% having food allergy, and 11% eczema in the natural allergen challenge limb, and 6% having urticaria and a further 5% having eczema in the repetitive allergen challenge arm (left grouped columns). Seven percent of total severe/ persistent allergic rhinitics in the natural allergen arm (dark red column) were smokers, 28% had asthma, 24% a food allergy, 33% urticaria and 22% had eczema. In the repetitive allergen challenge group (right purple column) there were no smokers or participants with urticaria, but 20% had asthma and 10% a food allergy or eczema.

A total of 11 nasal polyp patients were recruited. All 11 provided polyp biopsies with 10 providing turbinate biopsies. Recruitment of participants was a challenge as they needed to be off their topical

and systemic steroid treatment for at least 4 weeks prior to their biopsy. Furthermore, they needed to be identified prior to their surgery date posing logistical challenges of capturing these participants at the right time of their patient journey. Nasal polyposis participants showed almost completely absent skin responses and specific IgE levels, with total IgE of 74 IU/ml. None suffered co-existent eczema, but 13% were smokers, 38% were asthmatic (the highest in any group), 25% had a food allergy, and 13% had urticaria.

Table 3.1: Clinical Demographics- Non-atopic normal controls (left grouped columns), Severe/ Persistent Allergic Rhinitis (middle grouped columns) and nasal polyposis patients (right/ green columns), divided into natural allergen exposure (red), and repetitive allergen challenge (purple). The RAC group is further split into seasonal (pale red), perennial (red) and combined (deep red) groups.

	Non-atopic Controls		Allergic Rhinitics				Nasal Polyposis
	NAE n=19	**RAC n=16**	**NAE (SAR) n=23**	**NAE (PAR) n=23**	**NAE n=46**	**RAC n=10**	**n=8**
Age	30 (26-45)	29 (24.3-42)	30 (20-54)	34 (21-56)	30 (20-56)	27 (23.5-32.5)	30.5 (23.3-40.3)
Male: Female	11:8	8:4	12:11	10:13	22:24	6:10	6:2
spIgE Timothy Grass	0.01 (0.01-0.01)	0 (0-0.01)	60 (11-100)	29 (0-100)	45 (0-100)	17.1 (6.8-44.3)	<0.01 (0-0.12)
spIgE House Dust Mite	0.02 (0.01-0.06)	0 (0-0.03)	3 (0-16)	32 (0-89)	25 (0-16.7)	0 (0-0.02)	0 (0-0.02)
Total IgE	15 (4-49)	9 (3-44.5)	140 (3-760)	538 (16-1765)	335 (3-1765)	78 (34-175)	74 (10.75-602)
SPT: Timothy Grass	0 (0-0)	0 (0-0)	48 (6-183)	42 (0-200)	44 (6-200)	53.5 (33.5-70)	0 (0-4.5)
SPT: House Dust Mite	0 (0-0)	0 (0-0)	6 (0-47)	34 (0-70)	20 (0-70)	0 (0-0)	0 (0-0)
Comorbidity							
Smoker	1 (5%)	1 (6%)	2 (9%)	1 (4%)	3 (7%)	0 (0%)	1 (13%)
Asthmatic	2 (11%)	0 (0%)	7 (30%)	6 (26%)	13 (28%)	2 (20%)	3 (38%)
Food Allergy	2 (11%)	0 (0%)	5 (22%)	6 (26%)	11 (24%)	1 (10%)	2 (25%)
Urticaria	0 (0%)	1 (6%)	7 (30%)	8 (35%)	15 (33%)	0 (0%)	1 (13%)
Eczema	2 (11%)	1 (6%)	7 (30%)	3 (13%)	10 (22%)	1 (10%)	0 (0%)

Clinical symptoms were assessed using Quality of Life (QoL), Visual Analogue Scale (VAS) and Total Nasal Symptoms Scoring (TNSS) and are shown in figures 3.3 to 3.6. For each assessment type, top graph demonstrates natural allergen exposure study groups split into normal controls (far left), out of season Timothy grass allergic rhinitics (second column), in season Timothy grass allergic rhinitics (middle column), house dust mite allergic rhinitics outside of the pollen season (red columns), and nasal polyp positive controls (brown/ far right columns). The bottom graphs for each type of clinical assessment tool, show negative controls on the left, positive controls on the right, with the central column combining the in-season Timothy grass and the perennial house dust mite severe/ persistent allergic rhinitics.

For QoL, VAS and TNSS, grass allergic rhinitics were significantly more symptomatic in season than out of season and more symptomatic than their non-atopic counterparts. Nasal polyp sufferers were also significantly more symptomatic than normal controls but did not differ significantly from the amalgamated group of severe/ persistent rhinitics. Of the three assessment tools, TNSS yielded the highest p value for difference in clinical symptoms. Median values for TNSS, where the maximum is 12, were consistently higher in seasonal allergic rhinitis (9), nasal polyposis (8) and perennial rhinitis (4) than normal controls (0) and out of season allergic rhinitics (median=2). Figure 3.6 demonstrates TNSS data as a scatterplot to aid comparison with graphs in Chapters 4 and 5.

In the induced, repetitive allergen challenge model, nasal symptoms were monitored for 24 hours post challenge. Symptom severity increased with number of allergen challenges but did not change post diluent visits. Area under the curve analysis demonstrated priming of the early phase reaction (EPR) with increased total nasal symptom scores (TNSS) in the first 30 minutes when comparing day 1 and day 5 (i.e., visit 1 and visit 3) and day 1 and day 10 (i.e., visit 1 and visit 5). The late phase reaction (LPR) showed similar priming but this was evident between day 1 and day 10 (visit 1 and 5) only and not day 1 and day 5 (visit 1 and visit 3) Figures 3.7 and 3.8. No priming was evident with diluent challenges.

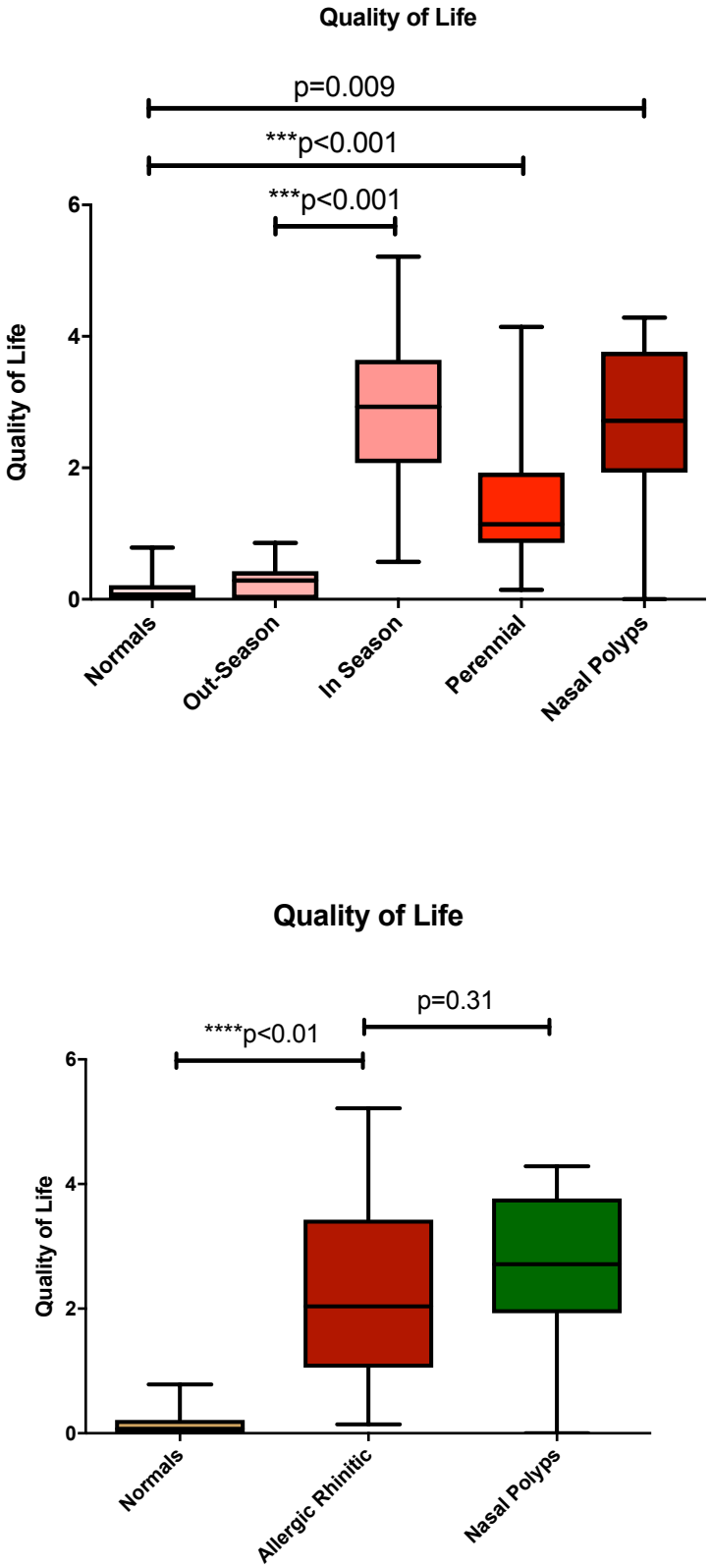


Figure 3.2:Quality of life (using mini RQLQ) at time of biopsy. Top graph demonstrates all subgroups. Bottom graph demonstrates grouped data. Significant differences were analysed using Mann-Whitney test.

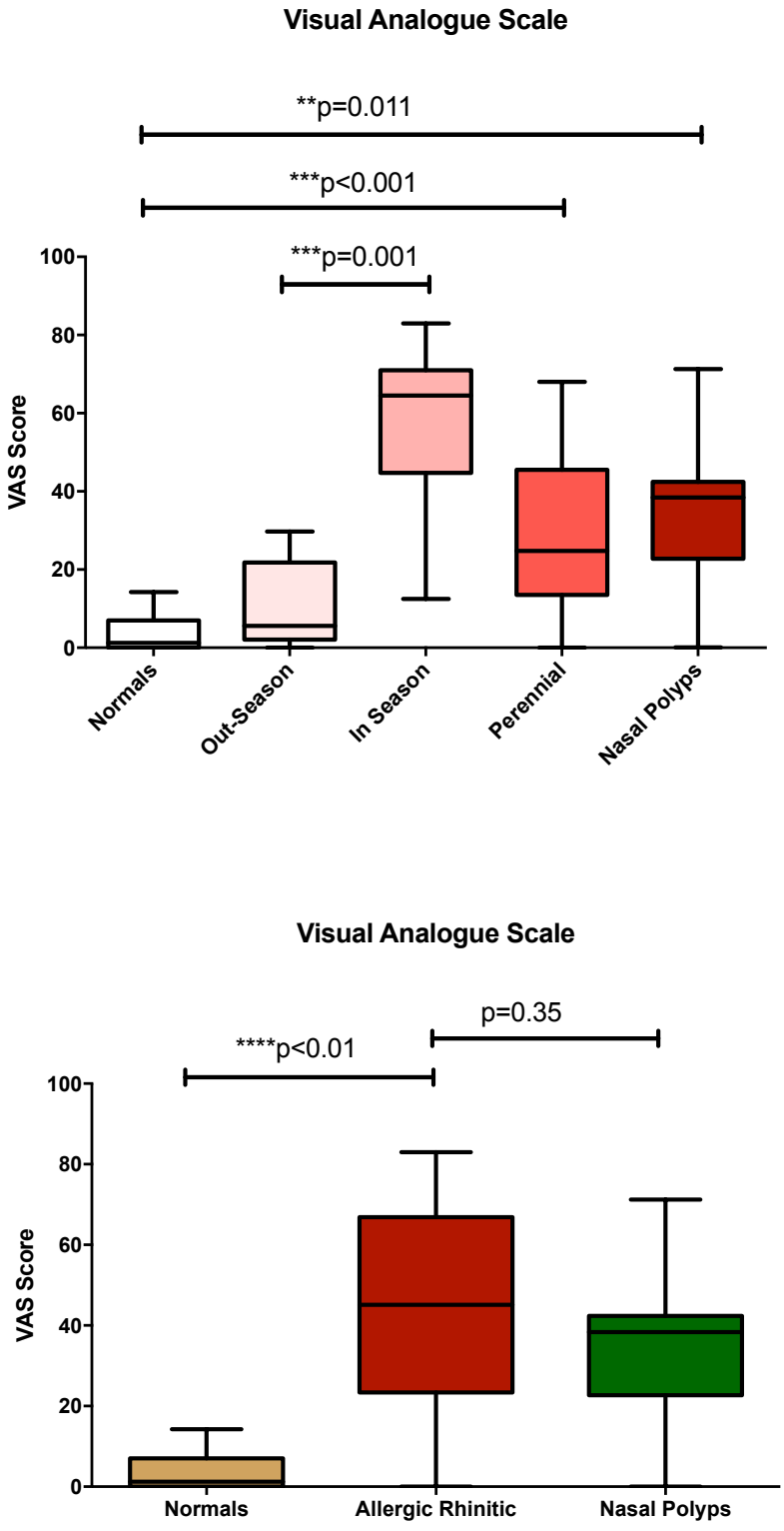


Figure 3.3: Visual Analogue Scale of nasal symptoms at time of biopsy. Top graph demonstrates all subgroups. Bottom graph demonstrates grouped data. Significant differences were analysed using Mann-Whitney test.

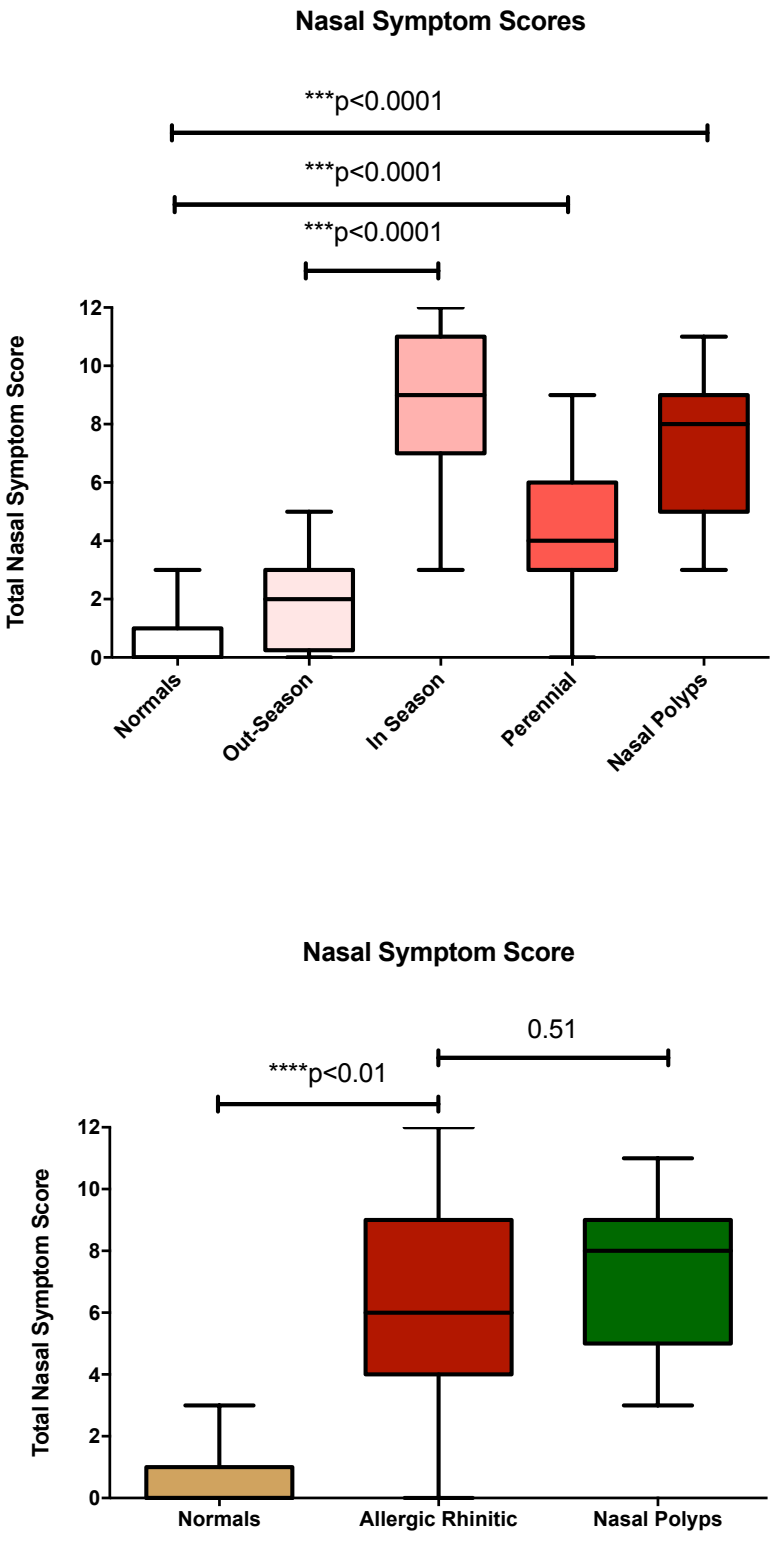


Figure 3.4: Nasal symptoms measured using Total Nasal Symptom Scores (TNSS) at time of biopsy. Top graph demonstrates all subgroups. Bottom graph demonstrates grouped data. Significant differences were analysed using Mann-Whitney test.

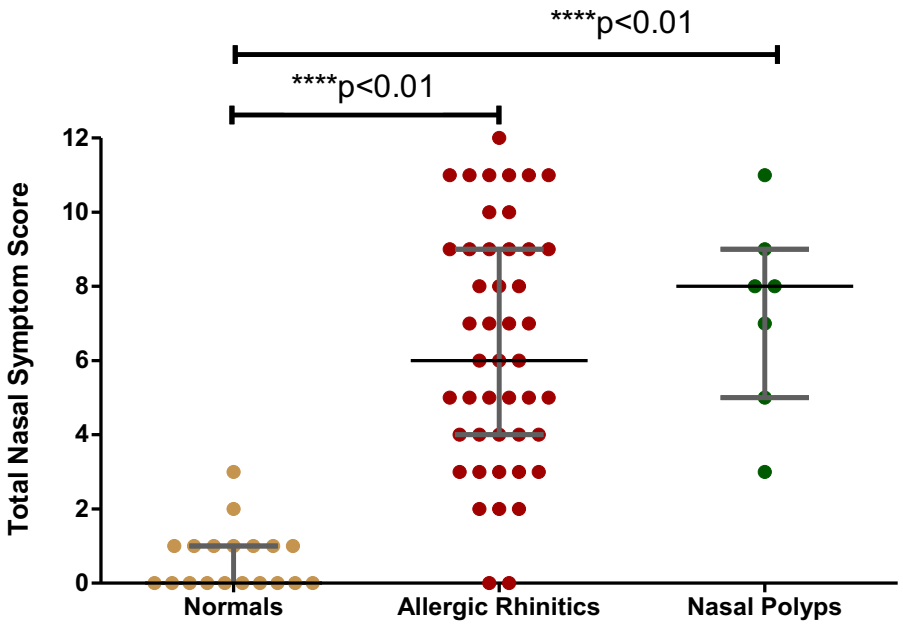


Figure 3.5: Scatter-plot graph of nasal symptoms measured using TNSS at time of biopsy. Significant differences were analysed using Mann-Whitney test.

Area under the curve analysis of the early phase reaction showed a significant increase between day 1 diluent and day 1 allergen, and day 11 diluent versus day 11 allergen in the allergic subjects (Figure 3.9). Normal volunteers had significantly reduced EPR comparing day 1 diluent and day 1 allergen challenge. Likewise, in the allergic group the late phase reaction was significantly increased on day 1 allergen compared to day 1 diluent whilst day 11 comparison between diluent and allergen revealed a tendency to increase which was not statistically significant ($p=0.06$, figure 3.9). LPR for Normal volunteers showed no difference between diluent and allergen challenge.

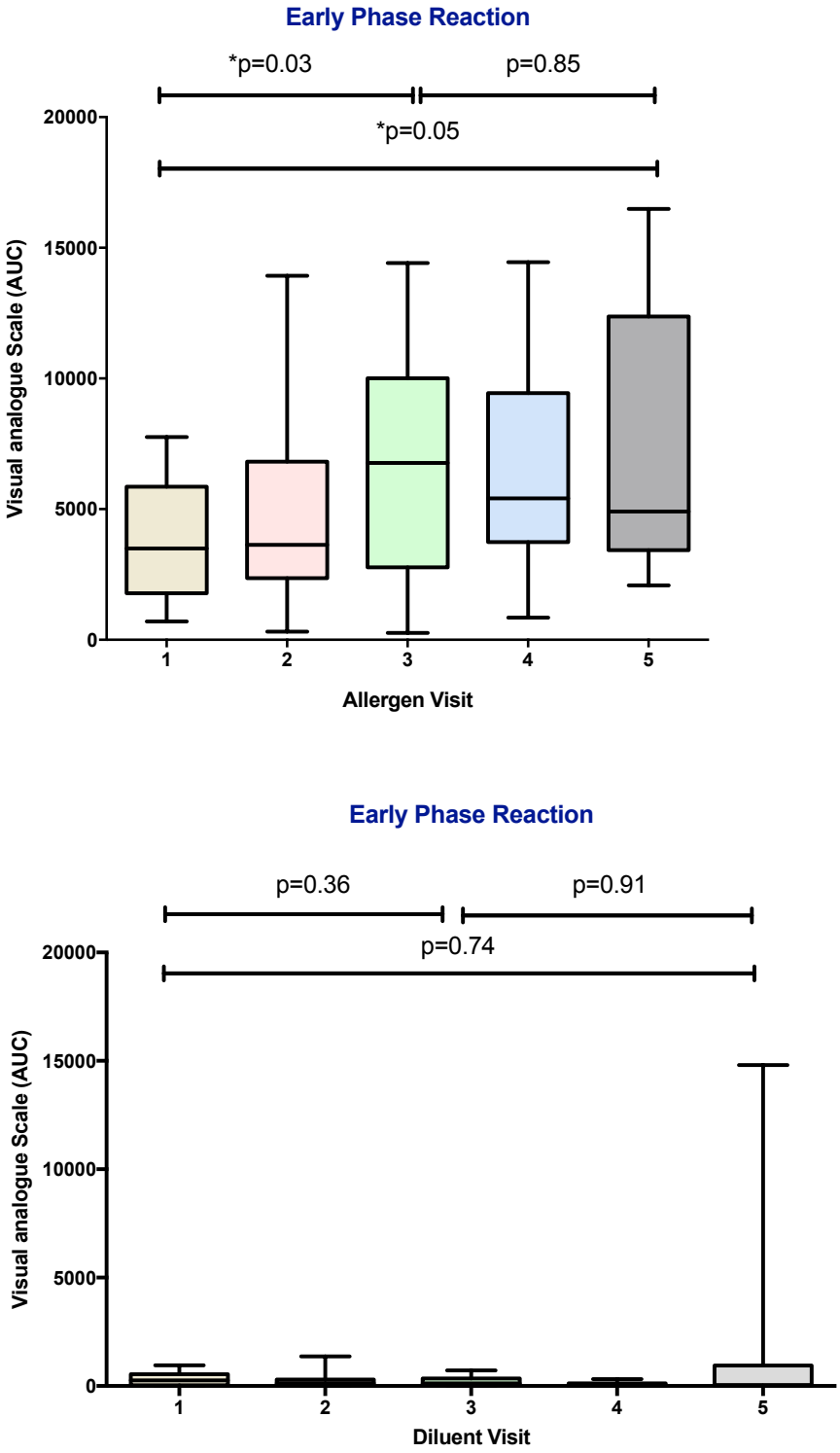
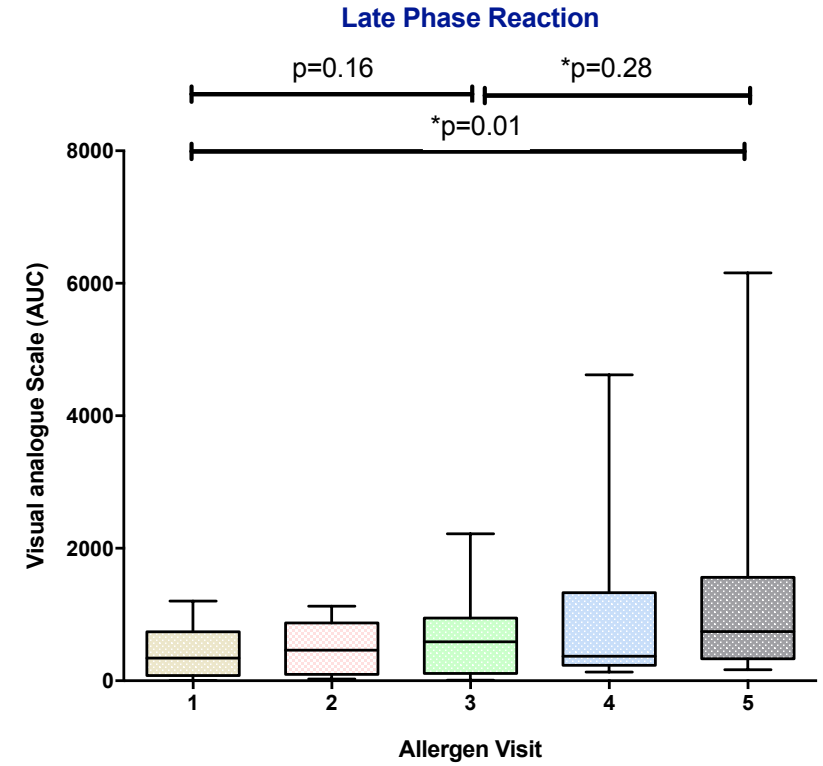


Figure 3.6: Total Nasal Symptom Scores (TNSS 0 -12). Area under the Curve (AUC) plotted against each of the 5 allergen visits. Early Phase Reaction (EPR) corresponds to AUC from 0-1 hours post challenge.



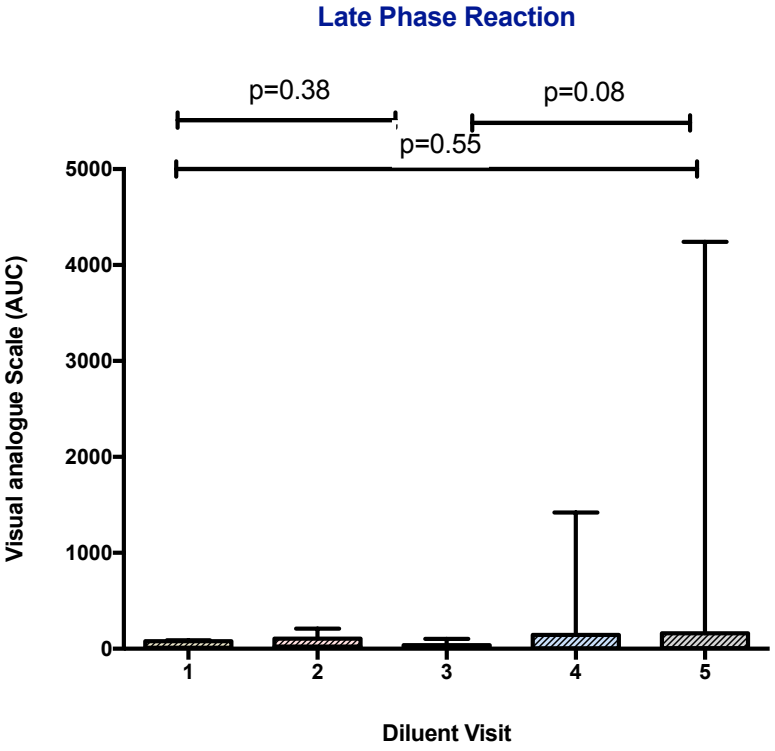


Figure 3.7: Nasal symptoms measured using Total Nasal Symptom Scores (TNSS). Area under the Curve (AUC) plotted against each of the 5 diluent visits. Late Phase Reaction (LPR) corresponds to AUC from 1-24 hours post challenge

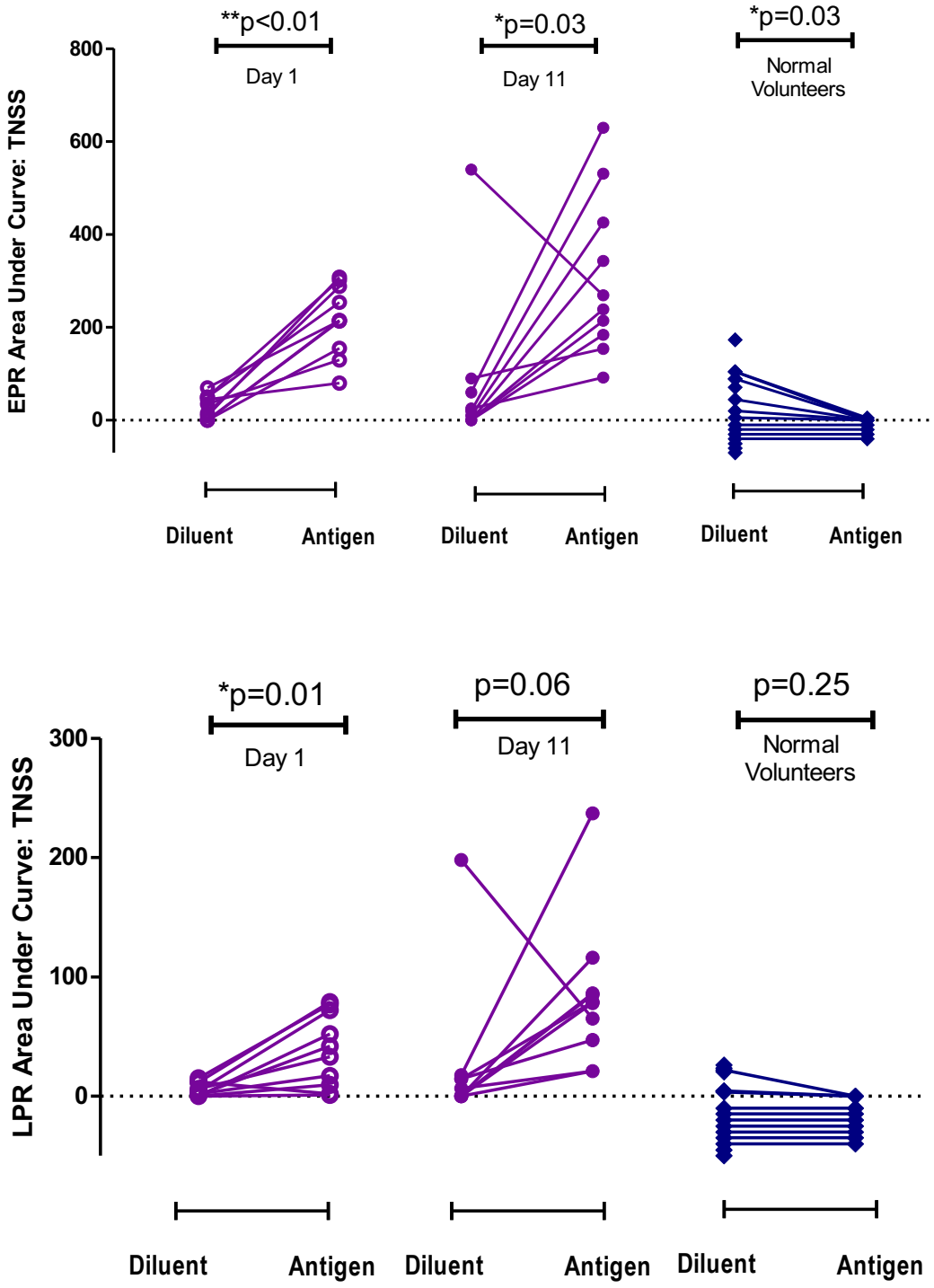


Figure 3.8: Nasal symptoms measured using Total Nasal Symptom Scores (TNSS). Area under the Curve (AUC) plotted Day 1 and Day 11 following allergen challenge, and Normal Non-atopic Controls. Columns show p values. Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05

Discussion

This chapter has shown that natural allergen exposure, whether seasonal or perennial, leads to significant escalation of rhinitic symptoms in those with disease classified by ARIA as persistent/ severe. Nasal polyposis patients were appropriate positive controls since they suffer symptoms that are both severe and persistent. Repetitive allergen challenge induced similar deterioration of clinical symptoms and revealed a priming effect for both early and late phase reactions, with normal non-atopic controls presenting lack of clinical response.

Patient characteristic and classification

Participant screening process included non-allergic, non-atopic controls, as demonstrated both clinically and by specific/ total IgE levels. Allergic rhinitics were suitably symptomatic during their allergen exposure window. In the UK with clear seasonal variation of grass pollen, Timothy grass is one of the most common seasonal allergens and elicits a marked clinical response in atopic individuals. Timothy grass allergic patients and house dust mite allergic individuals were included in the natural allergen exposure model. There was variability within and between these two groups as to the year-round duration of symptoms, with seasonal rhinitics suffering worse, more persistent symptoms (albeit for a shorter overall duration) than perennial patients. To circumvent patient phenotypic variability, all study AR patients classified as severe/ persistent as defined by ARIA, allowing the two groups to be united in representing natural allergen exposure.

At the time of biopsy, both grass and HDM patients should have had a requisite and comparable duration of exposure to enable study of the effect of persistent allergen exposure on remodelling. In the case of grass allergic patients, we biopsied after the very short-lived peak of the season when pollen levels were still high, and participants had had a minimum of a week of natural allergen challenge. House dust mite patients were all biopsied out of the pollen season to capture the effects of singular allergen exposure over a prolonged period. Total Nasal Symptom Scores (TNSS), VAS and QoL of our two study groups reflected comparable clinical severity enabling them to be grouped together to form a single study group of allergic rhinitics at the severe/ persistent end of the disease spectrum.

Our department has long experience utilising Timothy grass as allergen for single allergen challenge models and was therefore used in this repetitive allergen challenge protocol. The same single batch of allergen from a single supplier was used to prevent batch dose variability. The Bidose device is very similar in aerosolisation properties to many commercially available topical drug preparations and was favoured over allergen impregnated disks as a more even and reliable delivery system (584) (585).

Using single diluent and allergen batches with a highly reliable amount per puff, dosing allows for reproducible dosage minimising inter- and intra-participant dose variability (586). The positive control group comprised nasal polyposis patients who were captured under the clinical definition of chronic rhinosinusitis with nasal polyps (CRSwNP) as defined by EPOS criteria. They had all or some of a constellation of clinical symptoms (anosmia, congestion, rhinorrhoea, facial pain) compounded by CT and endoscopic evidence of nasal polyps (587). This group contained highest number of asthmatics at 38% but this did not relate to higher eosinophil counts in either inferior turbinate or polyp samples in this cohort. Co-existence of asthma does not influence overall symptoms scores following repetitive nasal allergen challenge in allergic rhinitis (588). Clinical symptoms are persistent in this positive control group who were also non-atopic and had not undergone previous sinus surgery. This abrogated any effect of allergic inflammation or fibrosis due to iatrogenic intervention on cellular profile or remodelling alterations in turbinate or polyp tissues.

Clinical symptoms

Subjective measurements using total nasal symptom scoring (TNSS) demonstrated significant differences during natural allergen exposure. TNSS uncovered priming or tolerogenic changes in the repetitive allergen group and a late phase reaction in both study groups (see below). In allergic rhinitis, above a certain threshold, all participants will have an early phase reaction with almost all exhibiting a late phase response. Although such delayed symptomatic responses peak around 6-8 hours post challenge, they are well maintained at 24 hours post provocation. For logistical purposes, biopsies were performed at this later timepoint, balancing adequate patient sample size with clinical and histological response.

The rhinosinusitis quality of life questionnaire (RQLQ) and its abridged version, the miniRQLQ, are both validated tools in the assessment of nasal symptoms individually, and against each other (575). One important distinction is the absence of nasal itchiness under the 'nose' domain in the miniRQLQ, but eye itchiness is accounted for. Some, but not all allergic rhinitics suffer from conjunctivitis, which is elicited by a combination of localised and direct ocular allergic inflammatory cellular infiltrate, a shared efferent neural input to both nasal and corneal mucosal surfaces, and the naso-ocular reflex whereby allergen stimulation to one nostril may elicit a response on the contralateral side as well as the eye (589). Over 20% of AR sufferers may bear the burden of ocular symptoms so the omission of nasal itchiness from the miniRQLQ is likely to underestimate the burden of disease under this specific domain (590). Despite this, results from miniRQLQ demonstrated strong discriminatory properties between study groups in both natural and induced AR protocols. The mini RQLQ was used to support the contemporaneous monitoring of symptoms using the visual analogue scale. By amalgamating four

major symptoms of AR (congestion, rhinorrhoea, sneezing and itching), it demonstrated good discriminatory and descriptive properties of symptoms during the season and in response to allergen challenge. Using the visual analogue version of the nasal symptom score permitted detailed monitoring of subjective clinical symptoms as area under the curve (AUC) measurements for early and late phase responses (EPR and LPR respectively).

Tests related to nasal patency may provide alternatives to clinical symptom monitoring. Rhinomanometry, acoustic rhinometry, (590) and peak nasal inspiratory flow (PNIF) are the current methods largely used in the context of clinical outcomes. Some studies demonstrate poor correlation between acoustic rhinometry and rhinomanometry although both tests and the visual analogue score exhibit significant changes in nasal patency pre and post decongestion (591). Attempts at coupling subjective to objective nasal obstruction measures have highlighted excessive variability and poor correlation with clinical symptoms (592) of both acoustic rhinometry (593) and rhinomanometry. Both tests possess high operator variability, are expensive and not easily portable (591). Although convenient, and easy to use, PNIF also correlates poorly with clinical symptoms (594). Such nasal airway measurements therefore do not replace symptom scores but can augment them. Robust subjective measurements such as nasal symptom scores remain optimal monitoring tools , responsive and clinically relevant.

Previous experience in our group has shown 320BU or just over 1000 SQ of allergen to be the minimum dose at which most participants show clinical response (156) (209). During the season, allergic rhinitic patients may become symptomatic after only a few minutes of allergen exposure, which equates to as few as 100 grains of pollen (with natural exposure being approximately 150 grains) (595).There are no known, well established conversion rates between natural pollen content and purified allergen dose (596) (597). In this study, individual thresholds were identified at the first visit and this personalized threshold was utilized for subsequent dosing.

In asthma, tissue eosinophilia is related to airway hyperreactivity, but such coupling of inflammatory cellular infiltrate to clinical severity may not always hold true for allergic rhinitis. As demonstrated in this study, symptoms are not completely ameliorated out of the season, even in mono-sensitised individuals. Despite cardinal lower respiratory symptom of airway hyperreactivity showing links to tissue eosinophilia (221), such link does not hold rigidly true for the upper airway where a number of mediators contribute to the clinical picture seen in allergic rhinitis (598). In further contrast to asthma, eosinophilic upregulation in allergic rhinitis is evidenced and linked to clinical severity on allergen

exposure, but such spikes in cellular infiltrate are transient and insufficient for maintenance of remodelling changes- such collagen deposition (599).

Clinical profile of allergic rhinitics out of the pollen season

Allergic rhinitics are not entirely symptom free out of the pollen season despite mono-allergic clinical profiles. Although not within the remit of this study, neurogenic inflammation may contribute to this finding. With in-seasonal inflammatory infiltrate manifesting clinically as allergic rhinitis, out of seasonal changes, although not linked to classical Th2 phenotypic alterations, still prevent complete abrogation of symptoms. Pertinacious differences of cytokine and cellular milieu in allergic rhinitis may drive short lived changes in neuro-excitability which, through enduring changes in gene expression and therefore neuronal phenotype, contribute to long term alteration in cellular behaviour and the persistent sensation of congestion, even in the absence of a classical Th2 characterised infiltrate. Local allergic rhinitis, where local specific IgE is found in the context of Th2 type mucosal cytokines without clinical manifestations of systemic atopy, also offers a potential avenue for out of season allergic rhinitic symptoms (600). Such differences may explain the disconnect between subjective decrease in nasal symptom scores and perception of dyspnea and objective measurements such as PNIF or FEV1. Out of season, rhinitics although symptomatic, do not demonstrate significant increases in allergic cellular profile but may have underlying cytokine changes in-keeping with maintenance of phenotypic drive.

Repetitive allergen challenge

Our repetitive allergen challenge model is in harmony with chronic allergen exposure murine models using house dust mite extract- 3 days a week for up to 3 weeks- to study airway remodelling (601) (602). With a paucity of evidence for induced models of inflammation and remodelling in allergic rhinitis via repetitive allergen challenge, our RAC protocol reflects and suitably applies what has been learnt and used in murine studies of allergic inflammatory airways disease.

Priming versus tolerance

The use of a repetitive allergen challenge protocol to establish the phenomena of priming was first trialled by Connell *et al* nearly 50 years ago (603) (604). This priming was not antigen specific but neither was it caused by a nonspecific irritant. Wachs et al used four ragweed challenges over a period of 17 days to demonstrate clinical priming (number of sneezes) and enhanced release of kinins and histamine- but not PGD_2. Nasal lavage cellular counts were, but epithelial cell numbers were not, increased in response to clinical evidence of priming (595). Since then there have been a limited number of studies attempting to faithfully replicate a natural pollen season (605).

Our model of repetitive allergen challenge (RAC) demonstrated sensitisation and priming at day 5 (3^{rd} allergen challenge) for the early phase reaction, and day 11 (5^{th} allergen challenge) for the late phase reaction as evidenced by total nasal symptom scoring (TNSS) and visual analogue scales. During the early phase non atopic normal controls displayed fewer symptoms as measured by TNSS post allergen challenge than post diluent challenge. This finding dovetailed into statistically significant rises in inflammatory cells in allergic rhinitics but not normal controls (see next chapter). This validated TNSS as a tool capable of accurately capturing priming during induced and natural allergen challenge in allergic rhinitis, and tolerance in non-atopic individuals.

Repetitive allergen challenge is known to cause priming (603). This effect is due to a combination of cellular infiltrate typically associated with Th2 response, with mast cell degranulation and acute mediators responsible for the early phase, and macrophages, lymphocytes and eosinophilic influx responsible for the late phase (606). Further contributing factors are neurotrophins released during the acute early phase response which enhance sensory hyperreactivity with subsequent allergen challenges; an effect that lasts at least 24 hours (607). In a mouse model of allergic rhinitis, endothelial and neuronal nitric oxide synthase is not, but inducible nitric oxide synthase is higher in allergics than controls (608).

Priming in the context of induced allergen challenge, is likely to need prolonged exposure. One week may not be sufficient (609). In addition, priming may be site specific with different epithelial surfaces demonstrating varying degrees of this phenomenon (610). Number of challenges, timing between challenge visits, and the total duration of exposure will all influence the final clinical and histopathological picture. Optimally balancing this triumvirate of variables is necessary for accurate, reliable and reproducible models of induced allergic rhinitis. This study offers an optimal model for manifestation of symptoms with clinical evidence of priming.

Summary of chapter

ARIA defined persistent/ severe allergic rhinitics are significantly more symptomatic during natural allergen exposure than non-atopic normal controls. Nasal polyposis patients are similarly so, demonstrating their clinically relevant categorisation as the positive control group. Allergic rhinitics were biopsied immediately after the highest peak in grass pollen counts. Reasons why Timothy grass allergic individuals are not entirely symptom free out of the pollen season may include neurogenic inflammation.

TNSS, VAS and QoL all provide reliable and reproducible measurements of symptom severity. Repetitive allergen challenge results in priming of the early phase response at day 5, and of the late phase response at day 11 of the RAC protocol as measured by total nasal symptom scoring. Normal non-atopic individuals do not exhibit an increase in early phase reaction after a single allergen challenge.

Chapter 4 : Inflammation during Natural Allergen Exposure and Repetitive Allergen Challenge Models of Allergic Rhinitis

Introduction

Priming refers to the tendency for mucosal surfaces that are exposed to an allergen or irritant to respond with an exaggerated response following re-exposure to the same or a different offending allergen or irritant. In trials of therapeutic intervention in allergic rhinitis this was first described by Connell (611) . It has subsequently been reproduced for the same individual allergen (612) (613) as well as in response to subsequent exposure to an alternative allergen (614) and also following ozone exposure (615). It is possible that this priming effect and the phenomenon of allergen induced hyperreactivity (mucosal irritability) overlap post allergen challenge. For this reason, in my studies the diluent challenge was always performed prior to the allergen provocation and an interval of at least 4 weeks was inserted between these two stages (616). One explanation for clinical priming (exaggerated symptoms following re-exposure after challenge and during the late pollen season in response to lower pollen concentrations) is recruitment of effector cells including primed mast cells and eosinophils, which degranulate in response to allergen re-exposure. Release of inflammatory cell derived mediators affect constant neuronal pathways to sustain clinical symptoms of rhinoconjunctivitis.

This chapter describes the changes in nasal secretion cytokines (Th2, Th1 and Treg) and alterations in epithelial and submucosal cell counts in NAE and RAC protocols. Comparisons are made between allergen versus diluent samples unless stated otherwise.

Results

Natural Allergen Exposure Protocol

Nasal secretion cytokine measurements demonstrate significant increases in Th2 cytokine markers (IL-4, IL-5 and IL-13) in allergic rhinitics when compared with normal non-atopic individuals (figure 4.1). No such increases were seen with Th1 markers (IL-2, IFN-γ) and Treg marker (IL-10) (figure 4.2). Participants with nasal polyps acted as positive controls. Nasal secretions from nasal polyp patients showed a significantly higher concentration of all the above cytokines- Th2, Th1 and Tregs (figure 4.1-4.3)

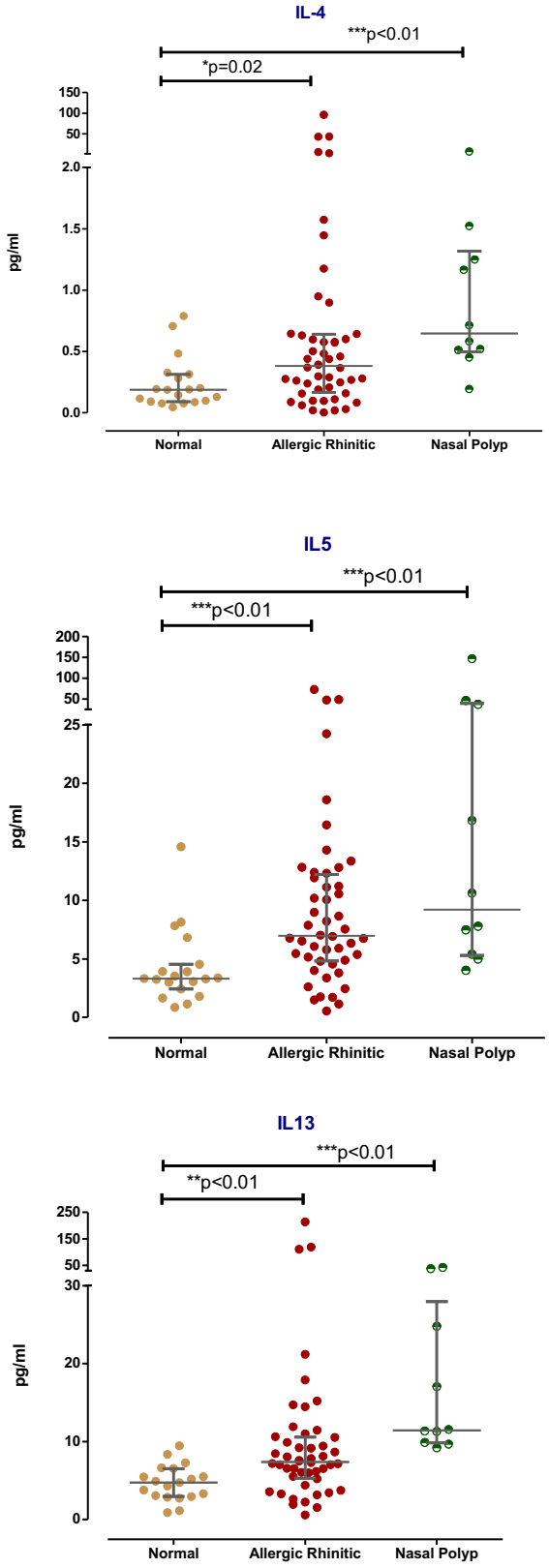


Figure 4.1: Nasal Secretion: Th2 cytokine measurements in pg/ml comparing persistent allergic rhinitics with healthy controls or secretions from CRSwNP and healthy controls. Graphs show median with interquartile range and comparisons are performed using Mann-Whitney test. P<0.05 was taken as significant.

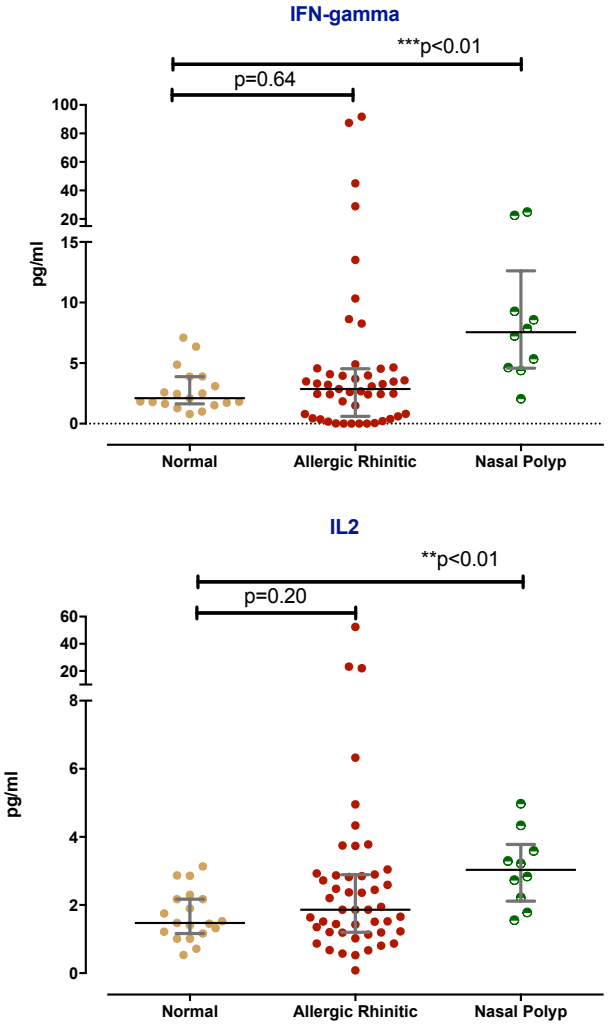


Figure 4.2: Nasal Secretion: Th1 cytokine measurements in pg/ml ml comparing persistent allergic rhinitics with healthy controls or secretions from CRSwNP and healthy controls. Graphs show median with interquartile range, comparisons performed using Mann-Whitney test. P<0.05 was taken as significant.

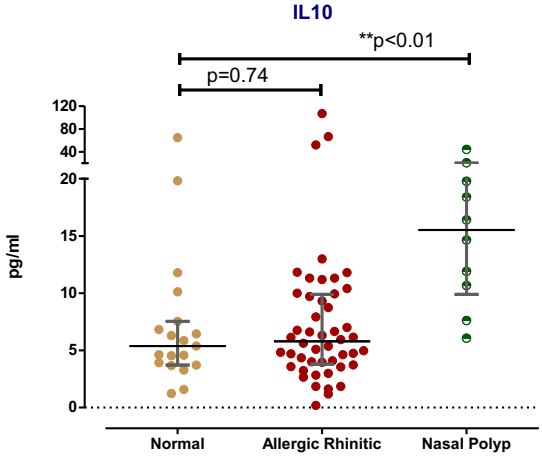


Figure 4.3: Nasal Secretion: T regulatory cytokine measurements in pg/ml comparing persistent allergic rhinitics with healthy controls or secretions from CRSwNP and healthy controls. Graphs show median with interquartile range, comparisons performed using Mann-Whitney test. P<0.05 was taken as significant.

In the epithelium, there was a tendency for an increase in epithelial eosinophils compared to non-atopic controls ($p=0.08$) whereas there were no changes in other inflammatory leukocytes (neutrophils, mast cells) (figure 4.4) (photomicrograph 4.1). Submucosal eosinophils also tend to be elevated in rhinitics compared to controls ($p=0.07$) (figure 4.5). In contrast submucosal neutrophils were lower ($p=0.06$) in allergic rhinitics compared to normal non-atopic controls. Epithelial and submucosal leukocyte counts were all significantly higher in NP participants. Mast cells were increased in nasal polyp epithelium ($p<0.01$) but not the submucosa ($p=0.38$). Submucosal basophil and macrophage counts were not statistically different from normal non-atopic controls (figure 4.6). In the nasal polyp submucosa significantly higher numbers of mast cells (p value) were observed with elevated basophils tending to be higher, but not reaching, significance ($p=0.06$).

No differences were observed in total T cell numbers (CD3), nor for CD4 T helper cells, nor CD8 suppressor T cells between allergic and non-atopic individuals. (figure 4.7) (photomicrograph 4.2).

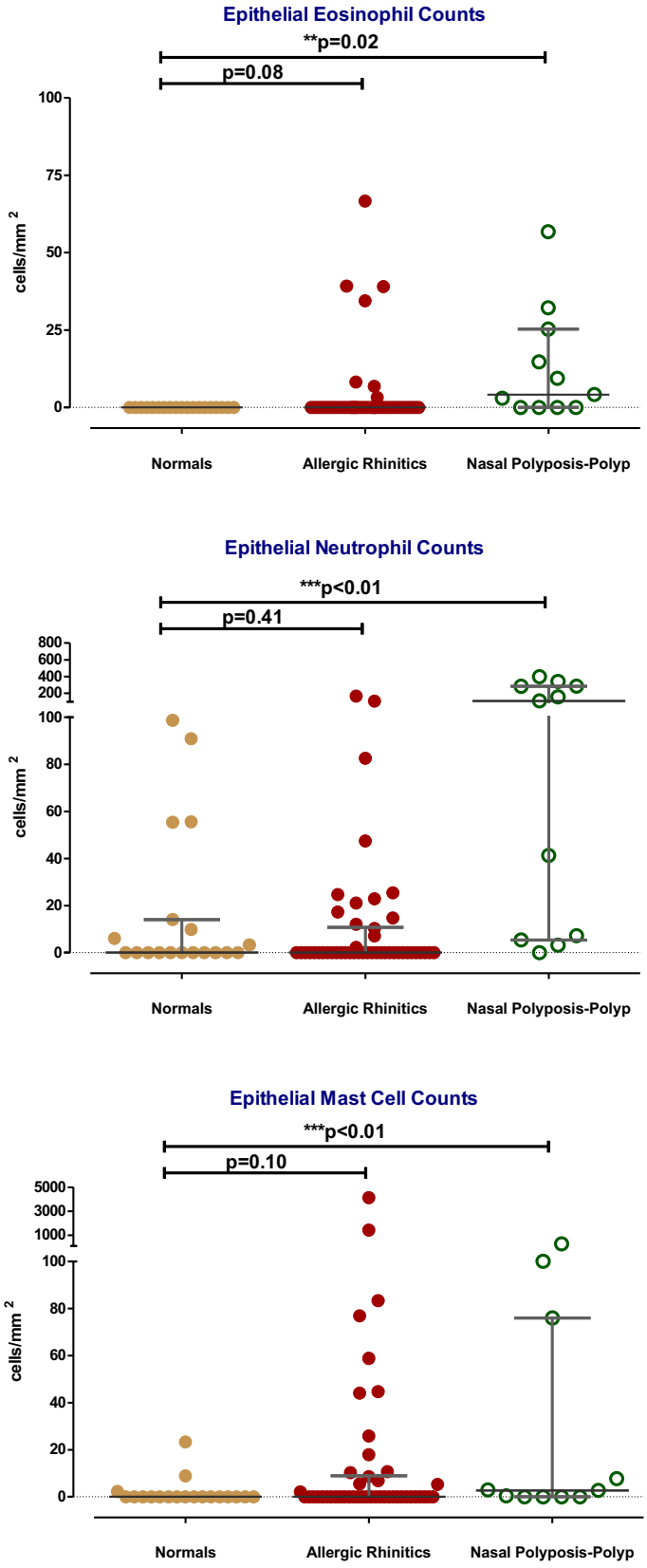


Figure 4.4: Natural allergen exposure. Epithelial Inflammatory Cell Counts (per mm²): comparing persistent allergic rhinitics (n=46) with healthy controls (n=19) or polyp tissue from CRSwNP (n=11) and healthy controls (n=19). Eosinophils (BMK13), neutrophils (anti-neutrophil elastase) and mast cells (AA1) stained using the APAAP technique. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05

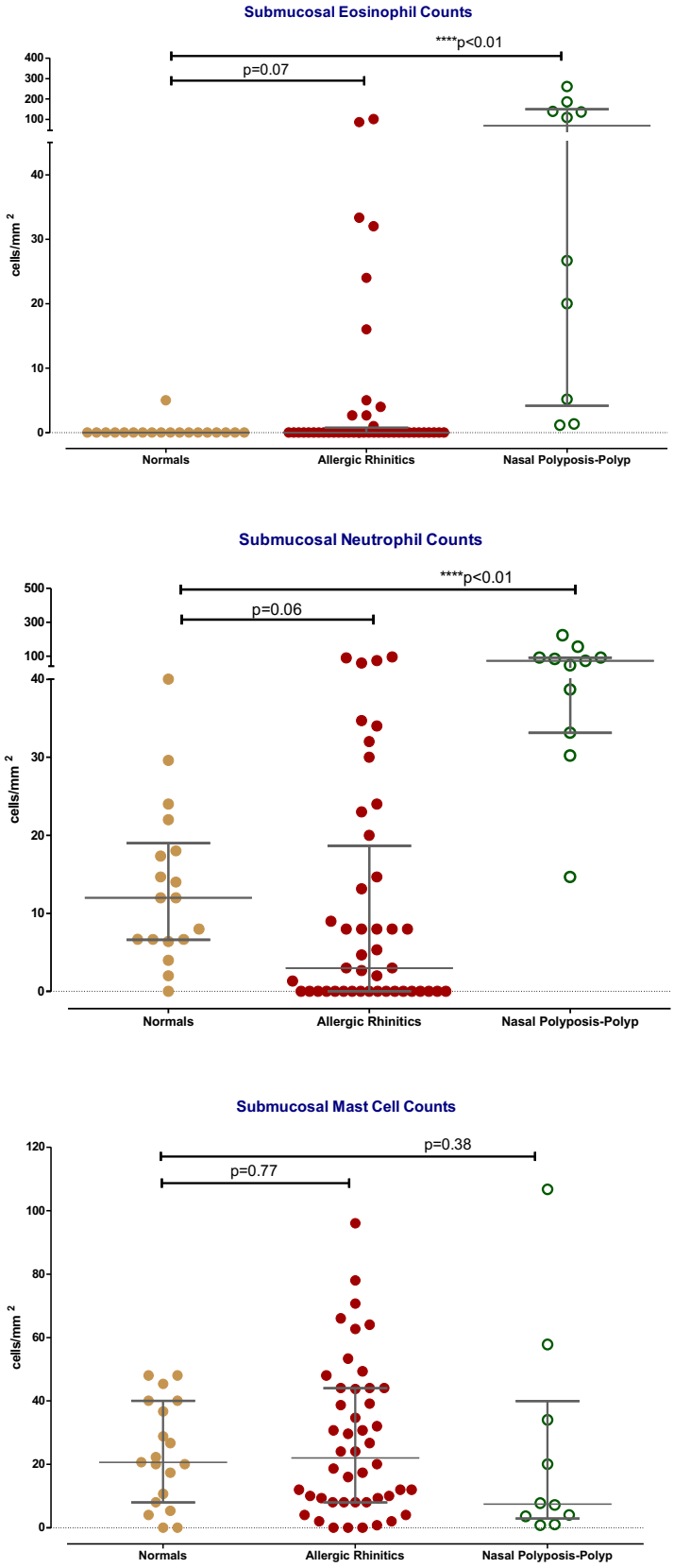
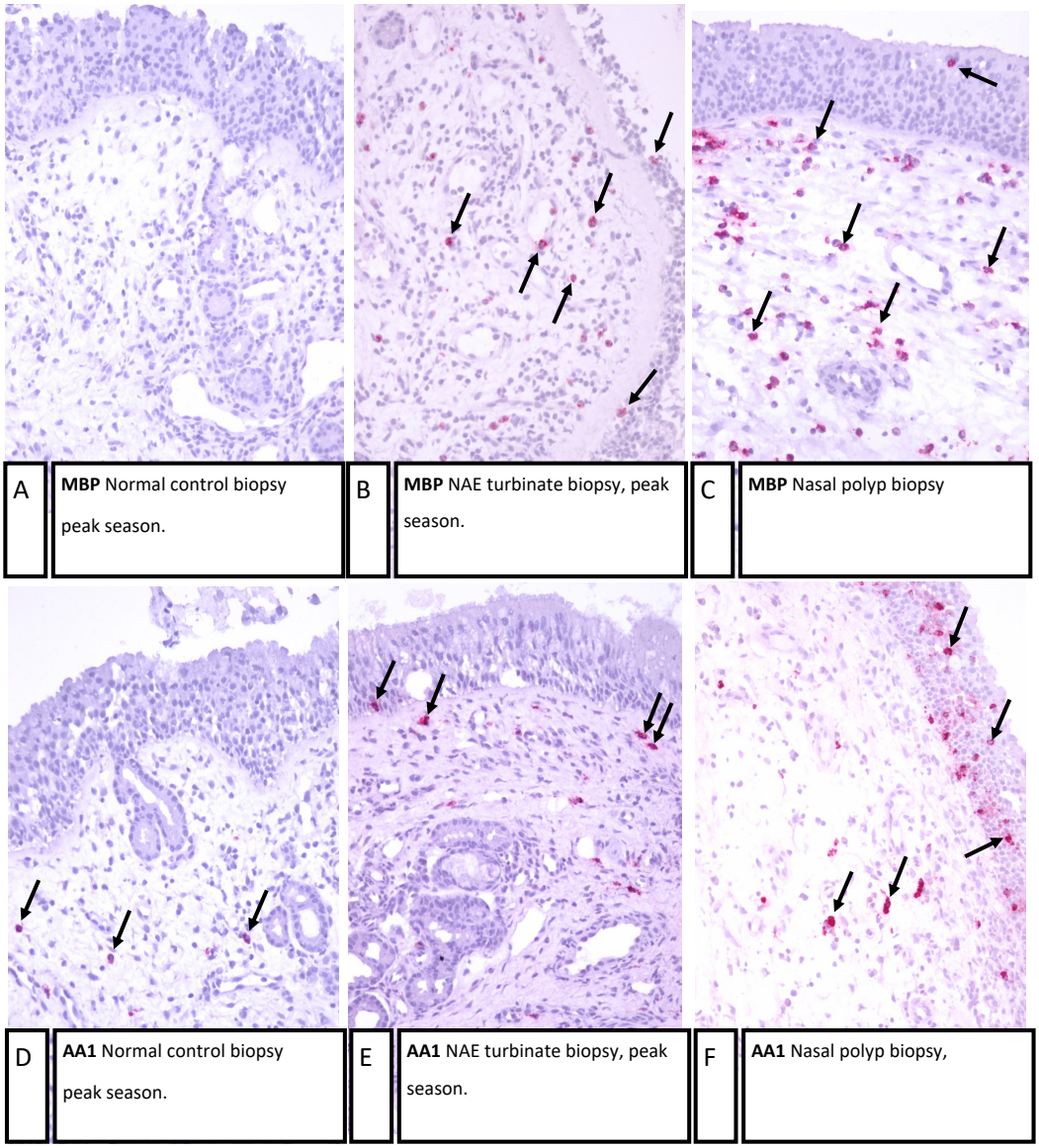


Figure 4.5: Natural allergen exposure. Submucosal Inflammatory Cell Counts (per mm²): comparing persistent allergic rhinitics (n=46) with healthy controls (n=19) or polyp tissue from CRSwNP (n=11) and healthy controls (n=19). Eosinophils (BMK13), neutrophils (anti-neutrophil elastase) and mast cells (AA1) stained using the APAAP technique. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05



Photomicrograph 4.1: MBP eosinophil staining using the monoclonal antibody BMK13 (A-C) and Mast cell tryptase staining using monoclonal antibody AA1 (D-F) with the APAAP technique. Eosinophils and mast cells visible as discrete red cells (black arrows) with haematoxylin (blue) nuclear counterstain. Eosinophils were absent in normal control tissue(B) but present in epithelium and submucosa NAE peak season (B) and throughout nasal polyp tissue (C). Similar numbers of mast cells were evident in submucosa of normal controls (D), NAE peak season (E) and in nasal polyps (F) whereas epithelial mast cells were only a feature of allergen challenge and more significantly nasal polyposis. Photographed using x20 lens.

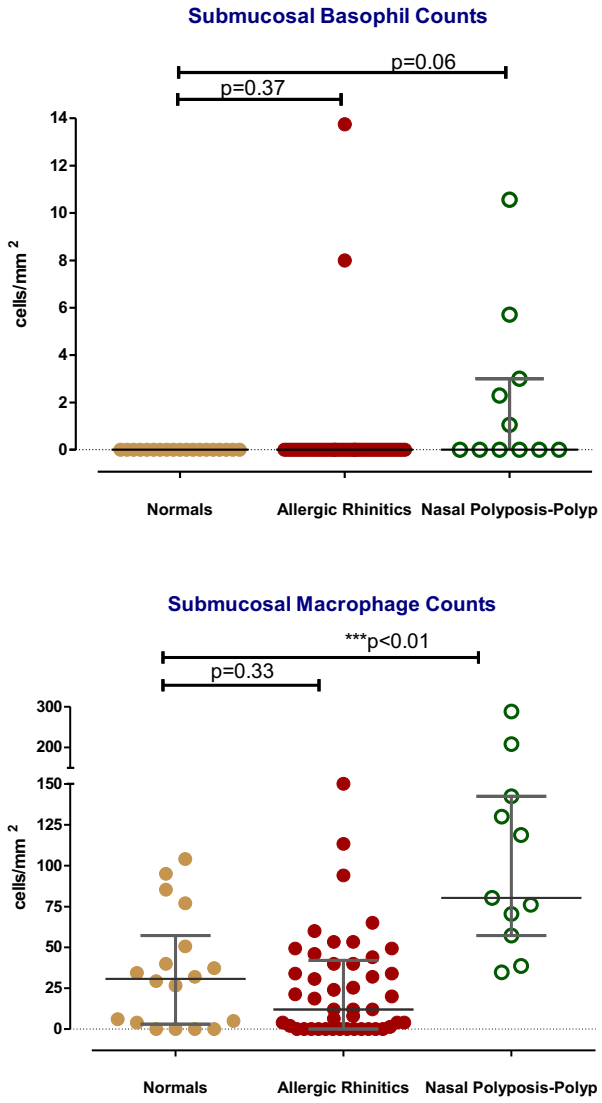


Figure 4.6: Natural allergen exposure. Submucosal Inflammatory Cell Counts II (per mm²): comparing persistent allergic rhinitics (n=46) with healthy controls (n=19) or polyp tissue from CRSwNP (n=11) and healthy controls (n=19). Basophils (2D7) and macrophages (CD68) stained using the APAAP technique. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05

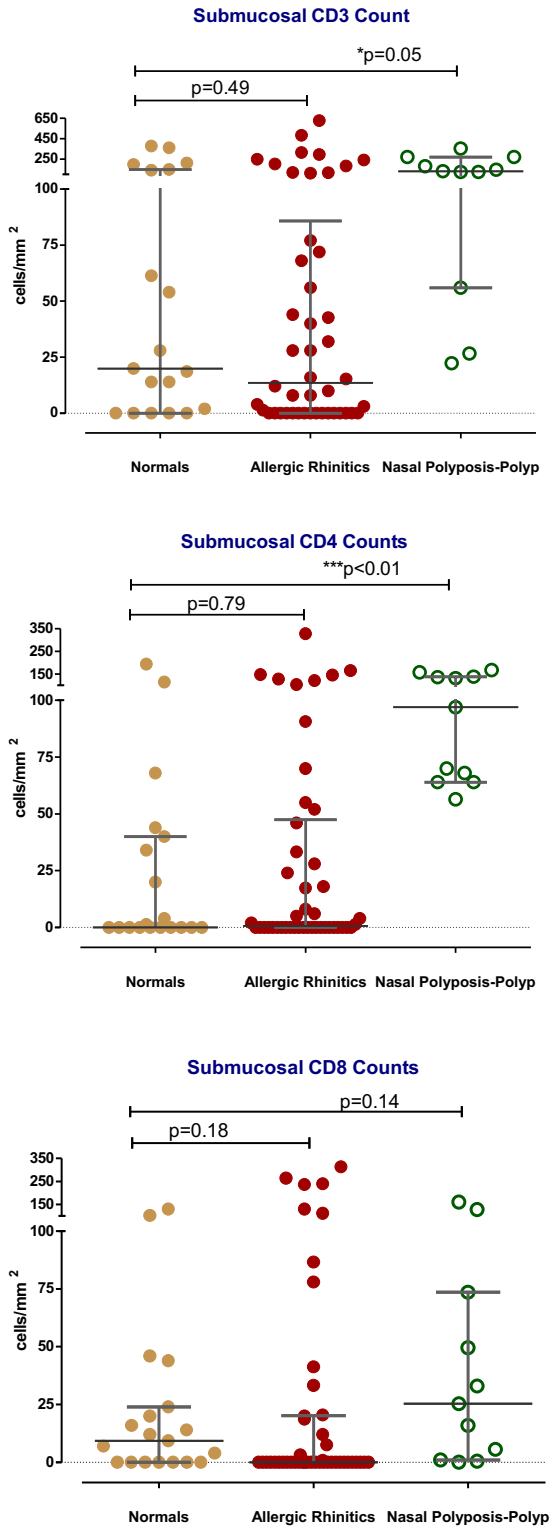
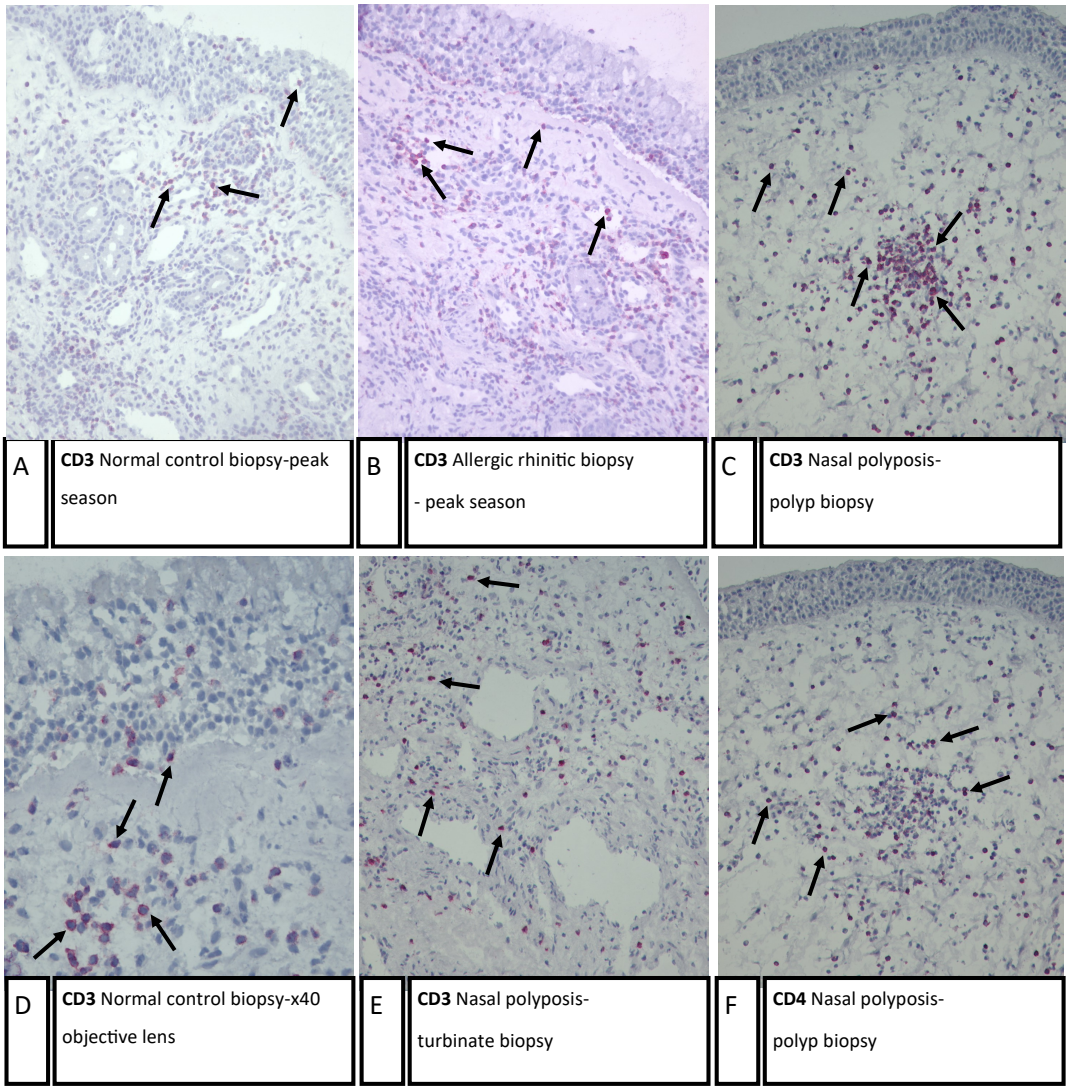


Figure 4.7: Natural allergen exposure. Submucosal T Cell Counts (per mm^2): comparing persistent allergic rhinitics (n=46) with healthy controls (n=19) or polyp tissue from CRSwNP (n=11) and healthy controls (n=19). CD3, CD4 and CD8 T cells stained using the APAAP technique. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05



Photomicrograph 4.2: T cell staining using anti-CD3 monoclonal antibody (A-E) and anti-CD4 monoclonal antibody (F) with the APAAP technique. T cells visible as discrete red cells (black arrows) with haematoxylin (blue) nuclear counterstain. CD3 and CD4+ T cells were significantly increased in nasal polyp tissue (C,F) compared to turbinate tissue from normal controls (A,D) and nasal turbinates (B,E).With the exception of (D) photographed using x20 lens.

Repetitive Allergen Challenge Model

RAC model consists of a diluent phase followed by an allergen phase. During each phase, 5 successive challenges were administered up to four days apart, and nasal biopsies taken 24 hours after the first challenge (day 1), and the last challenge (day11).

Of the Th2 profile of cytokine markers (IL4, IL-5 and IL-13), only IL-5 was significantly higher 11 days after allergen challenge when compared with diluent (figure 4.9). IL-4 and IL-13 did not change 11 days after repetitive allergen challenge (allergen v diluent). No differences were observed in all three Th2 cytokine measurements, IL4, IL5 and IL13, at 24 hours post allergen challenge Day 1 when compared with diluent. Normal non-atopic control samples showed a rise in IL-13 between allergen and diluent samples at 24hrs post challenge, with no change in IL-4 and IL-5.

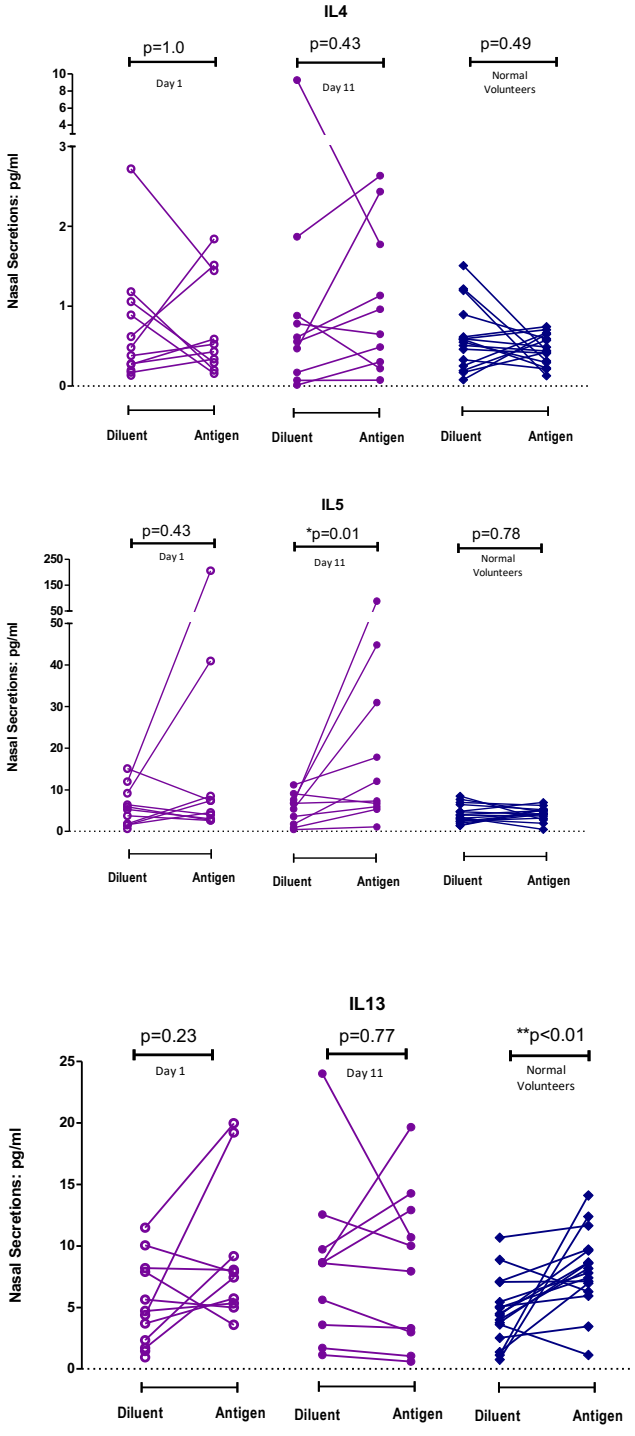


Figure 4.8: Nasal Secretion: Th2 cytokine measurements in pg/ml. Day 1 and Day 11 following diluent and allergen challenge in atopic allergic rhinitics (purple symbols) and diluent and allergen challenge Day 1 only for the normal non-atopic control subjects (dark blue symbols). Columns show p values. Paired data was analysed using the Wilcoxon matched-pairs signed-ranks test. Significance was accepted as p<0.05

Th1 cytokines IL2 and IFN-γ show no significant change at Day 1 or at Day 11 post allergen challenge when compared with diluent. Similarly, no change is seen in IL-2 and IFN-γ in non-atopic normal controls at 24hrs after allergen challenge when compared with diluent.

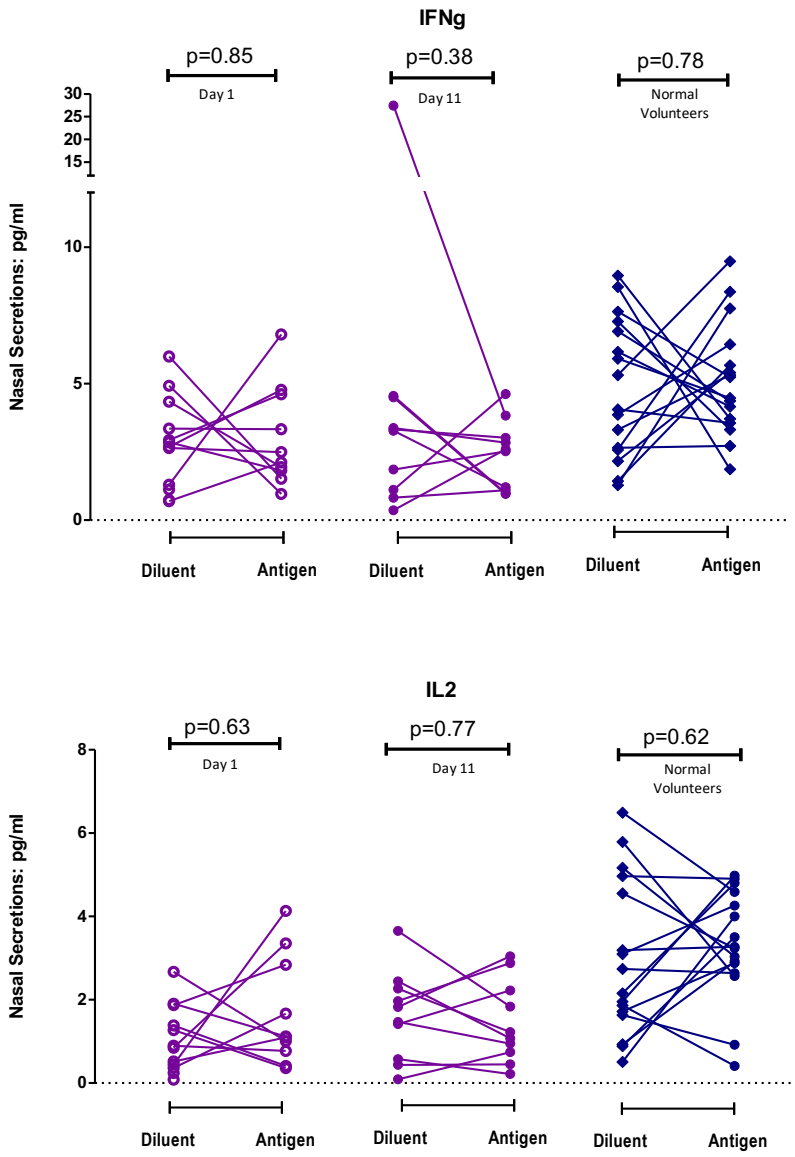


Figure 4.9:Nasal Secretions: Th1 cytokine measurements in pg/ml. Day 1 and Day 11 following diluent and allergen challenge in atopic allergic rhinitics (purple symbols) and diluent and allergen challenge Day 1 only for the Normal non-atopic control subjects (dark blue symbols). Columns show p values. Paired data was analysed using the Wilcoxon matched-pairs signed-ranks test. Significance was accepted as p<0.05.

T regulatory marker IL-10 showed no significant difference after allergen challenge when compared with diluent for either Day 1 or Day 11. Normal non atopic controls do show a near significant rise (p=0.07) at Day 1 following allergen challenge (figure 4.11).

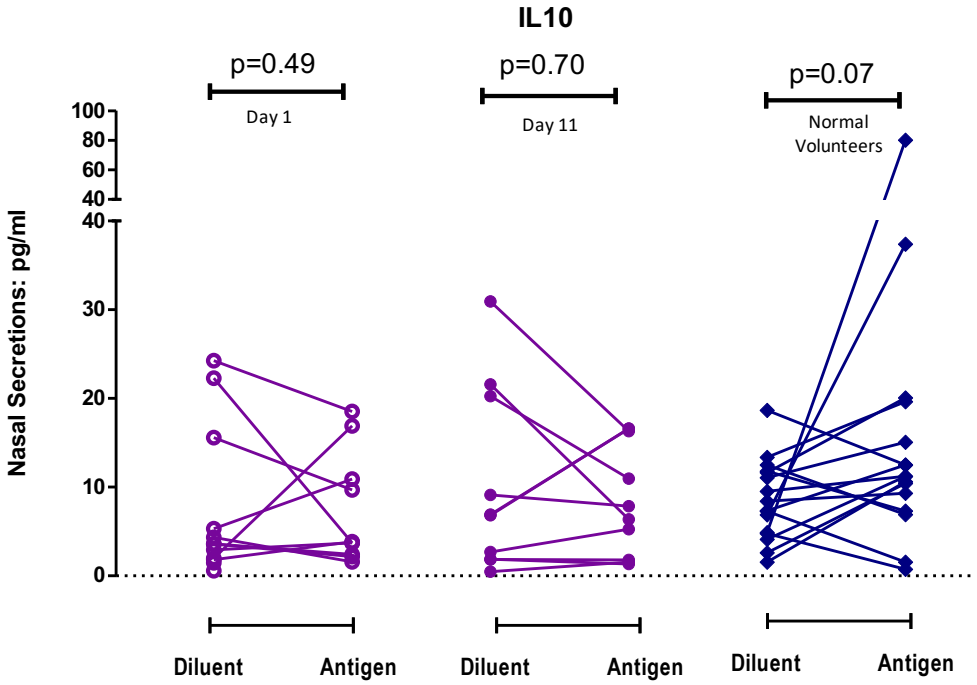


Figure 4.10: Nasal Secretion: T regulatory cytokine measurements in pg/ml. Day 1 and Day 11 following diluent and allergen challenge in atopic allergic rhinitics (purple symbols) and diluent and allergen challenge Day 1 only for the Normal non-atopic control subjects (dark blue symbols). Columns show p values. Paired data was analysed using the Wilcoxon matched-pairs signed- ranks test. Significance was accepted as p<0.05.

Nasal epithelial biopsies obtained following repetitive allergen challenge showed no difference in epithelial leukocytes (eosinophils and neutrophils) and mast cells between day 1 and day 11 allergen versus diluent samples (figure 4.12). Similarly, no statistical difference was demonstrated at 24 hours in epithelial eosinophils, neutrophils and mast cell counts in the normal non-atopic control subjects. Conversely, day 11 allergen vs diluent sample comparisons showed near significance for higher number of epithelial eosinophils (p=0.06) and neutrophils (0.08). Normal non-atopic participants demonstrate higher mast cell counts at day 1 post allergen versus diluent (p=0.06).

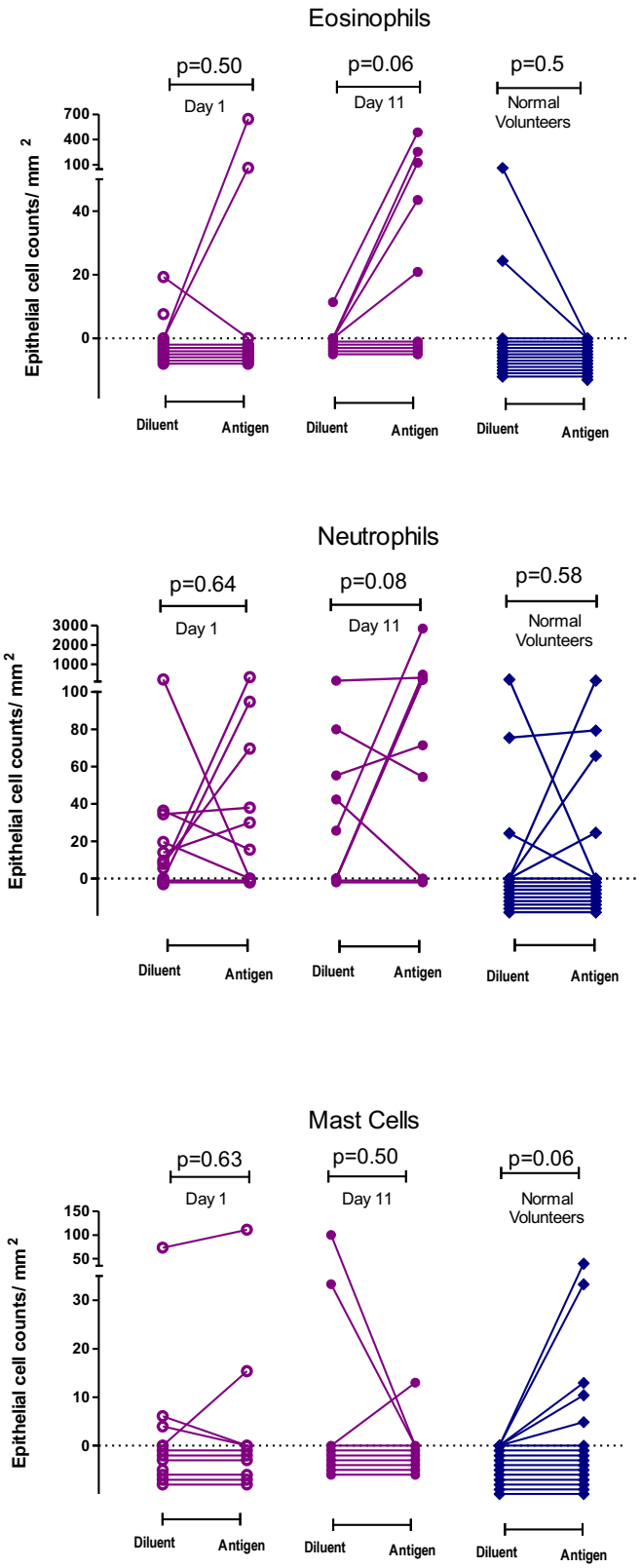


Figure 4.11: Epithelial Inflammatory Cell Counts: Day 1(left panel, purple) and Day 11(middle panel, purple) after diluent challenge and allergen challenge for participants with allergic rhinitis(n=12), and on day 1 after diluent and allergen challenge for Normal non-atopic controls (n=16) (right panel, Blue). Eosinophils (BMK13), neutrophils (anti-neutrophil elastase) and mast cells (AA1) stained using the APAAP technique. Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05

Submucosal eosinophil counts showed a marked increase after allergen challenge at both Day 1 (***p<0.01) and Day 11 (p=0.03). There was no significant change in submucosal eosinophils at 24 hours in non-atopic negative control participants (figure 4.13) (photomicrograph 4.3). For neutrophils, increases were noted in the submucosa at Day 11 after allergen compared to diluent for allergic participants but not at Day 1 for either the allergics or non-atopic controls. No differences were demonstrable with mast cells or basophils at either RAC timepoints or at 24 hours in normal non-atopic samples. Submucosal macrophages were higher after allergen compared to diluent at Day 1 (p=0.01), but not at Day 11 after repetitive allergen challenge. Macrophages were also elevated at 24 hours after allergen compared to diluent in non-atopic control samples (figure 4.14). Figures 4.16 shows representative photomicrograph of inflammatory nasal mucosal staining in allergic rhinitics.

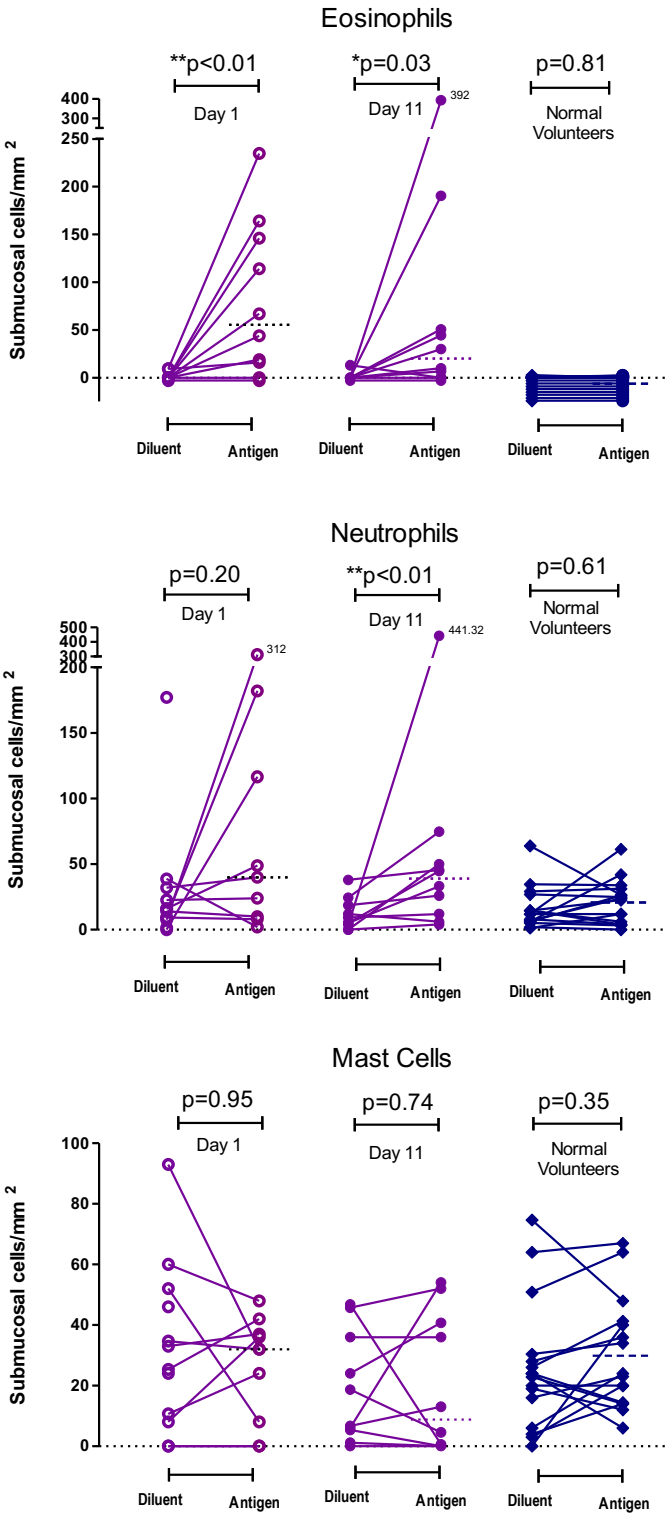
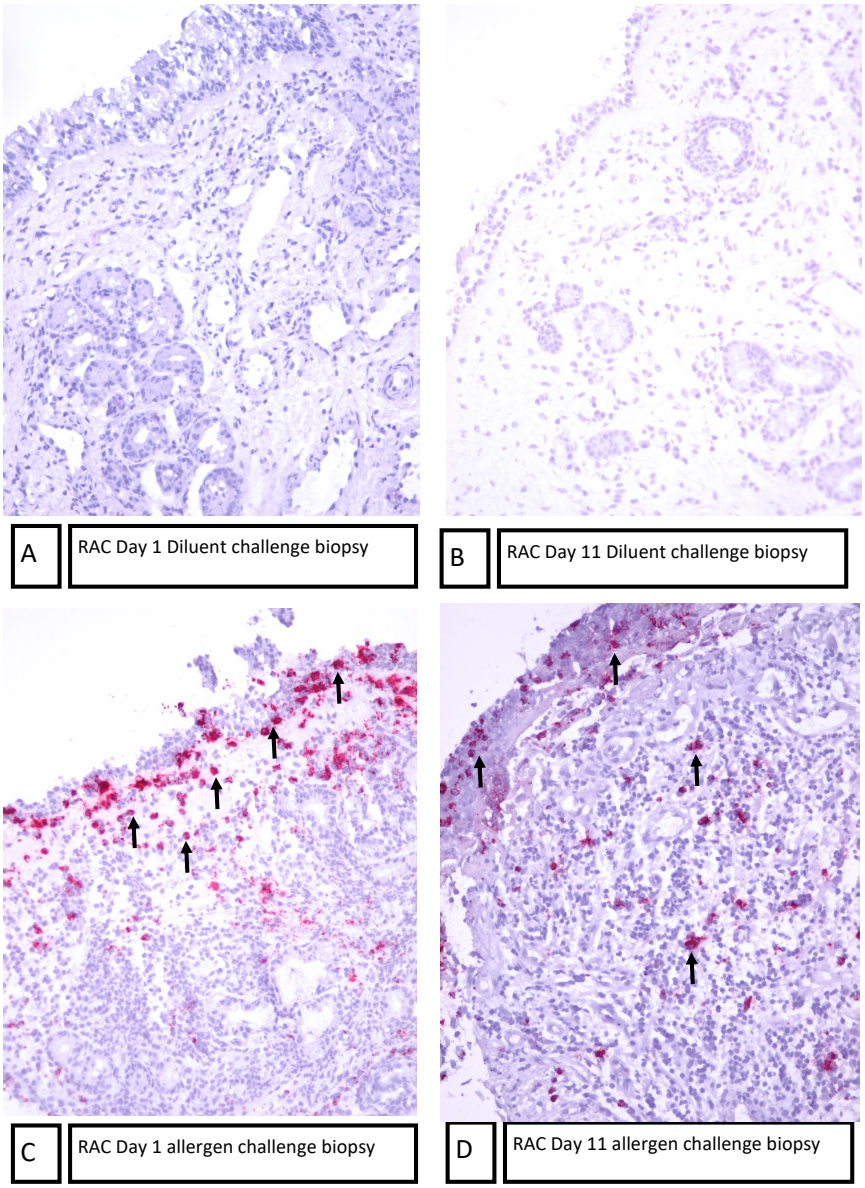


Figure 4.12: Submucosal Inflammatory Cell Counts. Day 1(left panel, purple) and Day 11(middle panel, purple) after diluent challenge and allergen challenge for participants with allergic rhinitis (n=12), and on Day 1 after diluent and allergen challenge for normal non-atopic controls (n=16)(right panel, Blue). Eosinophils (BMK13), neutrophils (anti-neutrophil elastase) and mast cells (AA1) stained using the APAAP technique. Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05



Photomicrograph 4.3: MBP eosinophil staining using the monoclonal antibody BMK13 with the APAAP technique. Eosinophils visible as discrete red cells (black arrows) with haematoxylin (blue) nuclear counterstain. Eosinophils were not found in nasal turbinate tissue Day 1(A) or Day 11(B) post diluent challenge but were significantly increased in both the epithelium and submucosa post allergen challenge, Day 1 (C) and Day 11 (D). Photographed using x20 lens.

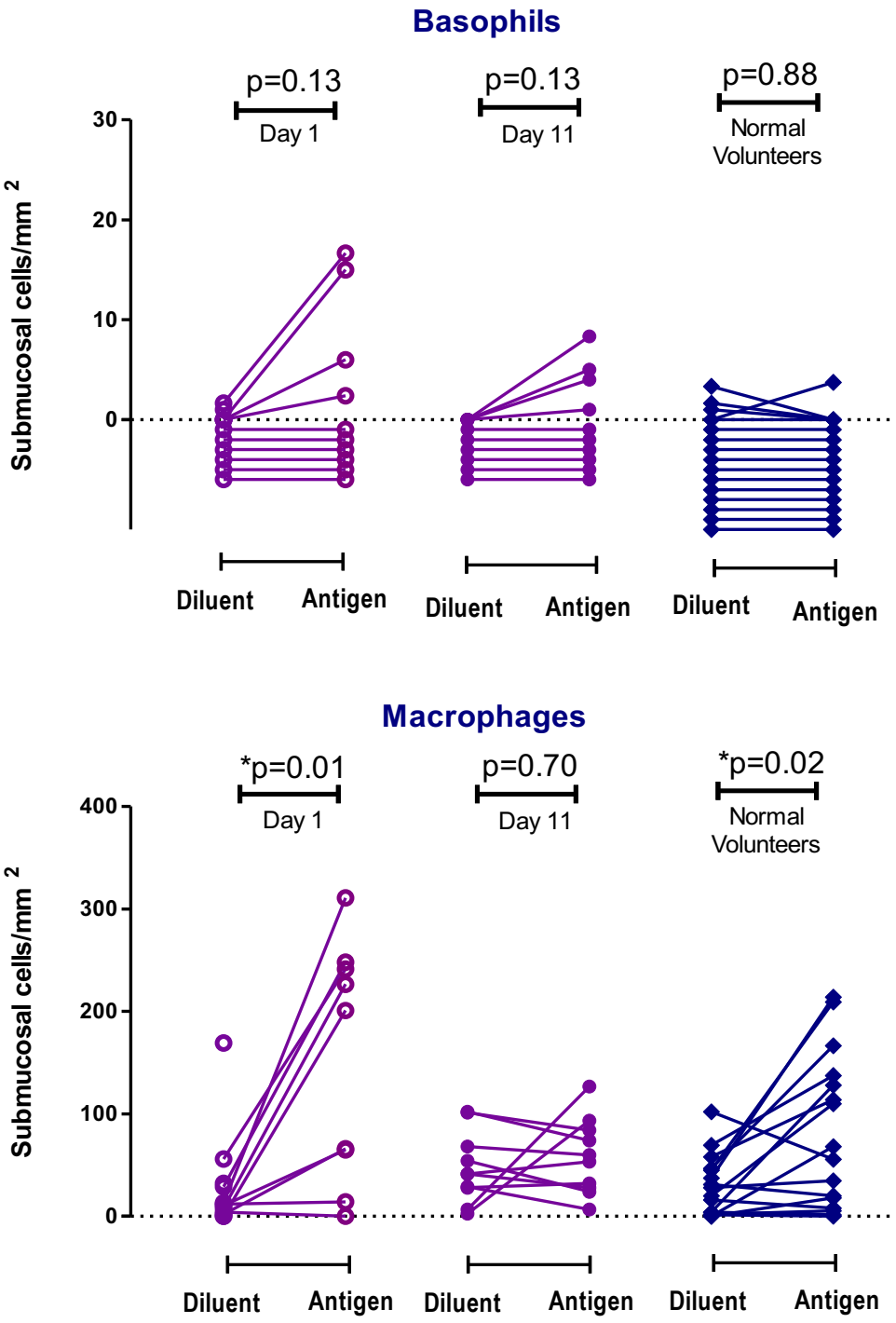


Figure 4.13: Submucosal Inflammatory Cell Counts II: Day 1 (left panel, purple) and Day 11(middle panel, purple) after diluent challenge and allergen challenge for participants with allergic rhinitis (n=12), and on Day 1 after diluent and allergen challenge for Normal non-atopic controls (n=16) (right panel, Blue). Basophils (2D7) and macrophages (CD68) stained using the APAAP technique. Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05

There was no significant change in CD3 positive cells after allergen challenge compared with diluent at either Day 1 or Day 11. CD4 counts were higher after allergen compared with diluent at Day 1 but not at Day 11, where there was no significant change. No changes in CD8 cells were observed in allergic rhinitic participants at either Day 1 or Day 11 post allergen challenge when compared with diluent. In normal non-atopic controls CD8 counts did increase at 24 hours post allergen challenge compared to diluent ($p=0.04$) (figure 4.15) with no significant difference in CD3 & CD4 numbers.

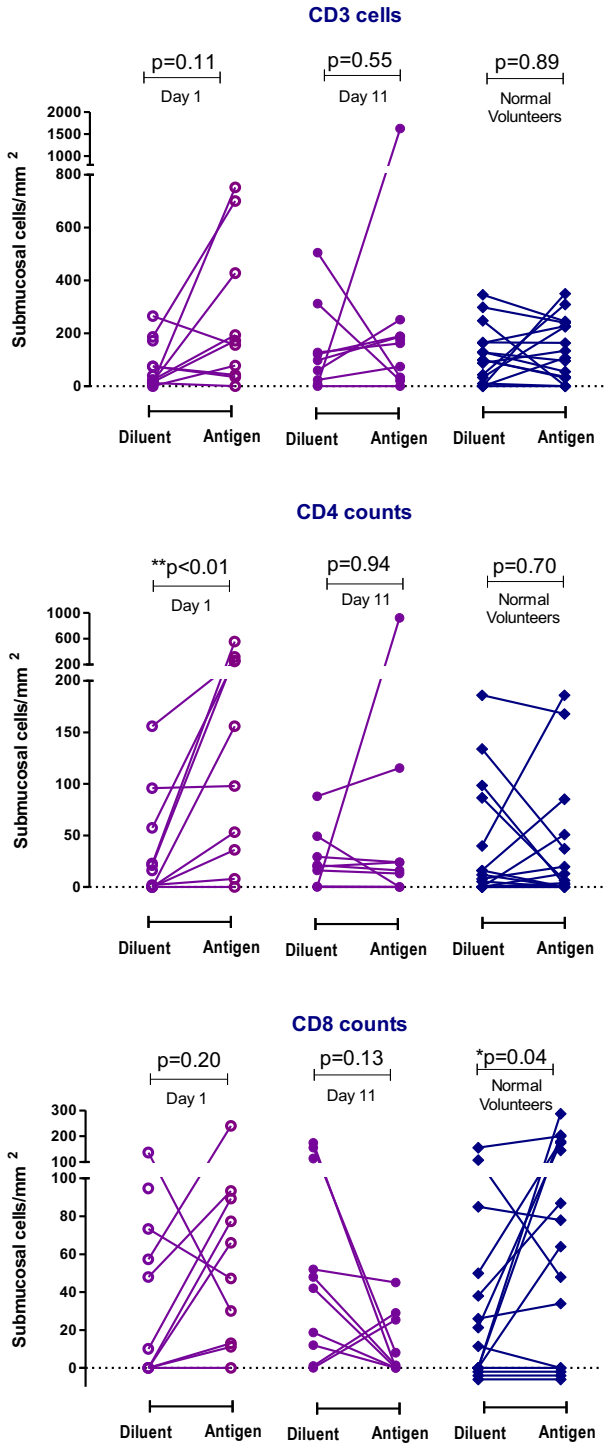


Figure 4.14: Submucosal T Cell Counts. Day 1(left panel, purple) and Day 11(middle panel, purple) after diluent challenge and allergen challenge for participants with allergic rhinitis (n=12), and on Day 1 after diluent and allergen challenge for Normal non-atopic controls (n=16) (right panel, Blue). CD3, CD4 and CD8 T cells stained using the APAAP technique. Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05

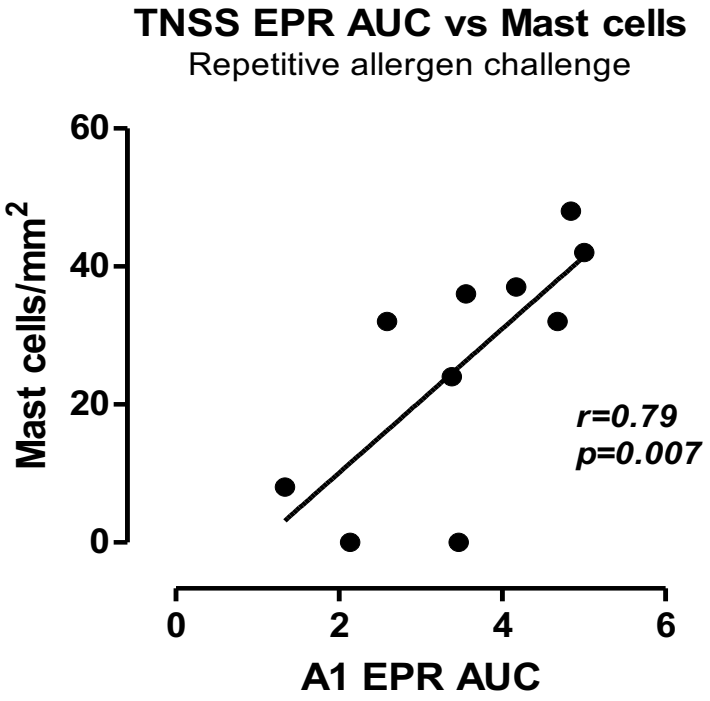


Figure 4.15: Significant correlation between early phase response (total nasal symptoms scores-AUC) post first dose of allergen challenge and mast cells in nasal submucosa

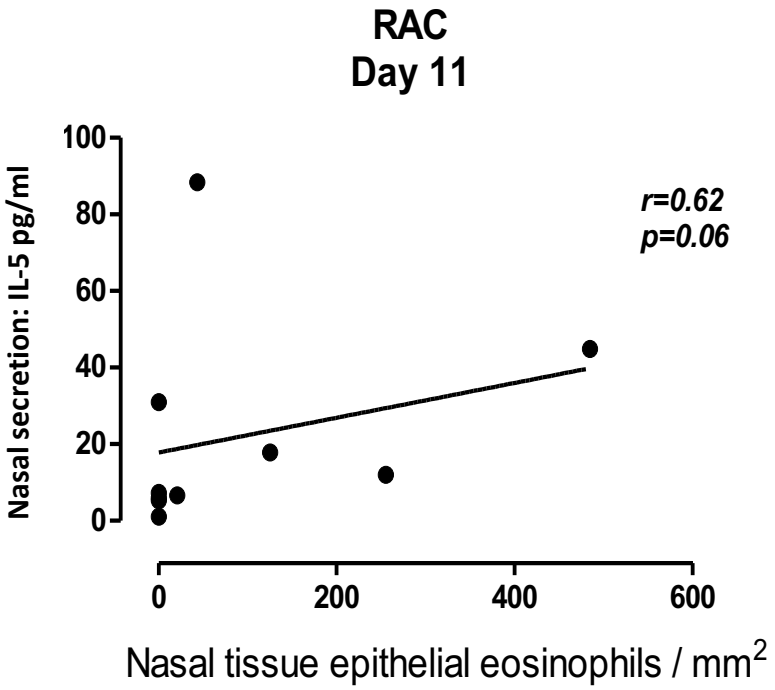


Figure 4.16: Correlation between IL-5 levels from nasal secretions and nasal tissue epithelial eosinophils post 5th dose of repetitive allergen challenge.

Discussion

In this chapter, changes in local nasal cytokines and inflammatory cells in the nasal epithelium and submucosa were reported during natural allergen exposure in participants with allergic rhinitis and at 24 hours following a single allergen challenge and at 11 days following a series of 5 repeated allergen challenges at 2-day intervals. During natural allergen exposure, comparison was made between allergic rhinitis in-season responses and normal non-atopic controls. In the induced model of allergic rhinitis, comparison was made 24 hours after allergen challenge versus 24 hours after diluent challenge at Day 1 and Day 11 of repetitive allergen challenge.

Cytokine profiles during natural exposure and induced models of allergic rhinitis

A number of multiplex analyses are available for cytokine measurements, with the meso-scale device (MSD) quantifying protein levels by electrochemiluminescence of bound substrates on a microtitre plate (617). Proteins to be detected may be multiplexed, as a single well may hold a number of detection ligands (618). With all samples performed on the same day by the same operator, and the same batch of plates, variability of the measurements was minimised (619).

Alterations in cytokine milieu occur as local effects in response to allergen challenge (620) (253). Relevant cytokines were measured in serum at pre-determined time points after challenge. No significant changes in the serum cytokines were noted either during natural exposure nor following nasal allergen challenge at 1 or 11 days after repeated challenge, for either allergic or normal control participants. (All cytokine measurements were above detection limit and reproducible). Seasonal exposure demonstrated statistically significant rises in Th2 nasal secretion cytokines: IL-4, IL-5 and IL1-3. In contrast, no changes in Th1 cytokines, INF-γ and IL-2 or cytokine IL-10 were observed in nasal secretions. This demonstrated that Th2 dominates the response in the allergic mucosa during natural allergen exposure and confirms findings of previous studies (209). Persistent elevation in Th2 cytokines in upper airway secretions has been shown to indicate on-going inflammation (621).

Repetitive allergen challenge (RAC) induced a significant rise in IL-5 in nasal secretions at 11 days without concomitant rise in Th1 cytokines nor IL-10. Local nasal Th2 cytokines were higher in allergic rhinitics compared to normal controls (Figure 4.9). As such, further increases post allergen challenge, may be anticipated to be modest and may not achieve statistical significance, even though the post challenge levels were numerically higher than for the normal control participants. Indeed, comparison in and out of season reveals no significant increase in IL-4 ($p=0.43$), IL-5 ($p=0.10$) or IL-13 ($p=0.95$) (data not shown). A further influencing variable may be the time delay between the de novo transcription of a cytokine and its detection in nasal secretions (mRNA may be detected at 24 hours post challenge

via co-localisation techniques which demonstrate an increase in IL-4 mRNA in 2D7 stained basophils post challenge) (253). Transit across the nasal mucosa may result in a time lag before detection in nasal fluid after allergen challenge. Reliable measurement of IL-4 is difficult as it is produced in relatively small quantities, binds rapidly to its receptor (soluble or membrane bound) and is catabolised promptly (622).

Inflammatory cells during natural exposure and induced models of allergic rhinitis

Coupled to Th2 positive cytokine profile of allergic rhinitics, epithelial and submucosal allergic inflammatory granulocytes are seen to rise. Both epithelial ($p=0.08$) and submucosal ($p=0.07$) eosinophil counts are enhanced in allergic rhinitic biopsies compared with normal controls, mirroring the change in nasal secretion Th2 dominant cytokine profile. Repetitive allergen samples show increased epithelial eosinophils at Day 11 (over tenfold), with significant rise in submucosal eosinophil numbers at both Day 1 and Day 11 (over 20x greater) (Table 4.1). As tissue eosinophil levels represent the balance between influx, survival and luminal exit, cell counts may return to relatively normal levels despite continued detection in lavage fluid (206) (623). Epithelial migration and loss may prevent significant rises in cell counts within the mucosa as was also noted by Wachs et al. They demonstrated that inflammatory cells in nasal lavage do, but epithelial inflammatory cell infiltrate does not, increase with repetitive allergen challenge (595). Rises in epithelial eosinophils at two weeks with immediate rise in submucosal cell counts at both 24 hours and two weeks is likely to reflect the kinetics of epithelial accumulation and loss (624). With luminal exit greater than epithelial accumulation at 24 hours, it can be postulated that at two weeks, a stable state is reached where cell rises are seen in both epithelium and submucosa. During natural allergen exposure (in both seasonal and perennial allergic rhinitics), day-to-day fluctuations in allergen concentrations as frequently occur may explain why, in a cross-sectional study where biopsies are taken at a single time point, eosinophil counts may also vary and in particular wane when allergen, such as pollen counts are low. Although such fluctuations are likely to be more pronounced in seasonal rhinitics, perennial allergens may also vary with time (625) (626). This may explain eosinophil counts that are higher but just short of significance in epithelium ($p=0.07$) and submucosa ($p=0.08$) in allergic rhinitics versus normal controls.

As few as 6 participants are needed to achieve statistically significant differences in tissue cellular and cytokine markers following an adequate pollen season (627). This finding was used to inform the decision of group size for nasal polyposis where eosinophilic infiltrate is pronounced as part of disease histology. Meticulous recruitment and screening ensured large study population groups with robust statistically significant differences. These studies recruited numbers in excess of requirement. Nasal polyp samples provided a positive control with marked enhancement of IL-5 and of eosinophil numbers

(both epithelial and submucosal) when compared to non-atopic controls. Atopic nasal polyp patients have been shown to have significantly more IL-5 and ECP in their nasal secretions and more submucosal eosinophils than non-atopic polyp patients or those with allergic rhinitis. However, allergic rhinitis sufferers were shown to have increased eosinophils in their submucosa when compared to non-atopic polyp samples (212). In asthma, airway hyperreactivity (AHR) may persist beyond the resolution of allergic inflammation (628). The role of allergic inflammatory cells is likely to rely on and interact with resident structural cells (such as myofibroblasts) in the maintenance of symptomatic deterioration, such as bronchoconstriction or nasal congestion. Clinical manifestation of allergic rhinitis is not only related to an absolute rise and number/concentration of IL-5 and eosinophils but will be also affected by tissue architecture and structural cell type.

IL-5 was seen to rise significantly after 11 days of allergen challenge, with very little presence in normal non-atopic controls. Elevations in eosinophils following repetitive challenge occurred in parallel to changes in Th2 cytokines figures 4.9, 4.12, 4.13. In a birch pollen study of priming, eosinophil adhesion to VCAM and ICAM increased in season and altered with immunotherapy (629). Clinical symptoms persisted despite waning allergic inflammation. IL-5 dependent and independent mechanisms are crucial for eosinophil mediated remodelling. In support of this, reduction in blood and sputum eosinophil count will reduce asthma exacerbations but will not improve bronchial hyperreactivity and FEV1 as seen with anti-IL-5 therapy (87).

Absolute mast cell numbers showed no increase with seasonal or induced allergen exposure. This may be explained by the possibility that primed and sensitized mast cells degranulate rapidly on antigen stimulation (630) and will not be seen in higher numbers at 24 hours post challenge. In lower airway disease mast cell degranulation reflects disease severity (631). In rhinitis, histamine release is associated with severity of clinical symptoms (632). Further loss of mast cells occurs via luminal exit as both mast cells and histamine are detectable in bronchoalveolar lavage (BAL) (633). Mast cells, under complex chemotactic control (634), contribute to less than 1% of BAL cells and luminal exit may return to normal as quickly as 1 week after a single allergen exposure (635). This mechanism is likely to play a secondary role with ongoing degranulation the main reason behind no observable rise in intact mast cell counts in my studies of both natural allergen exposure and repetitive allergen challenge. The short survival of intact previously primed mast cells after exposure to allergen makes this cell an unlikely candidate to adequately reflect disease severity in allergic rhinitis.

No changes in neutrophil counts were observed during natural allergen exposure in allergic rhinitics compared to normal controls. Following repetitive allergen challenge neutrophils were only elevated

at 11 days following 5 repetitive challenges. This is in harmony with previous studies where lower airway disease may demonstrate a rise in BAL granulocytes (156), but this is not reflected in the upper airways where eosinophils but not neutrophils are increased in nasal washes of asthmatic children. This study demonstrated weak correlation between neutrophil counts and FeNO2 but did not show any link with infective exacerbations such as positivity to rhinovirus on PCR (636) indicating that neutrophils may not necessarily rise during viral illness although evidence to the contrary (rise in IL-8 and neutrophil myeloperoxidase in nasal aspirates) also exists (637). With no significant rise in IL-8 either in seasonal or induced allergen exposure, neutrophil counts fail to demonstrate an increase in the pollen season or indeed following single allergen challenge. In other inflammatory non-allergic rhinological conditions such as chronic rhinosinusitis, neutrophils may not correlate with nasal symptoms, CT or endoscopic findings (638). Following 10 days of repetitive allergen challenge, neutrophil recruitment was more sustained with rise in neutrophil counts in the nasal mucosa, confirming earlier findings (156). Neutrophil numbers show individual variability and may be related to endotoxin concentration of the allergen or possibly an irritant response to repetitive challenge. Although no endotoxin level is available for the allergen used in our study, ALK-Abello emphasise endotoxin levels in grass allergens is extremely low. (639). Also, on mixing the grass allergen extract with mouse spleen cells *in vitro*, no interferon gamma was detected following incubation, arguing against significant endotoxin contamination (S Durham, personal communication).

Macrophage numbers were unaltered in natural seasonal allergen exposure and after 11 days of the RAC protocol but rose on Day 1 24 hours after single allergen challenge. Such quick but transient rise in macrophage number may relate to their ability to function as antigen presenting cells (640). Although not subcategorised into M1 or M2 macrophages, these cells may contribute to a pro-fibrotic/ pro-angiogenic environment linked to their temporal association of TGF-β production and secretion (641). Macrophage ability to phagocytose apoptotic cells via recognition of membrane phosphatidylserine will underscore their relevance during active resolution of inflammation and cell death (642) (643). It has previously been shown that nasal macrophage numbers increase with local allergen challenge and seasonal allergen exposure and decrease in response to glucocorticoid therapy versus placebo control (265) but such changes in cell numbers are not correlated to clinical symptoms.

Basophils are sparsely present in non-challenged mucosa and are not a prominent cell type in asthmatic airways. Their numbers rise in upper airway post bronchial segmental challenge (644). When basophil surface FcεRI receptors are cross-linked by allergen, they play a significant role in allergic late phase reactions, an effect that is likely to be influenced by their adhesion and migration, and strongly influenced by IL-33 (645). Shiraishi et al have shown that post mast cell degranulation,

histamine receptor activation (via H4) on basophils will result in degranulation and further release of IL-4 and IL-13 perpetuating Th2 type inflammation (646). The role of basophils is highlighted by decreases in cell numbers post immunotherapy - subcutaneous immunotherapy decreased epithelial but not sub mucosal basophil numbers. Furthermore, early suppression of basophil activation (647) and clinical relief is linked to reduced basophil sensitivity post immunotherapy (648). In our model of natural allergen exposure, basophils are likely to play an important role in contributing to symptoms. During repetitive allergen challenge, basophils are likely to contribute to late reaction histamine release and contribute to skewing toward a Th2 cellular and cytokine tissue profile.

T cells exhibited no significant alteration in their CD4 or CD8 subsets following natural allergen exposure, in keeping with previous observations in nasal mucosae (181). Following single allergen challenge (Day 1) there was a temporary significant rise in CD4 T cell subsets at 24 hours, as previously shown by our group (649). This significance is lost after two weeks of repetitive allergen challenge (day 11 diluent versus allergen). Dendritic cells show Th2 polarising properties with repeated stimulation (650); an effect that is mediated through temporal changes in a number of cytokines such as IL-4, IL-12 and TSLP (651) (652). Lymphocytes work in synergy with such cytokines, for example TSLP enhances CD80 and CD40 expression on dendritic cells and TSLPR is expressed by T cells. Furthermore TSLP activates B cells via STAT5 and such local tissue resident B cells persistently produce IgE in ex-vivo transplants even out of season, providing evidence of local IgE synthesis and tissue priming (653). Mast cell and T cell derived IL-25 is capable of enhancing Th2 cytokine production via T cell independent mechanisms (654). Furthermore, IL-25 may exacerbate airway hyperresponsiveness in the absence of Th2 cytokines, directly induce eosinophilia, activate DCs directly and drive allergic inflammation via Th2 cell independent mechanisms. Initial T cell increases may be of sufficient magnitude to continually drive allergic inflammation and symptomatic deterioration without the need for persistent elevation in absolute T cell numbers.

Repetitive allergen challenge faithfully replicates natural allergic inflammation

The purpose of the repetitive allergen challenge (RAC) protocol was to establish how faithfully it can, both clinically and histologically, replicate natural allergen exposure (NAE) Table 4.2. This repetitive allergen protocol demonstrated priming of the early phase at day 5 and of the late phase at day 11 via clinical symptom scoring. No biopsies were taken at day 5 as the biggest difference was expected to be at the start and the end of a multiple challenge protocol. As a result, comparisons were made between diluent versus allergen Day 1 and Day 11. The clinical priming observed was likely linked to persistent recruitment of mast cells into sensitised tissue where degranulation and constant neuronal pathways sustain clinical symptoms of rhinoconjunctivitis. Day 11 IL-5 levels are consistent with

ongoing eosinophilic recruitment, migration and epithelial shedding. After a time such migration may slow up as epithelial eosinophils are increased at two weeks, at which point such trafficking is likely to have reached a steady state (655). A cardinal feature of allergic inflammation is the accumulation of epithelial and submucosal eosinophils following both single and repetitive allergen challenge. Increases in macrophages endowed with both phagocytic as well as antigen presenting capacity, in the context of possible TGF-β secretion (see Chapter 5), enables coordination of clearance of apoptotic cells and possibly encourages remodelling via TGF-β family signalling (such as activin, TGF-β and BMPs). Proteinase 3 (PR3) and azurocidin boost monocyte recruitment and, in concert with other neutrophil granule contents, may strengthen macrophage phagocytic action (656) providing a continuous feedback loop between granulocytes during allergic inflammation.

There is an overlapping picture of T cells and granulocytes from 6 hours onwards post allergen challenge. The repetitive allergen challenge protocol was better able to demonstrate a CD4+ cell change with such cells increased at day 1 post allergen challenge. T cells contribute to late asthmatic reactions (657), late phase reactions in the skin (658) and late phase reaction in the nose (659), with a significant rise seen in repetitive allergen challenge but not in natural allergen exposure protocols. This study did not examine if such differences were linked to allergen specific T cells. Nonetheless, this study confirms that persistent and repetitive contact with allergen is better able to demonstrate a significant rise in overall CD4+ T cell numbers.

Absence of Th2 profile and elevated Th1 profile in nasal fluid in non-atopic controls

In normal non-atopic controls during the repetitive allergen challenge protocol, there was very little IL-5 signal detected at baseline and no increase post challenge. In addition, normal controls had few if any eosinophils in their nasal mucosa. In contrast a rise in IFN-γ (*p=0.01) and IL-2 (*p=0.01) cytokines in nasal fluid at 24hrs post single allergen challenge in normal controls was significant compared to allergic rhinitics. This may represent a 'natural' preferential skewing of the immune response in favour of a Th1 response as a 'normal' response to exposure to foreign proteins including grass pollen extract after challenge.

The increase in epithelial mast cells 24 hours after challenge in non-atopic controls but not in allergic rhinitics is more difficult to explain. It may possibly reflect 'absence of degranulation' in normal individuals, whereas the lack of an increase in mast cells in the allergic rhinitics may reflect a relative absence of mast cell staining due to degranulation and loss of tryptase staining. It has previously been shown that nasal mast cell numbers increase with local allergen challenge, seasonal allergen exposure and decrease in response to glucocorticoid therapy versus placebo control (265) but such changes in

cell numbers are not correlated to clinical symptoms. In normal non-atopic participants these mast cells are not primed and will not release inflammatory cytokines on contact with allergen resulting in absence of clinical symptoms.

Increases in macrophage numbers may reflect macrophage ability to function as an antigen presenting cells, and to trigger altered T cell responses with CD8+ T cells and IL-13, with IL-10 also increased at 24 hours post allergen challenge in normal non atopic controls. Allergen sensitised rats show enhanced DC antigen presentation following allergen challenge (266) and increased IgE when combined with depletion of alveolar macrophages. The authors conclude that such macrophages are at least partly responsible for immunological tolerance. On its own IL-13 does not drive Th2 differentiation as naïve T cells lack the IL-13 receptor α chain (660). Evidence of the anti-inflammatory property of IL-13 (661) demonstrates its ability to reduce monocyte derived IL-1, IL-8 and TNF-α (662). In addition, IL-13 may induce TGF-β secretion by epithelial cells (663) and this may occur in the absence of inflammation. In non-atopic individuals IL10 and IL13 may work in harmony to prevent monocytes acquiring an inflammatory secretory profile and differentiating into effective antigen presenting DCs (664).

	Seasonal allergic rhinitis				Normal Healthy control		*p values*		
	1st dose Diluent	1st dose Allergen	5th dose Diluent	5th dose Allergen	Diluent Normal	Allergen Normal	1st Dose Diluent v Allergen	5th Dose Diluent v Allergen	Healthy controls Diluent v Allergen
	Epithelial Inflammatory cells (medians 25-75 centile)								
Eosinophils	0 (0)	0 (0-16)	0 (0)	10 (0-157)	0 (0)	0 (0)	0.5	0.06	0.5
Mast cells	0(0-3)	0(0-3.8)	0 (0-8.3)	0 (0)	0 (0)	0 (0-9)	0.63	0.5	0.06
	Submucosal Inflammatory cells (medians 25-75 centile)								
Eosinophils	0 (0)	53 (12-150)	0 (0)	20 (0-85)	0 (0-0.6)	0 (0-0.6)	**<0.01**	**0.03**	0.81
Mast cells	29 (2-50)	32 (13-38)	12 (4-38)	9 (0-37)	24 (8-45)	23 (14-41)	1.00	0.57	0.61
Basophils	0	0(0-8)	0	0 (0-4)	0	0	0.12	0.12	0.8
	Total epithelial and submucosal Inflammatory cells (medians 25-75 centile)								
Eosinophils	0 (0-9)	53 (12-166)	0 (0-2.8)	32 (5-292)	0 (0-1)	0 (0-0.6)	**0.01**	**<0.01**	0.57
Mast cells	28.5 (5-50)	34 (13-49)	12.6 (4-51)	9.5 (0-46)	24 (8.5-45)	35 (23-48)	0.91	0.57	0.20
	Nasal secretion cytokines (medians 25-75 centile)								
IL-4	0.4(0.2-1)	0.5(0.3-1.5)	0.6(0.1-1.1)	0.8(0.3-2)	0.6(0.3-0.8)	0.4(0.3-0.6)	0.9	0.4	0.4
IL-5	4.5(1.5-8)	6(3-16)	6(1.5-8)	9.6(5.7-34)	3.7(2.4-6)	4.3(3-5.2)	0.4	**0.01**	0.7
IFN-γ	3(1-4)	2(1-5)	3(1-4)	2.5(1-3)	4.6(2.6-7)	4.8(3-6)	0.85	0.38	0.78
IL-10	3.5(1.8-13)	3.7(2.3-12)	6.8(1.8-20)	7.1(1.7-16)	7.8(4.8-12)	11(8-18)	0.49	0.7	0.07
	Serum secretion cytokines (medians 25-75 centile)								
IL-4	12 (1-54)	28 (1-137)	20 (1-106)	26 (2-132)	14 (0-72)	4.5 (1-33)	0.7	0.2	0.04
IL-5	8 (0-81)	12 (0-210)	7 (1-88)	9 (1-183)	5 (0-111)	4 (0-43)	0.4	1.0	0.2
IFN-γ	50 (4-322)	97 (4-921)	61 (2-481)	70 (4-854)	40 (1-449)	22 (0-154)	0.31	0.72	0.76
IL-10	15 (2-113)	21 (2-334)	14 (2-195)	16 (2-255)	14 (3-141)	12 (2-61)	0.67	0.57	0.31

Table 4.1: Nasal tissue cells and secretion inflammatory cytokines in RAC model of allergic rhinitis

	Seasonal allergic rhinitis RAC				Seasonal allergic rhinitics NAE		*p values*		
	1st dose Diluent	1st dose Allergen	5th dose Diluent	5th dose Allergen	Out of season	In season	1st Dose RAC Diluent v Allergen	5th Dose RAC Diluent v Allergen	NAE Out v in season
	Epithelial Inflammatory cells (medians 25-75 centile)								
Eosinophils	0 (0)	0 (0-16)	0 (0)	10 (0-157)	0 (0)	0 (0)	0.5	0.06	0.84
Mast cells	0(0-3)	0(0-3.8)	0 (0-8.3)	0 (0)	0 (0-9)	0 (0-14)	0.63	0.5	0.41
	Submucosal Inflammatory cells (medians 25-75 centile)								
Eosinophils	0 (0)	53 (12-150)	0 (0)	20 (0-85)	0 90-0.13)	0 (0-5)	**<0.01**	**0.03**	0.55
Mast cells	29 (2-50)	32 (13-38)	12 (4-38)	9 (0-37)	38 (27-58)	17.3 (8-46)	1.00	0.57	0.06
Basophils	0	0(0-8)	0	0 (0-4)	0 (0)	0 (0)	0.12	0.12	0.87
	Total epithelial and submucosal Inflammatory cells (medians 25-75 centile)								
Eosinophils	0 (0-9)	53 (12-166)	0 (0-2.8)	32 (5-292)	0 (0)	0 (0)	**0.01**	**<0.01**	0.62
Mast cells	28.5 (5-50)	34 (13-49)	12.6 (4-51)	9.5 (0-46)	18.3 (0-39)	10 (0-40)	0.91	0.57	0.4
	Nasal secretion cytokines (medians 25-75 centile)								
IL-4	0.4(0.2-1)	0.5(0.3-1.5)	0.6(0.1-1.1)	0.8(0.3-2)	0.4 (0.2-0.6)	0.4 (0.1-1)	0.9	0.4	0.43
IL-5	4.5(1.5-8)	6(3-16)	6(1.5-8)	9.6(5.7-34)	5.9 (3.6-8.5)	7.9 (4.7-12.3)	0.4	**0.01**	0.11
IFN-γ	3(1-4)	2(1-5)	3(1-4)	2.5(1-3)	2.4 (0.5-4.5)	2.6 (0.3-4.5)	0.85	0.38	0.89
IL-10	3.5(1.8-13)	3.7(2.3-12)	6.8(1.8-20)	7.1(1.7-16)	7.9 (4.4-11.7)	5.5 (4.1- 9.8)	0.49	0.7	0.22
	Serum secretion cytokines (medians 25-75 centile)								
IL-4	12 (1-54)	28 (1-137)	20 (1-106)	26 (2-132)	5 (2-29)	8 (2.31)	0.7	0.2	0.16
IL-5	8 (0-81)	12 (0-210)	7 (1-88)	9 (1-183)	4 (1.5-14.5)	2 (1-14)	0.4	1.0	0.61
IFN-γ	50 (4-322)	97 (4-921)	61 (2-481)	70 (4-854)	26 (10-128)	17 (13.5-94)	0.31	0.72	0.09
IL-10	15 (2-113)	21 (2-334)	14 (2-195)	16 (2-255)	12 (4-33)	5 (3-27)	0.67	0.57	0.62

Table 4.2 Nasal tissue cells and secretion inflammatory cytokines in RAC versus NAE model of allergic rhinitis

Summary of chapter

Both natural allergen exposure and repetitive allergen challenge protocols cause Th2-mediated eosinophilic inflammation that coincides with increases in nasal symptoms. Natural exposure in our hands was able to elucidate a Th2 cytokine profile with concomitant rise in epithelial and submucosal eosinophils. The repetitive allergen challenge protocol developed an eosinophilic inflammatory infiltrate and was appropriately capable of reproducing seasonal findings. Normal non-atopic controls develop a tolerogenic cytokine and cellular profile where macrophages are likely to play a central role as antigen presenting cells.

Chapter 5 : Remodelling in Natural Allergen Exposure and Repetitive Allergen Challenge models of Allergic Rhinitis

Introduction

In this chapter the effect of allergen during natural and induced models of allergic rhinitis was investigated. A significant body of evidence is present to substantiate remodelling processes in the lung on allergen exposure and resultant Th2 exemplified allergic inflammation. In the context of asthma, dissociation of inflammation and remodelling is seen with the latter thought to be responsible for prolonged clinical deterioration even in the absence of inflammation. Evidence that remodelling significantly contributes to the disease process is lacking in the nose.

Analyses of upper airway mucosal topographical alterations show conflicting evidence. Whilst changes in remodelling markers are suggestive of remodelling changes resulting from allergen exposure in sensitised individuals, heterogeneity of comparison groups makes for very difficult interpretation of the absolute presence of remodelling (665). The previous chapters have demonstrated the rigour of patient selection criteria and have shown how both natural and induced models precipitate a Th2 driven allergic inflammation. With significant Th2 characterised cytokine responses in both models and eosinophilic inflammation, this chapter examined the presence and degree of structural remodelling. As any alterations in tissue topography will require coexisting interplay between a number of markers, this chapter examined possible tissue markers with well-established roles in lower airways remodelling and looked at their presence during upper airway allergic inflammation.

Results: Natural Allergen Exposure Model

Allergic rhinitic samples did not demonstrate increased epithelial shedding and breakdown of epithelial integrity compared with normal non-atopic controls. Inferior turbinate samples (tNP) from nasal polyp patients similarly failed to demonstrate loss of epithelial continuity. Basement membrane thickness did not change in AR participants but was significantly less ($p<0.01$) in tNP. The percentage area occupied by mucus/ filled goblet cells and the staining intensity of epithelial mucus content was no different between normal controls, AR, or NP participants (figure 5.1, 5.2) (photomicrograph 5.1).

Allergic rhinitis did not affect epithelial shedding, basement membrane thickness or epithelial mucus content. Nasal polyp participants had thinned basement membranes in the absence of either epithelial shedding or significantly different epithelial goblet cell and mucus content.

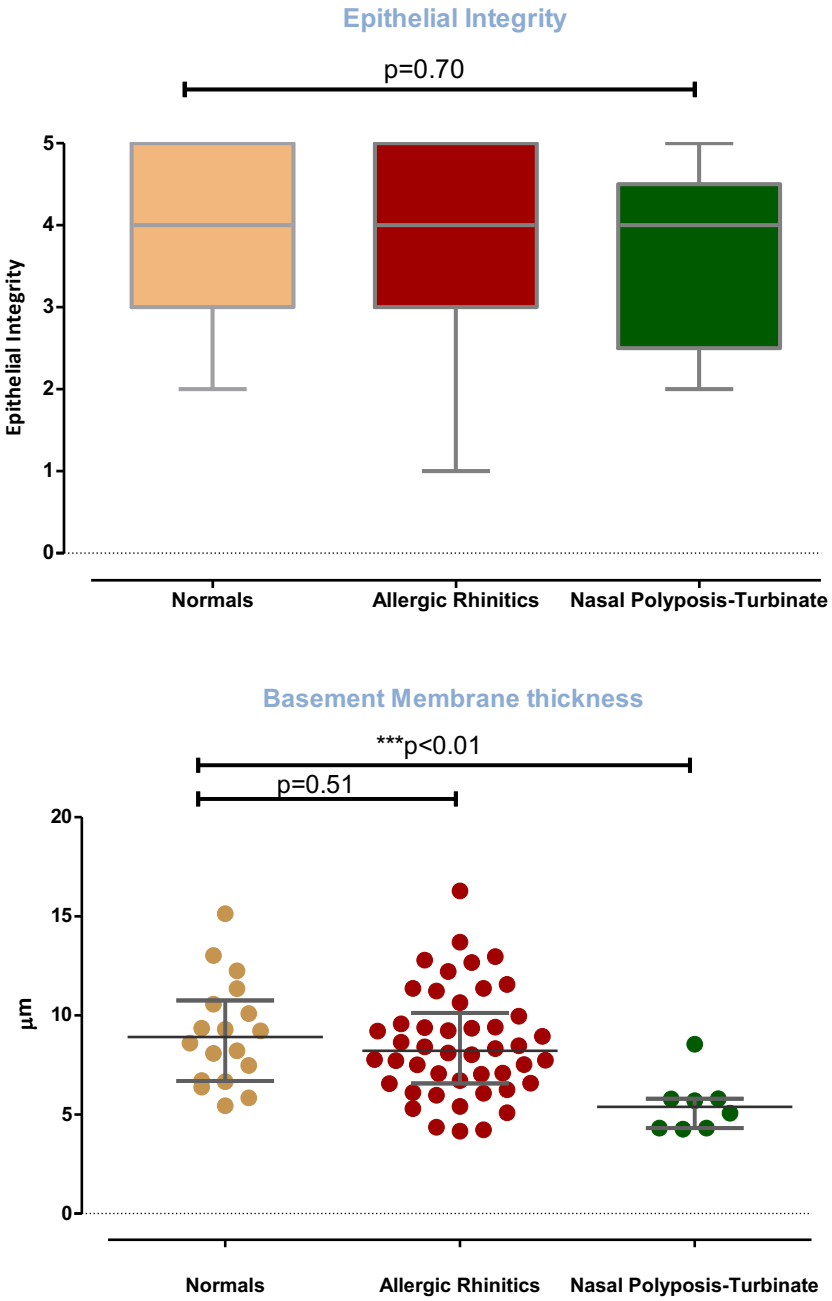


Figure 5.1: Epithelial Integrity and Basement Membrane thickness: comparing persistent allergic rhinitics (n=46) with healthy controls (n=18) or turbinate tissue from CRSwNP (n=8) and healthy controls (n=18). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05

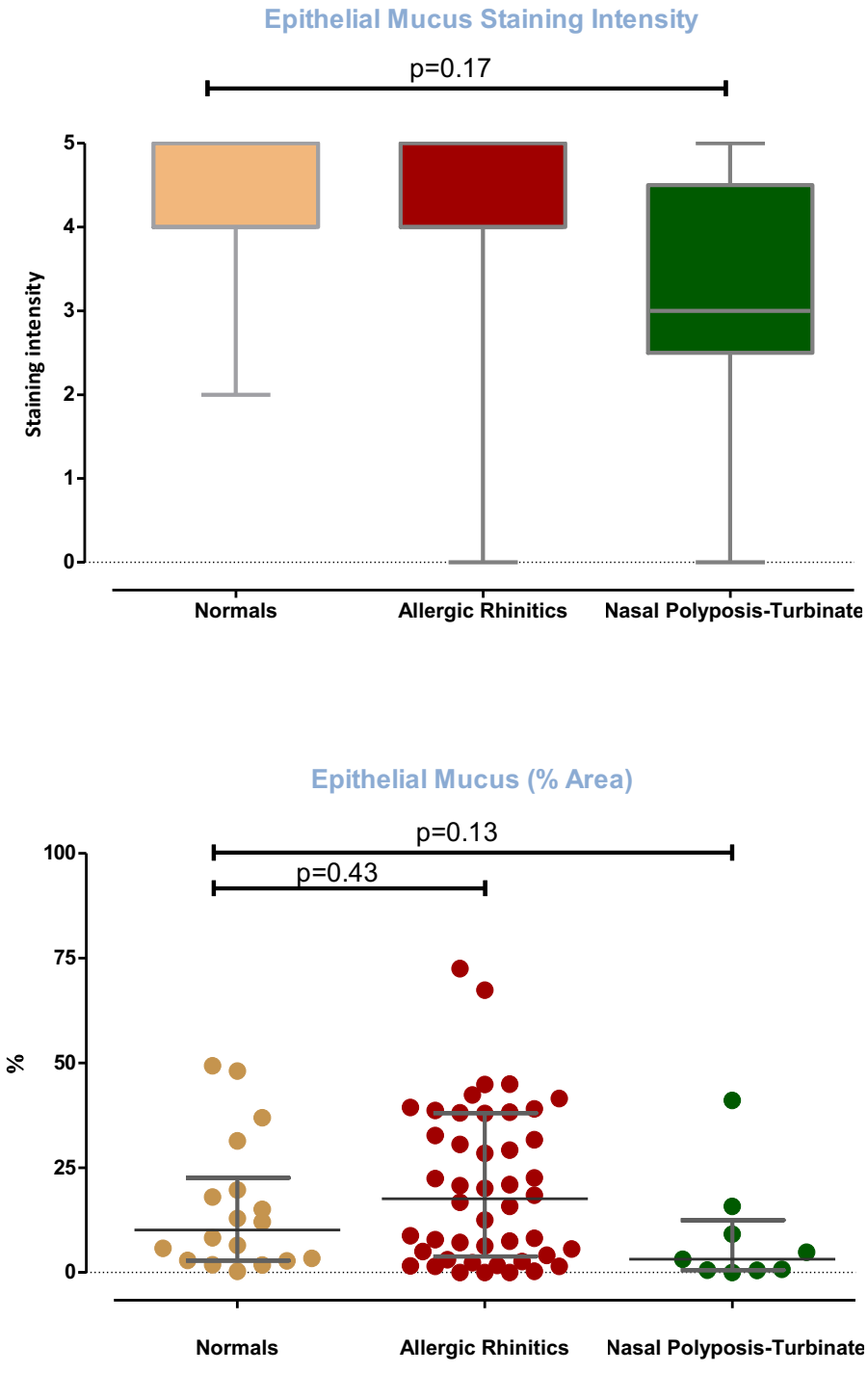
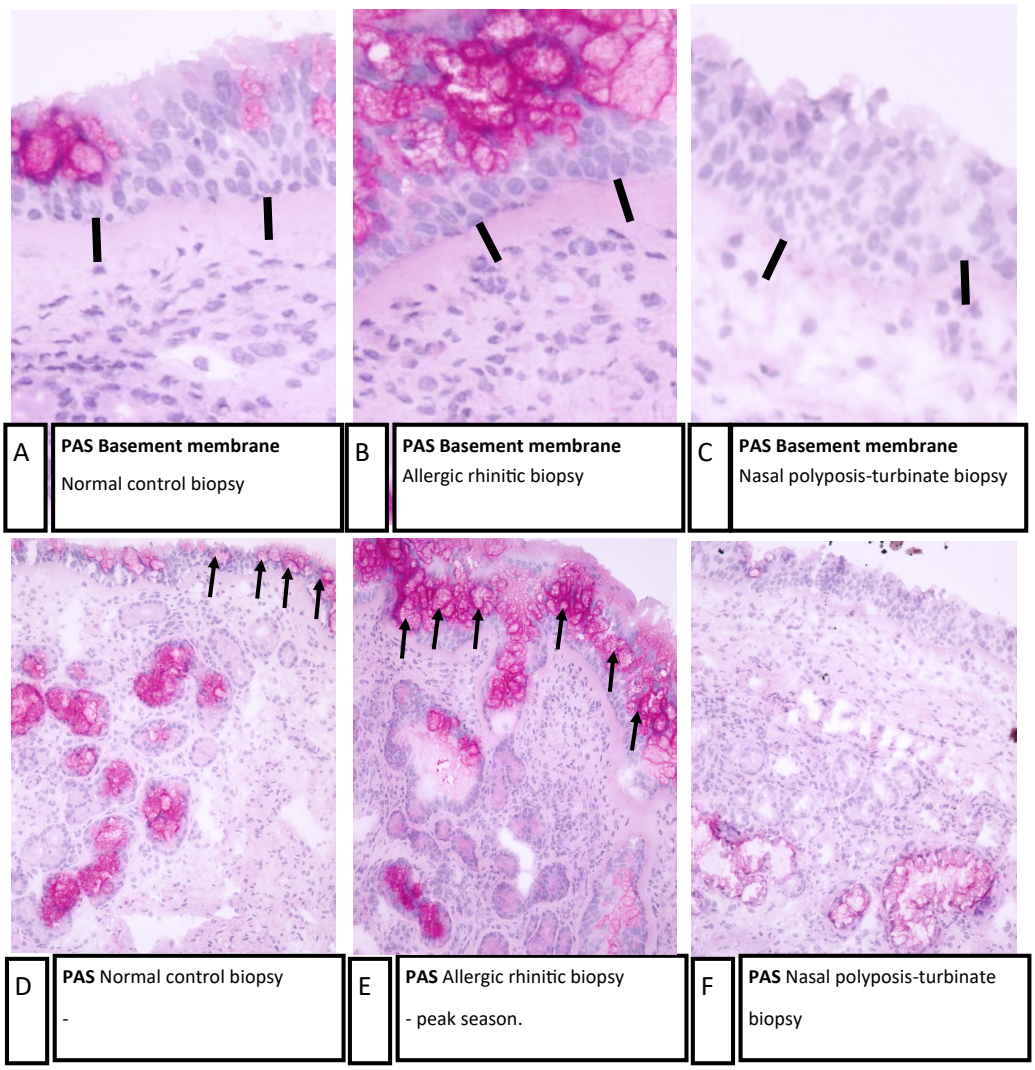


Figure 5.2: Intensity of epithelial mucus staining and Epithelial mucus content (% of total epithelial area): comparing persistent allergic rhinitics (n=46) with healthy controls (n=18) or turbinate tissue from CRSwNP (n=9) and healthy controls (n=18). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05



Photomicrograph 5.1: Periodic Acid Schiff staining (PAS) with haematoxylin counterstain. Basement membrane thickness was significantly reduced in nasal polyposis turbinate biopsies (C) compared to turbinates from normal controls (A) and allergic rhinitics (B). Black bar indicates the width of the membrane in normal control biopsies, the same sized bar extends into the submucosa of nasal polyposis turbinate biopsies. Epithelial integrity was similar in normal control (D), allergic rhinitic (E) and nasal polyposis turbinates. Photographed using x20 lens.

The four markers used for lymphatic vessels and lymphangiogenesis were total number of D2-40 positive cells, total number of D2-40 positive vessels, percentage submucosal area occupied by lymphatic vessels, and size of lymphatic vessels within the submucosa. All four markers failed to demonstrate significant differences between allergic rhinitics and normal non-atopic controls (figures 5.3a and b). Three of the markers (total number of D2-40 positive vessels, percentage lymphatic area, and lymphatic vessel size) were statistically increased in nasal polyps (tNP) with the fourth marker- total number of D2-40 positive cells- tending to but not reaching significance ($p=0.07$).

Altered lymphatics are not a feature of allergic rhinitis under natural allergen exposure conditions. Turbinate samples from nasal polyposis patients do demonstrate increased lymphatics and this appears to be a feature of the disease in this clinical group.

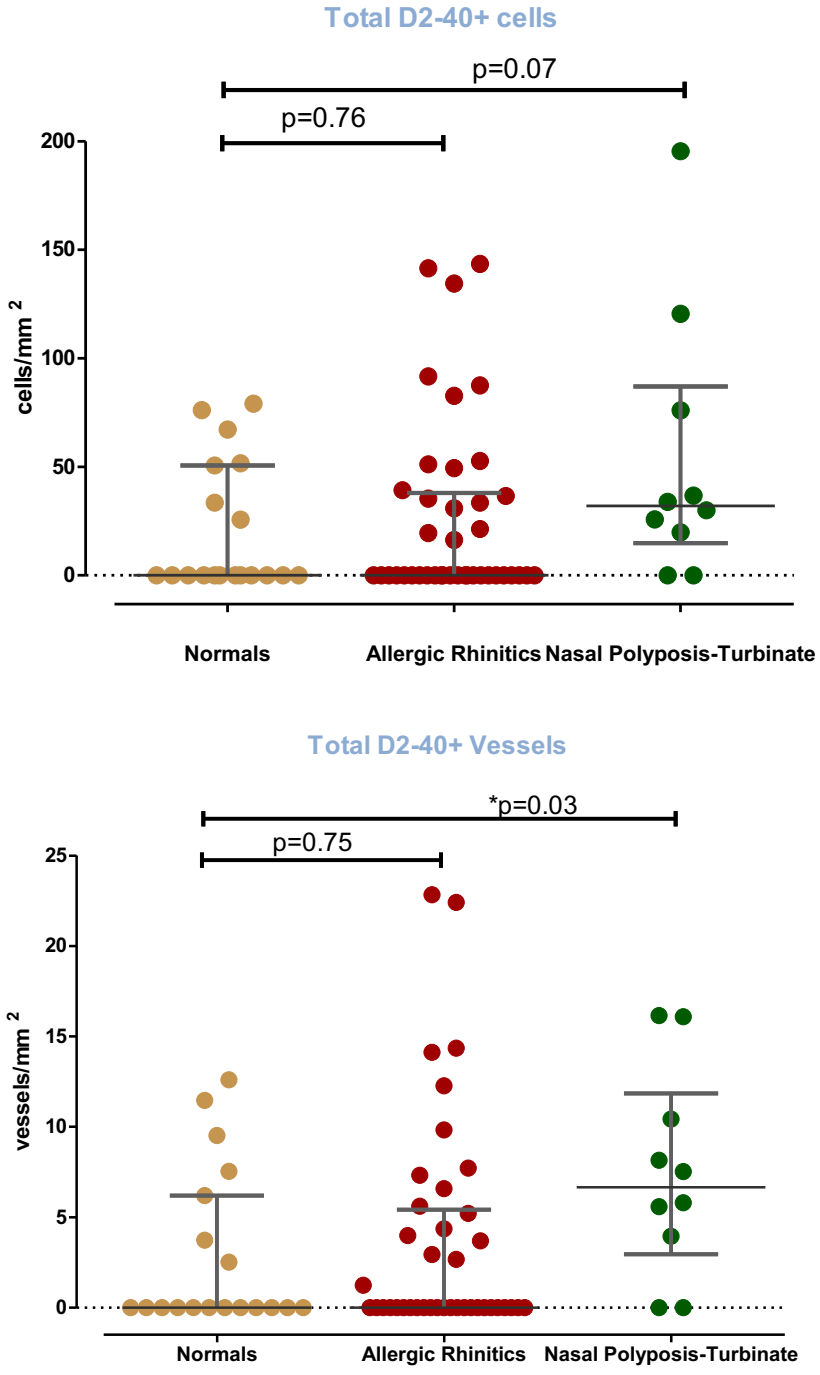


Figure 5.3a: Lymphangiogenesis: using D2-40 as lymphangiogenic marker. Counted as total number of D2-40 +ve cells and D2-40 +ve vessels/mm^2: comparing persistent allergic rhinitics (n=41) with healthy controls (n=19) or turbinate tissue from CRSwNP (n=10) and healthy controls (n=19). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05

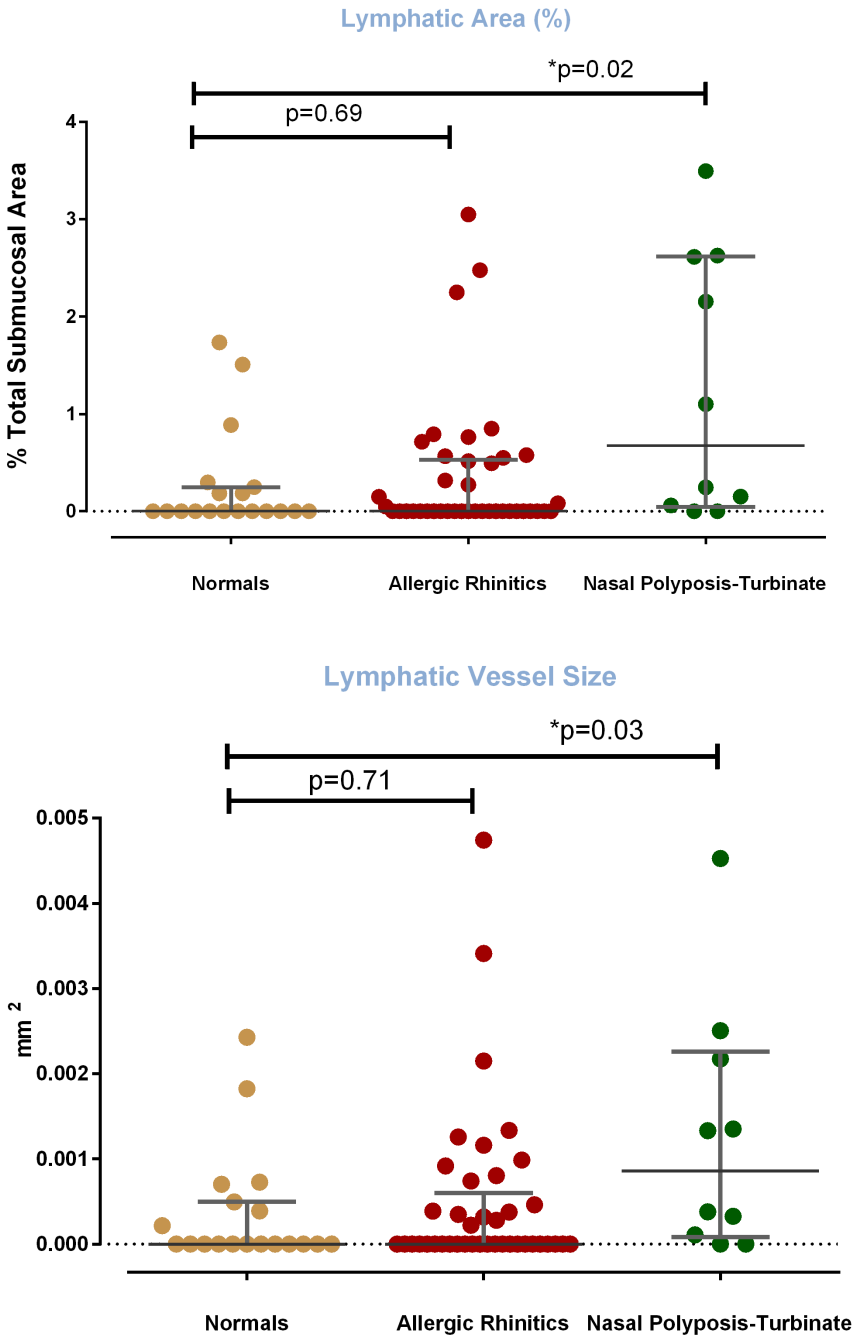


Figure 5.4b: Lymphangiogenesis: using D2-40 as lymphangiogenic marker. Lymphatic area (% total submucosal area) and lymphatic vessel size mm²: comparing persistent allergic rhinitics (n=41) with healthy controls (n=19) or turbinate tissue from CRSwNP (n=10) and healthy controls (n=19) Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05

Submucosal glands were quantified by three parameters, expressed as percentage of the total submucosal area: total glandular area, total intra-glandular area and total gland tissue area. All three measurements failed to demonstrate a significant difference between allergic rhinitics and normal non-atopic controls. No difference was seen between negative controls and nasal polyp samples (figure 5.4).

There was no significant difference in secretory submucosal gland tissue between allergic rhinitics and non-atopic normal controls. Alteration of submucosal gland tissue was not a feature of nasal polyposis.

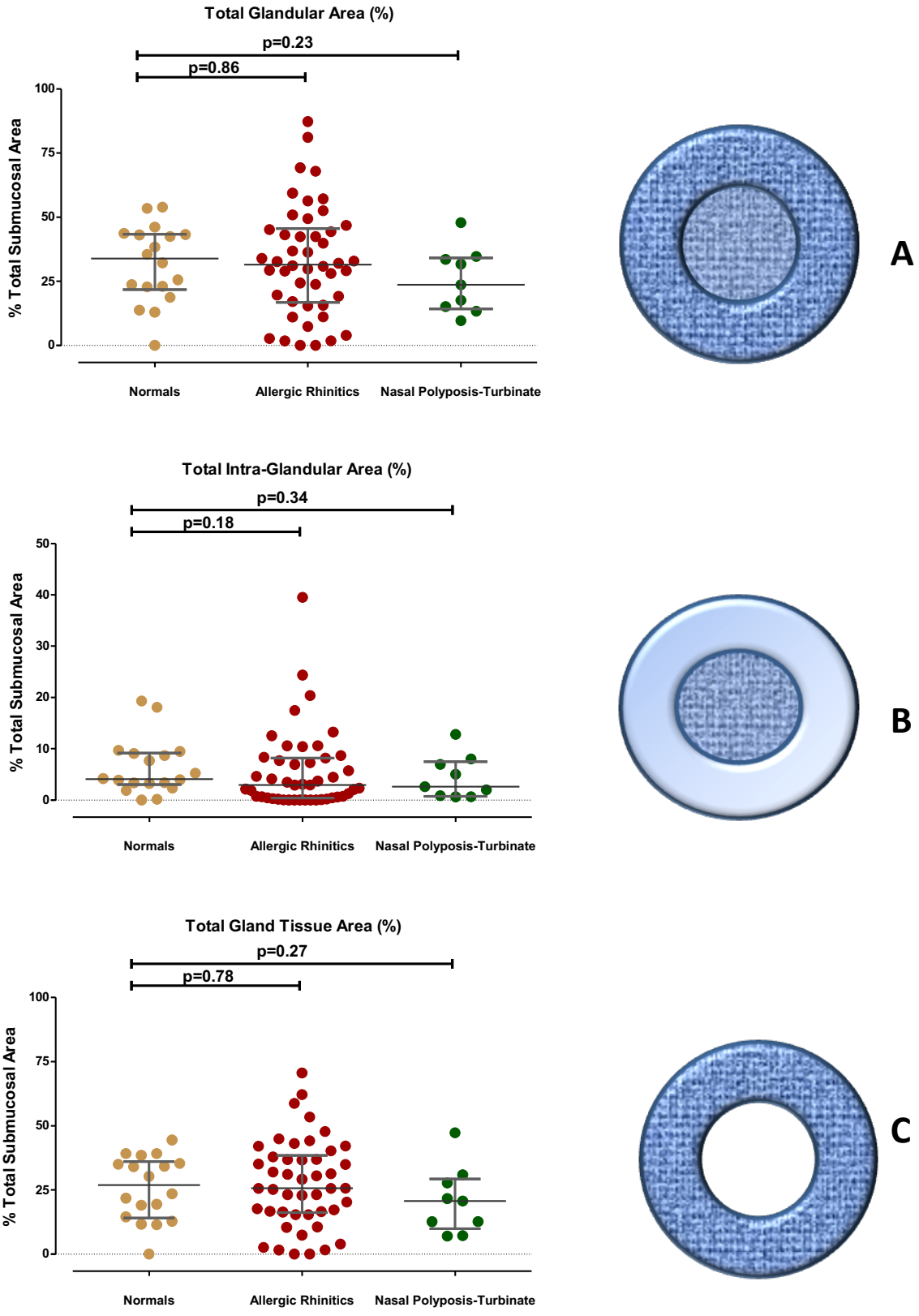


Figure 5.5: Submucosal Glandular Area: measured area is represented by textured blue background on schematic gland drawing, representing (A) gland tissue and secretory contents, (B) secretory content area only and, (C) glandular tissue only. Comparing persistent allergic rhinitics (n=46) with healthy controls (n=18) or turbinate tissue from CRSwNP (n=9) and healthy controls (n=18). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

Differences in angiogenesis and presence of established blood vessels was measured using four criteria reflecting angiogenic markers: total number of CD31+ve cells, total number of CD31+ve vessels, percentage vascular area, and absolute vascular vessel size (figures 5.5a and b). All four markers demonstrated no significant difference between allergic rhinitics and normal controls. Percentage of submucosal area occupied by blood vessels ($p=0.05$) and the total number of CD31 +ve cells ($p=0.08$) were higher in nasal polyposis turbinate samples than non-atopic normal controls.

VEGF staining was measured by four parameters: total number of VEGF positive cells, total number of VEGF positive vessels, percentage of VEGF positive submucosal vascular area (as compared to total submucosal vascular area) and VEGF positive vessel size. VEGF negative measures included: total number of VEGF negative vessels, percentage of VEGF negative submucosal vascular area (as compared to total submucosal vascular area) and VEGF negative vessel size. There was no significant difference between allergic rhinitis and normal non-atopic controls in any of the above VEGF positive or VEGF negative markers for increased submucosal vascularity.

Increased submucosal vascularity as per CD31 staining measurements is not a feature of allergic rhinitics during natural allergen exposure. Nasal polyposis turbinates demonstrated increased vascularity as per increase in total submucosal vascular area using CD31 as a marker. VEGF positive and VEGF negative vascular staining was not significantly different in allergic rhinitics during natural allergen exposure.

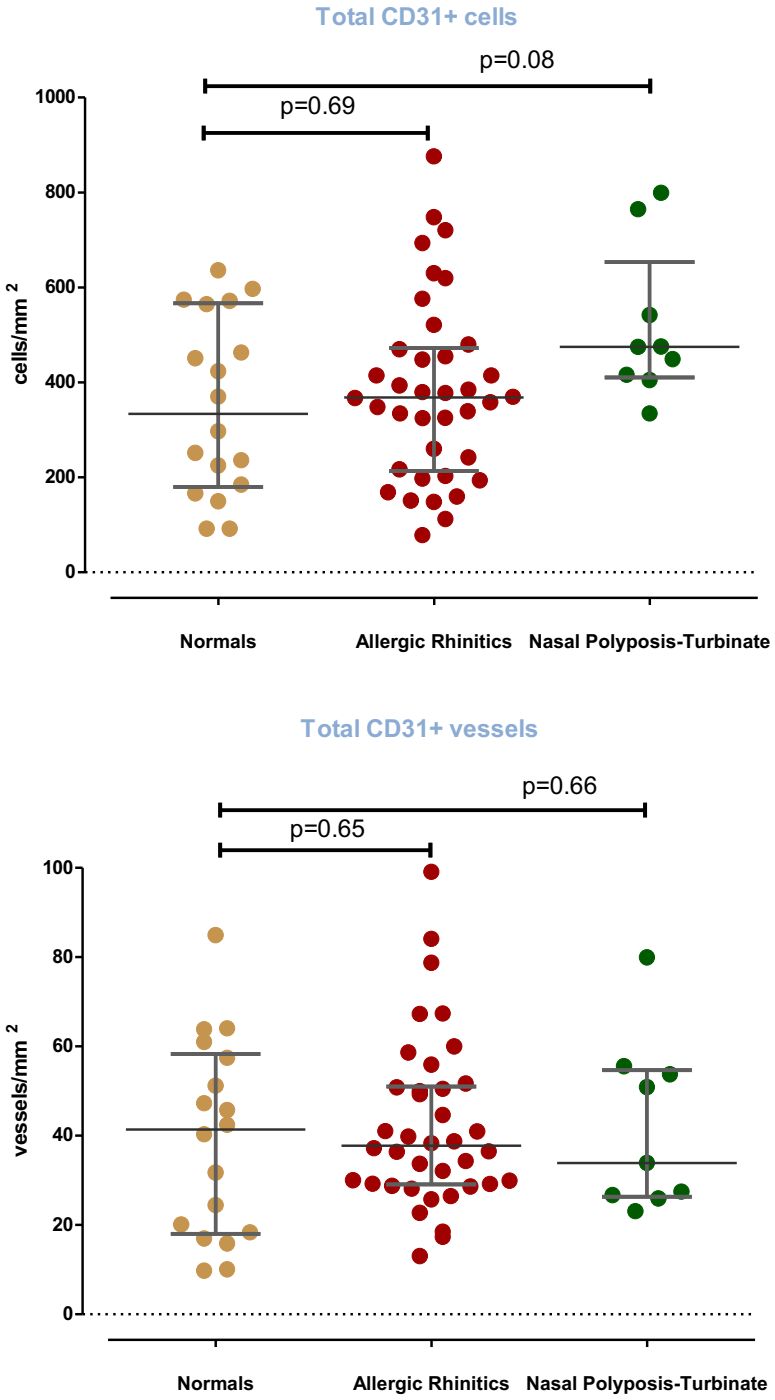


Figure 5.6a: Angiogenesis: CD31 as angiogenic marker. Counted as total number of CD31+ve cells and vessels/mm². Comparing persistent allergic rhinitics (n=38) with healthy controls (n=18) or turbinate tissue from CRSwNP (n=9) and healthy controls (n=18). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

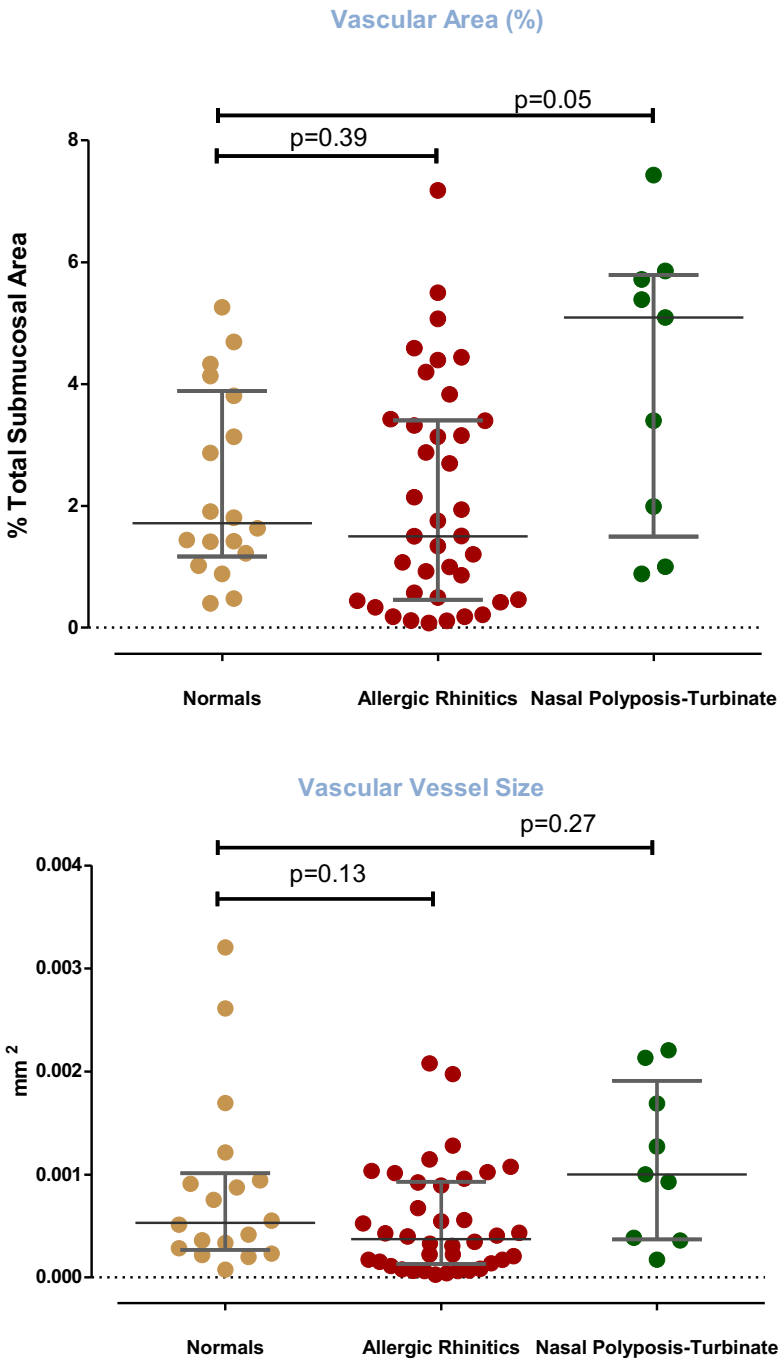
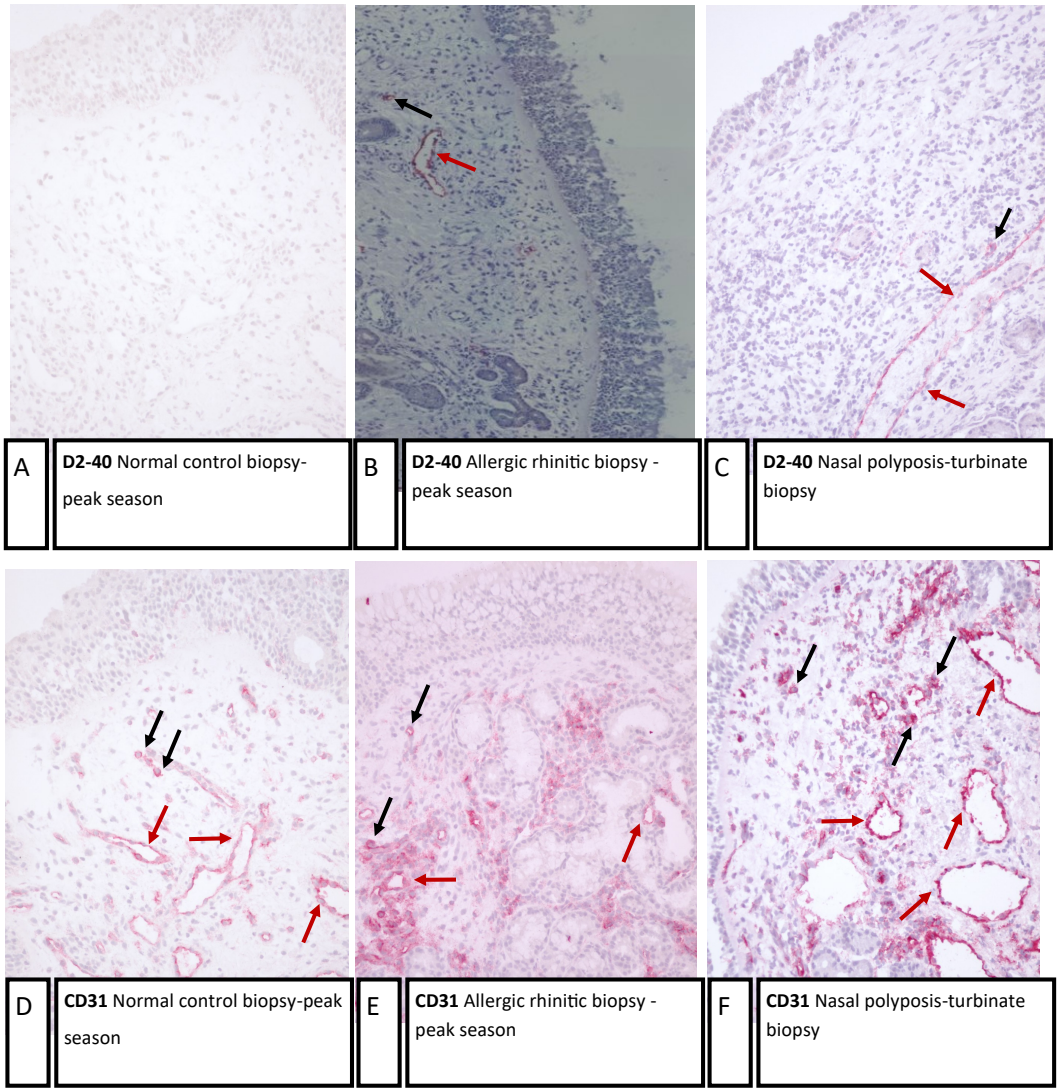
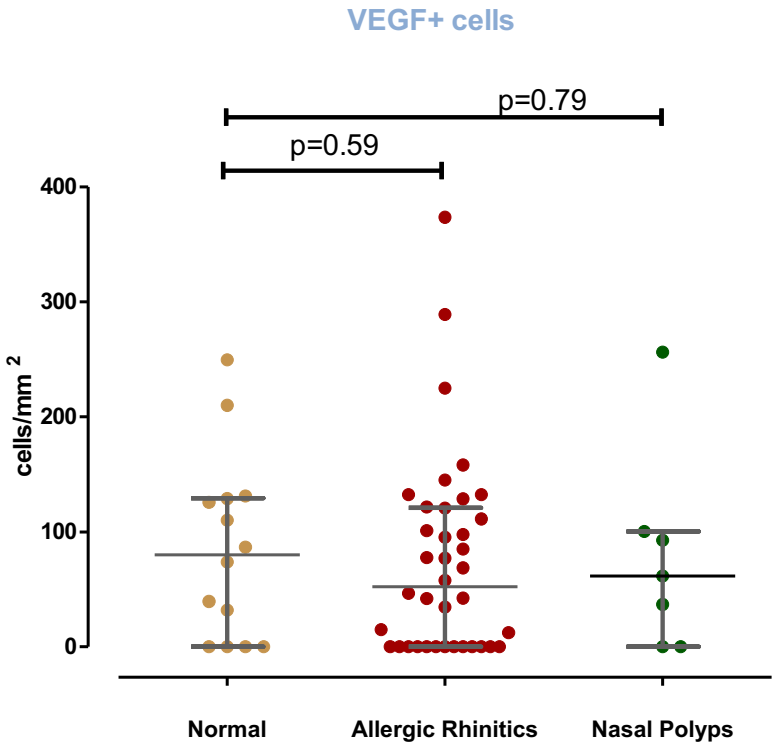


Figure 5.7b: Angiogenesis: using CD31 as angiogenic marker. Measured as vascular area (% total submucosal area) and vascular vessel size mm^2. Comparing persistent allergic rhinitics (n=38) with healthy controls (n=18) or turbinate tissue from CRSwNP (n=9) and healthy controls (n=18) Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.



Photomicrograph 5.2: Lymphangiogenesis staining using monoclonal antibody D2-40 (A-C) and angiogenesis staining with the monoclonal antibody CD31 (D-F) with the ABC-AP technique. Discretely stained red cells (black arrows) and vessels (red arrows) with haematoxylin (blue) nuclear counterstain. D2-40+ vessels were significantly larger and more numerous in turbinate biopsies from polyposis patients(C) than normal control subjects (A) whilst natural seasonal allergen exposure had no effect on D2-40+ vessels or cell numbers. CD31+ vascular area was significantly increased in nasal turbinate biopsies from polyposis patients (F) compared to biopsies from normal control subjects (D) whilst natural seasonal allergen exposure had no effect on CD31+ vessels or cell numbers (F). Photographed using x20 lens.



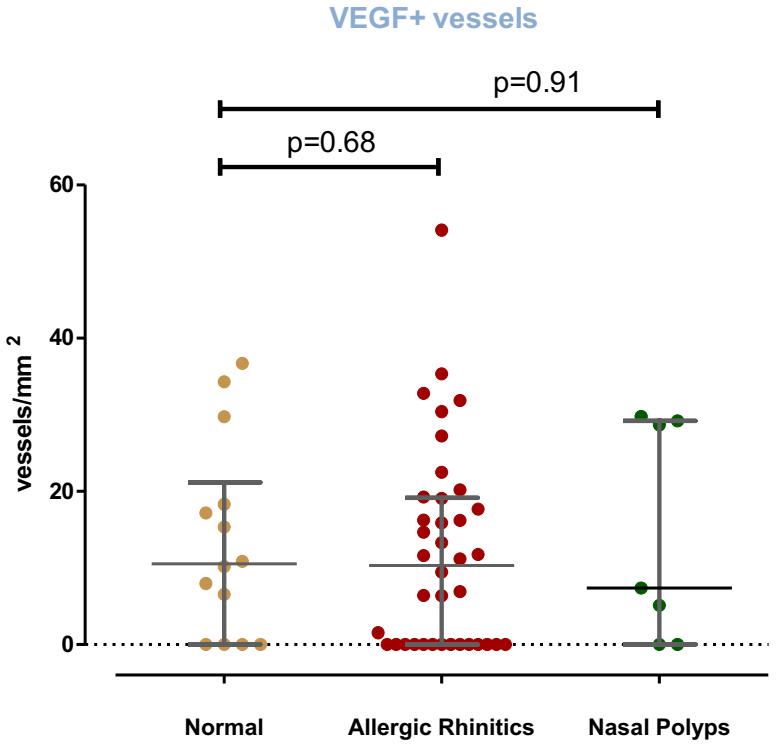


Figure 5.8a: VEGF positive submucosal signal- cells and vessels/mm². Comparing persistent allergic rhinitics (n=38) with healthy controls (n=18) or polyp tissue from CRSwNP (n=7) and healthy controls (n=18). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

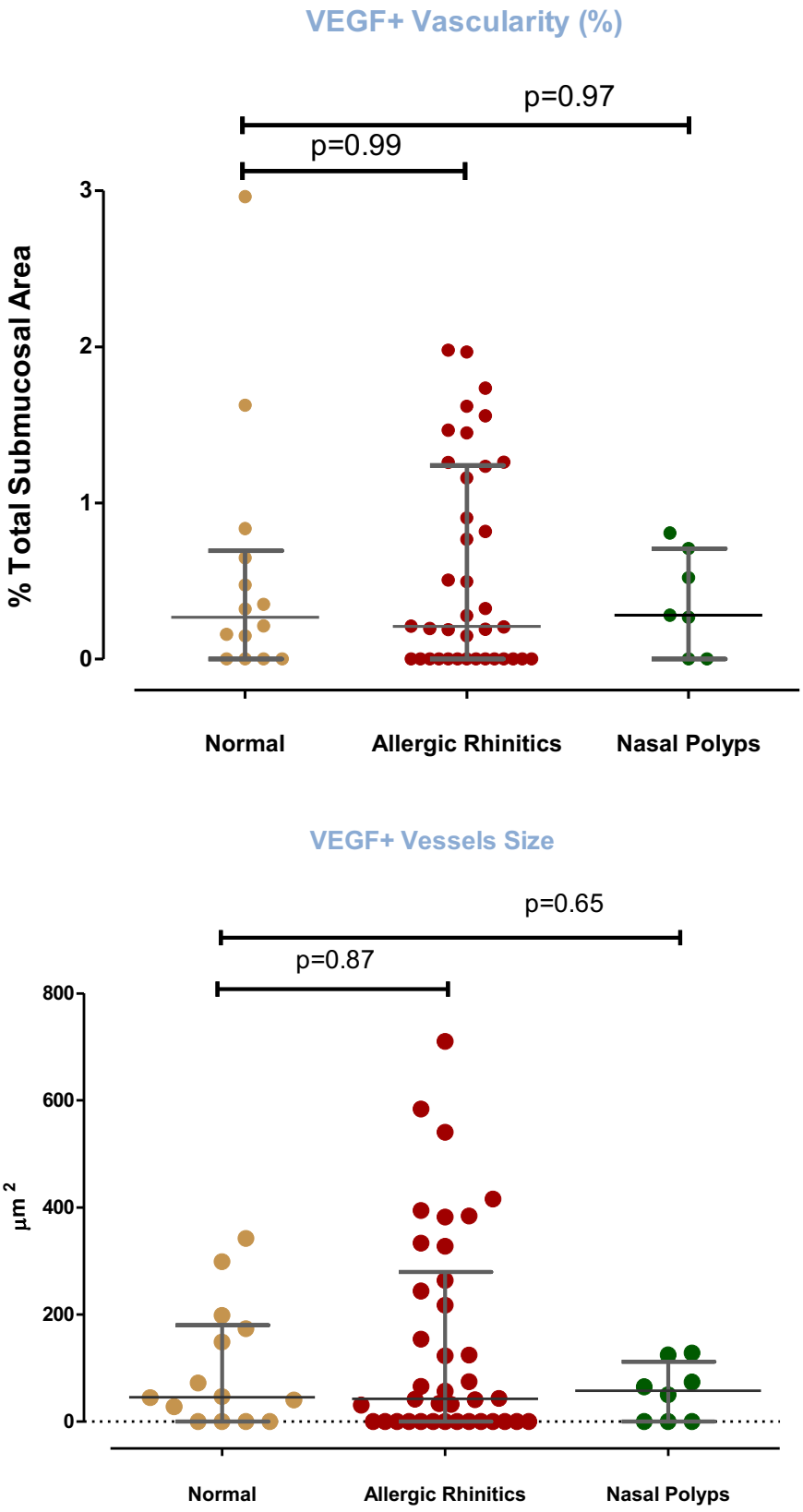


Figure 5.9b: VEGF positive submucosal signal- vessel area as % of total submucosal area and vessel size μm^2. Comparing persistent allergic rhinitics (n=38) with healthy controls (n=14) or polyp tissue from CRSwNP (n=8) and healthy controls (n=14). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

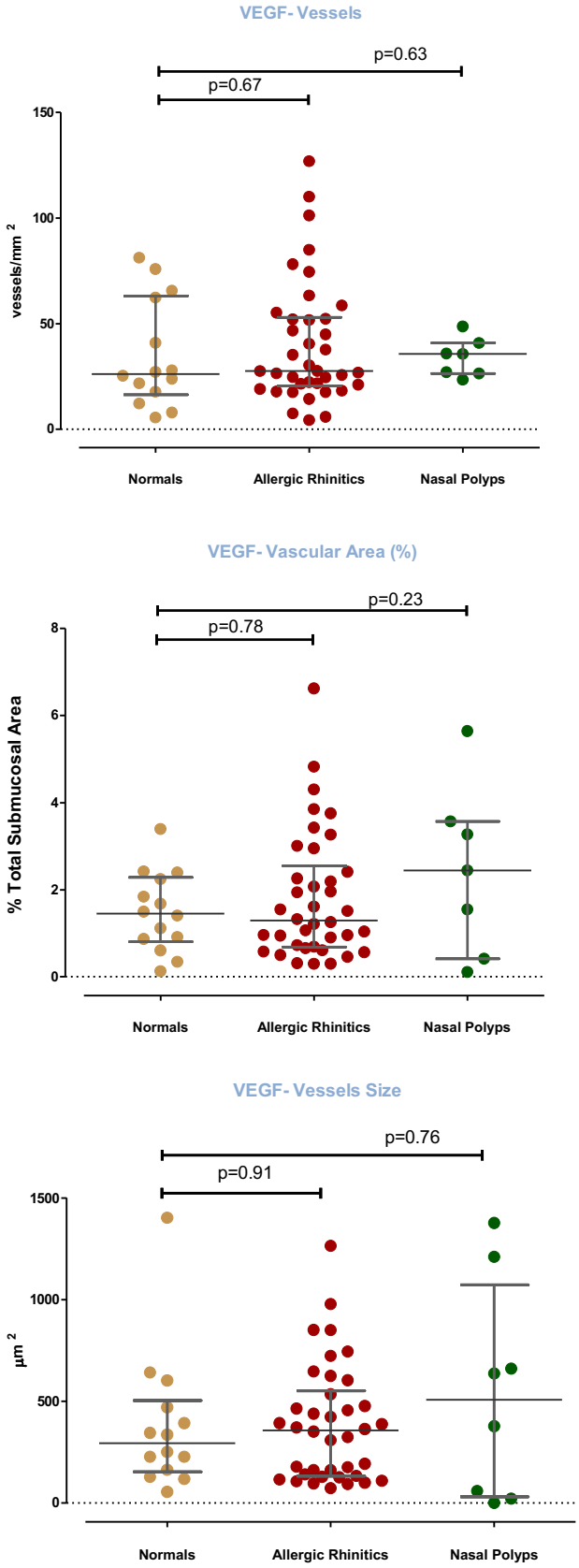


Figure 5.10: VEGF negative submucosal signal- vessel number/mm², % surface area, size µm². Comparing persistent allergic rhinitics (n=38) with healthy controls (n=14) or polyp tissue from CRSwNP (n=8) and healthy controls (n=14). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

Matrix metalloproteases (MMP7, MMP9) and their inhibitor (TIMP1) showed no difference between AR and normal non-atopic controls. The ratio of MMP7: TIMP1 and MMP9: TIMP1 was not statistically different in allergic rhinitics in Natural Allergen Protocol when compared to normal controls. The intensity of TIMP1 staining in submucosal gland mucus was unchanged in allergic rhinitics when compared to non-allergic tissue (figure 5.8) (photomicrograph 5.3).

MMP9 staining was less ($p=0.08$) in turbinates of nasal polyp patients when compared with normal controls with significant increase in TIMP1 staining ($p=0.01$). As a result, MMP7: TIMP1 ($p=0.06$) and MMP9:TIMP1 ($p=0.04$) staining ratio was less in polyposis than normal controls. The intensity of TIMP1 staining in submucosal gland mucus was significantly more in polyp tissue when compared to non-atopic samples (figure 5.9).

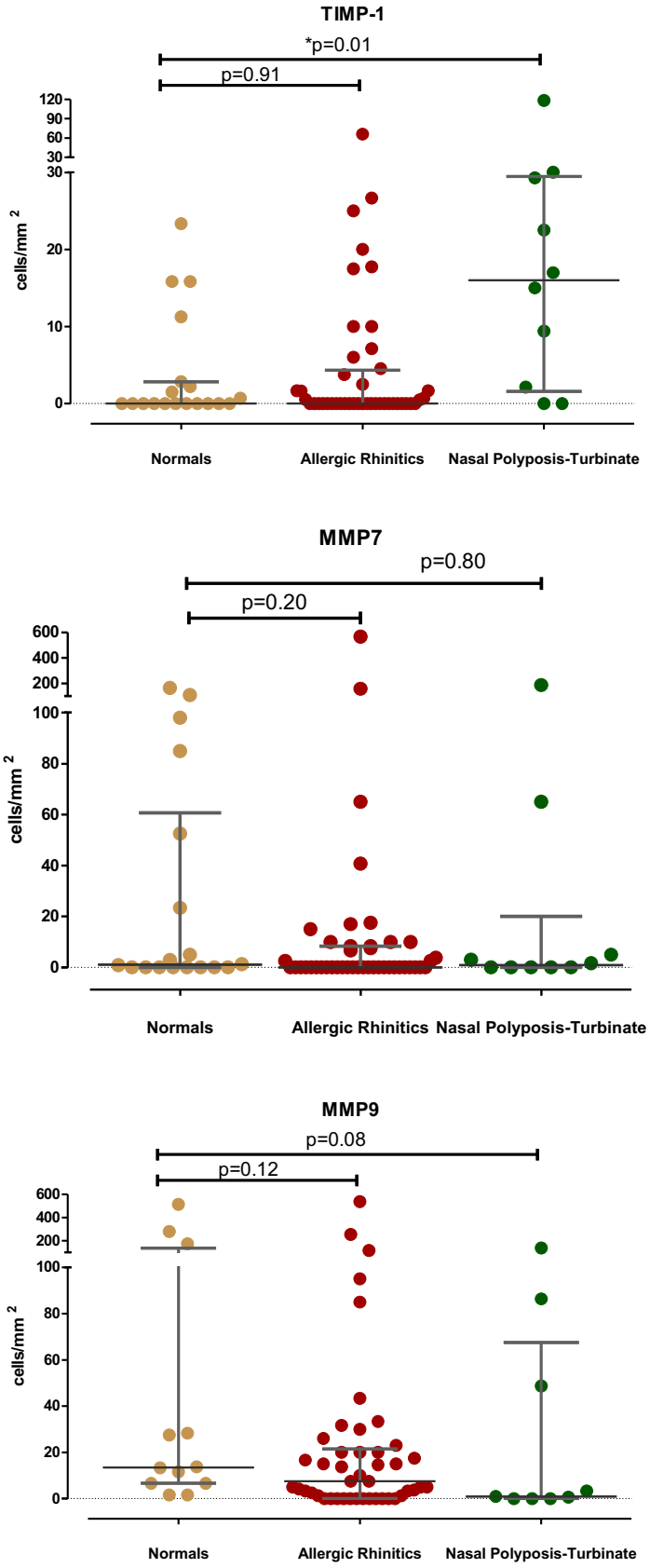


Figure 5.11: Matrix metalloproteases 7 and 9 with their inhibitor TIMP-1/mm². Comparing persistent allergic rhinitics (n=44) with healthy controls (n=19; except MMP9 n=12) or turbinate tissue from CRSwNP (n=9) and healthy controls (n=19; except MMP9 n=12). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

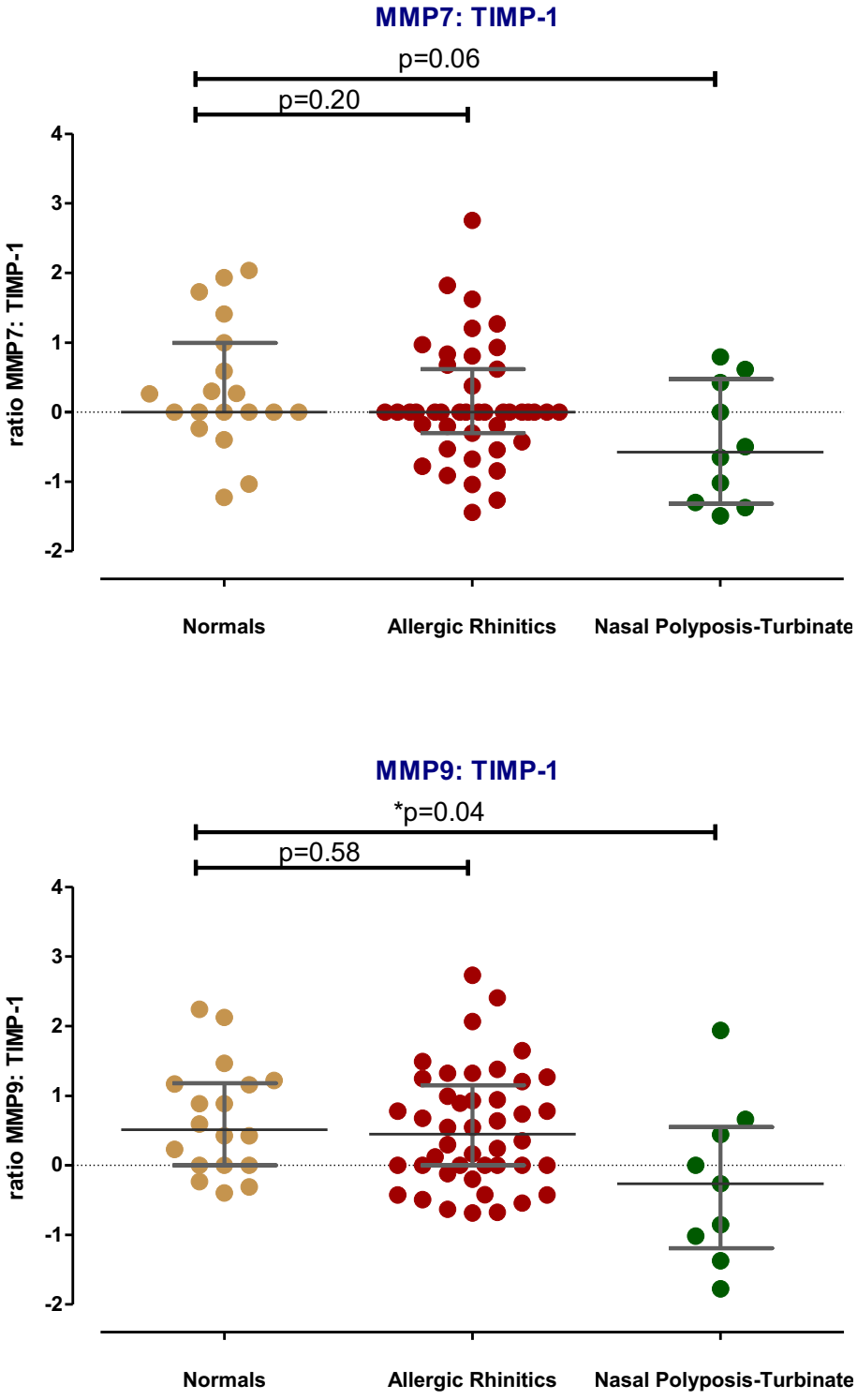
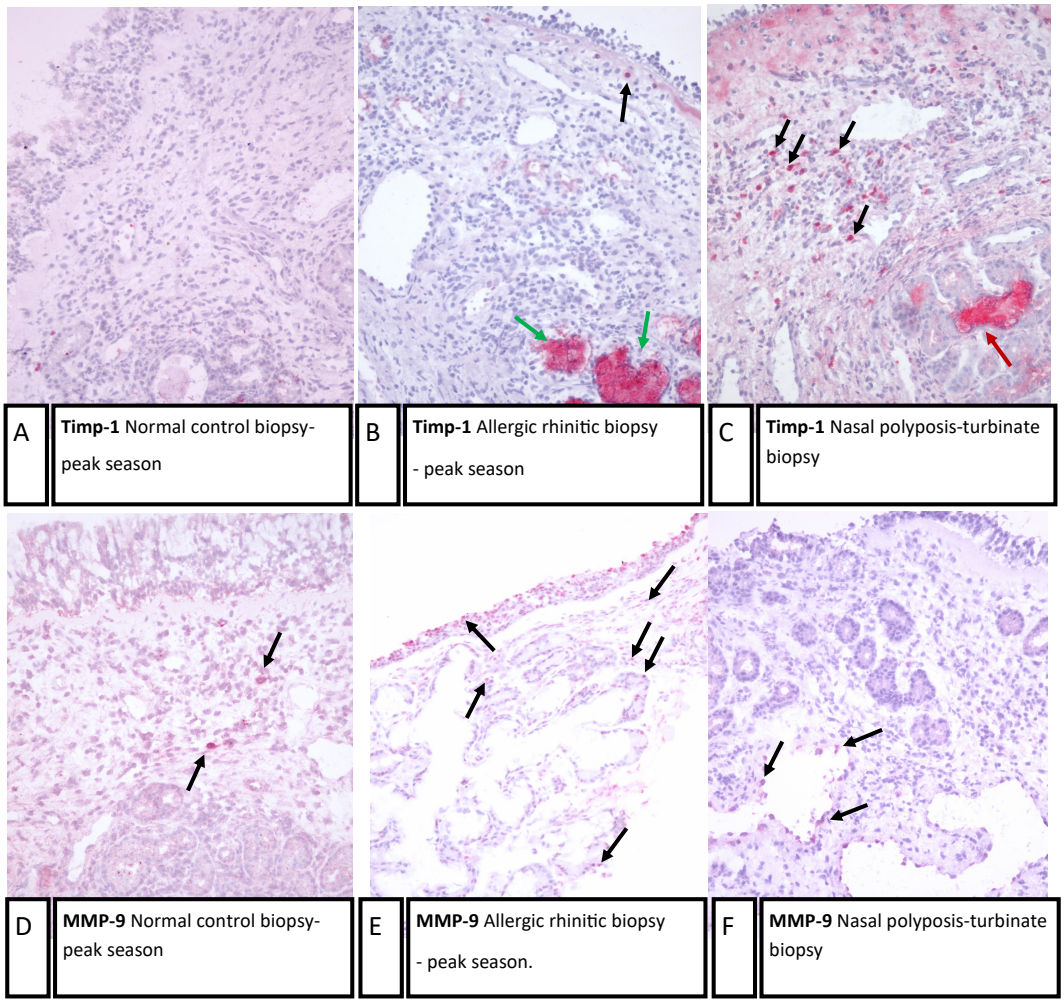


Figure 5.12: Matrix metalloproteases 7 and 9 ratio to TIMP-1. Comparing persistent allergic rhinitics (n=44) with healthy controls (n=19) or turbinate tissue from CRSwNP (n=9) and healthy controls (n=19). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.



Photomicrograph 5.3: Timp-1 staining using monoclonal antibody (A-C) and MMP-7 staining using monoclonal antibody (D-F) with the ABC-AP technique. Timp-1+ and MMP-7+ visible as discrete red cells (black arrows) with haematoxylin (blue) nuclear counterstain. Timp-1+ mucus indicated with green arrows. Significantly more Timp-1+cells in nasal polyposis turbinates (C) than in normal control biopsies (A) or turbinates from allergen exposed allergic rhinitics (B). MMP-9+ cells were found in similar levels in submucosa of normal controls (E), turbinates from allergen exposed allergic rhinitics (E) and nasal polyposis turbinates (F) although the ratio of MMP-9: Timp-1 was significantly reduced in polyposis turbinates. Photographed using x20 lens.

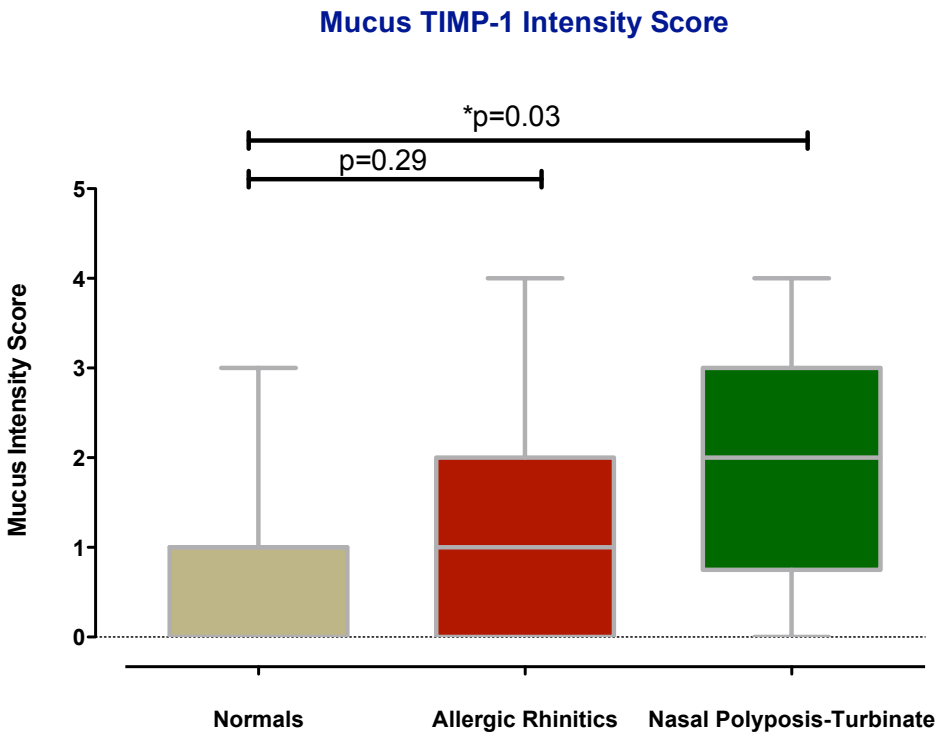


Figure 5.13: Tissue inhibitor of matrix metalloprotease TIMP-1 mucus intensity score. Comparing persistent allergic rhinitics (n=44) with healthy controls (n=19) or turbinate tissue from CRSwNP (n=9) and healthy controls (n=19). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

Smooth muscle actin staining showed no difference between allergic rhinitic and normal groups. Furthermore, nasal polyps also failed to demonstrate significant differences when compared with normal non-atopic controls. Heat shock protein 47, similarly failed to change significantly in allergic rhinitics, either as a total cell count or when co-localised to fibroblasts. At p=0.06 HSP-47 bearing fibroblasts in allergic rhinitics, tended to, but failed to reach significantly reduced staining. Nasal polyp samples demonstrated no difference in SMA, HSP-47 in all cells or HSP-47 isolated to fibroblasts when compared to normal non-atopic controls (figure 5.13).

Alteration in HSP-47 and smooth muscle actin was not demonstrated in allergic rhinitics with natural allergen exposure.

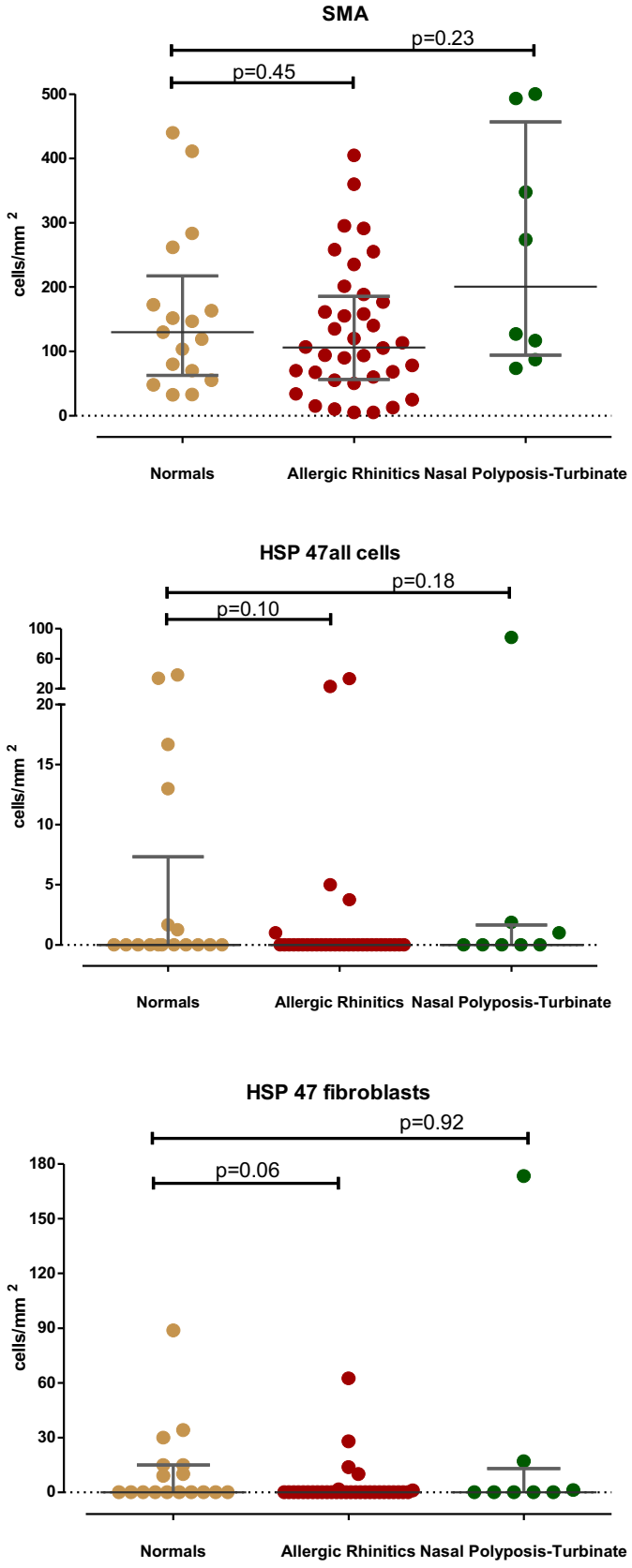


Figure 5.14: Smooth Muscle Actin and Heat Shock Protein-47 (measured in all cells and in a fibroblast cell subset) /mm^2. Comparing persistent allergic rhinitics (n=36) with healthy controls (n=17) or turbinate tissue from CRSwNP (n=8) and healthy controls (n=17). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

Submucosal, but not epithelial, Transforming Growth Factor β was significantly less in allergic rhinitics than non-atopic controls (p=0.02). There was no demonstrable difference between nasal polyps and normal controls for epithelial or submucosal TGF-β (figure 5.14).

ALK-4 bearing cells were significantly more numerous, whilst pSMAD-2 bearing cells were significantly fewer in allergic rhinitics when compared to normal controls. ALK-4 was significantly more numerous but pSMAD-2 was unchanged in tNP. There was no difference in levels of activin positive cells in allergic rhinitis (figure 5.15).

TGF-β and its signalling molecules were altered in allergic rhinitics during natural allergen exposure.

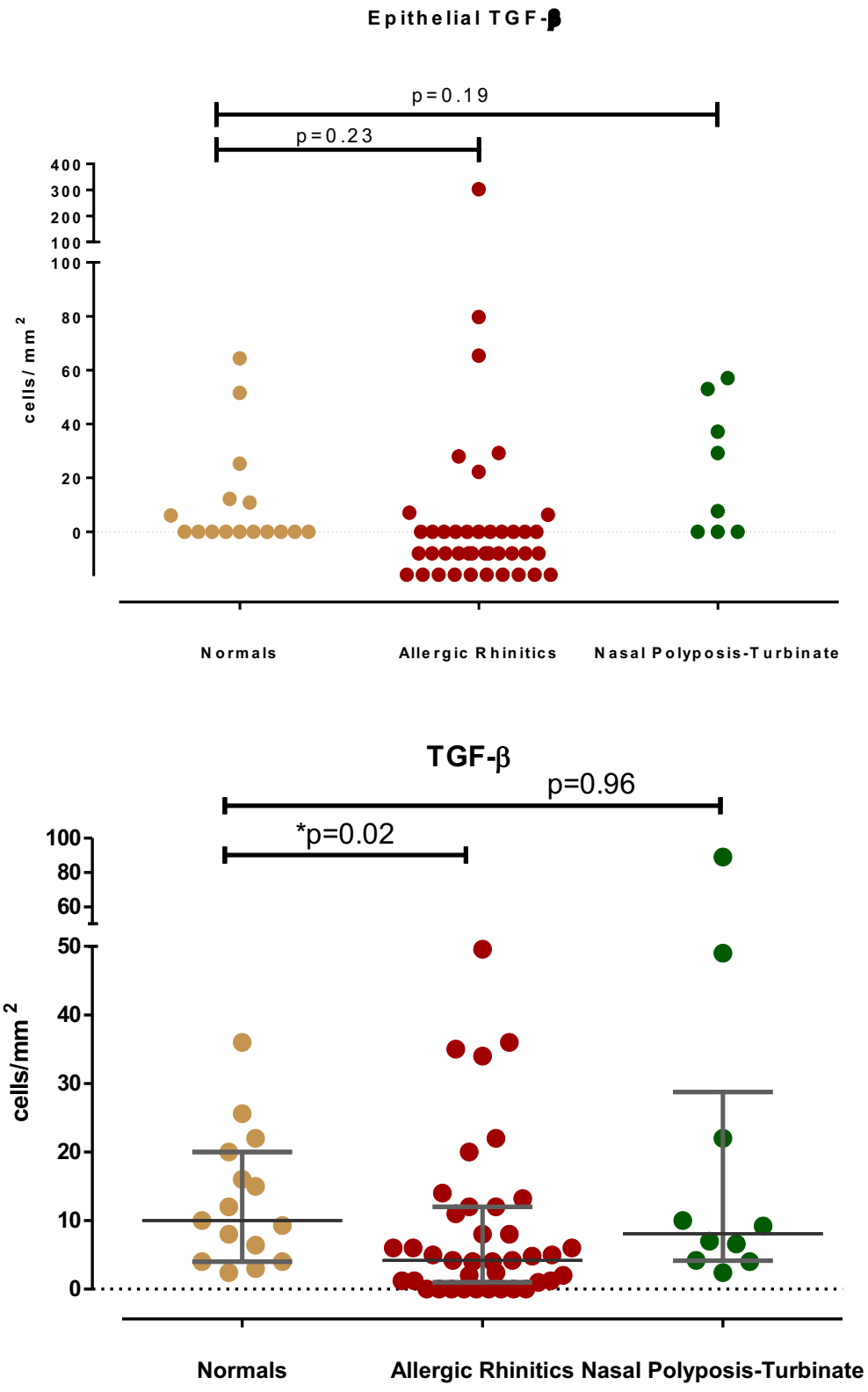


Figure 5.15: TGF-β: epithelial and submucosal cell counts/mm². Comparing persistent allergic rhinitics (n=41) with healthy controls (n=16) or turbinate tissue from CRSwNP (n=8 epithelium; n=10 submucosa) and healthy controls (n=16). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

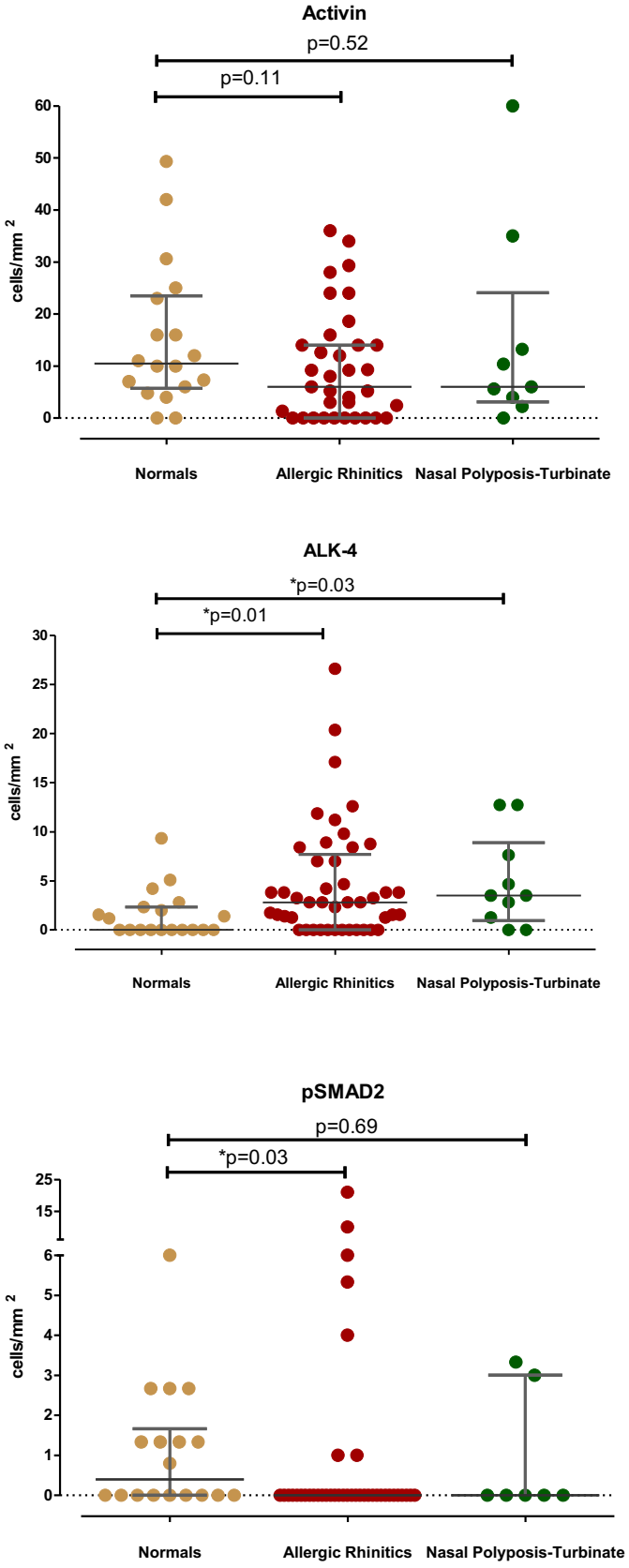


Figure 5.16: Submucosal activin, ALK-4 and pSMAD-2 cell counts /mm². Comparing persistent allergic rhinitics (n=35 activin; n=45 Alk-4) with healthy controls (n=18) or turbinate tissue from CRSwNP (n=9 activin; n=10 Alk-4;n=7 pSMAD-2) and healthy controls (n=18). Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

Repetitive Allergen Challenge Protocol

Epithelial integrity decreased significantly (p=0.03) at 24 hours (diluent versus allergen) but not at 11 days. Basement membrane thickness was unchanged at both day 1 and day 11 in the repetitive allergen challenge (RAC) protocol (figure 5.16). Intensity of epithelial mucus staining decreased significantly (p=0.03) at 24 hours but not at day 11 (diluent versus allergen). Percentage epithelial area occupied by mucus decreased at 24 hours (diluent versus allergen) but did not reach significance (p=0.06) (figure 5.17).

During this induced model of allergic rhinitis, epithelial injury was evidenced by epithelial shedding evident at 24 hours but not 11 days.

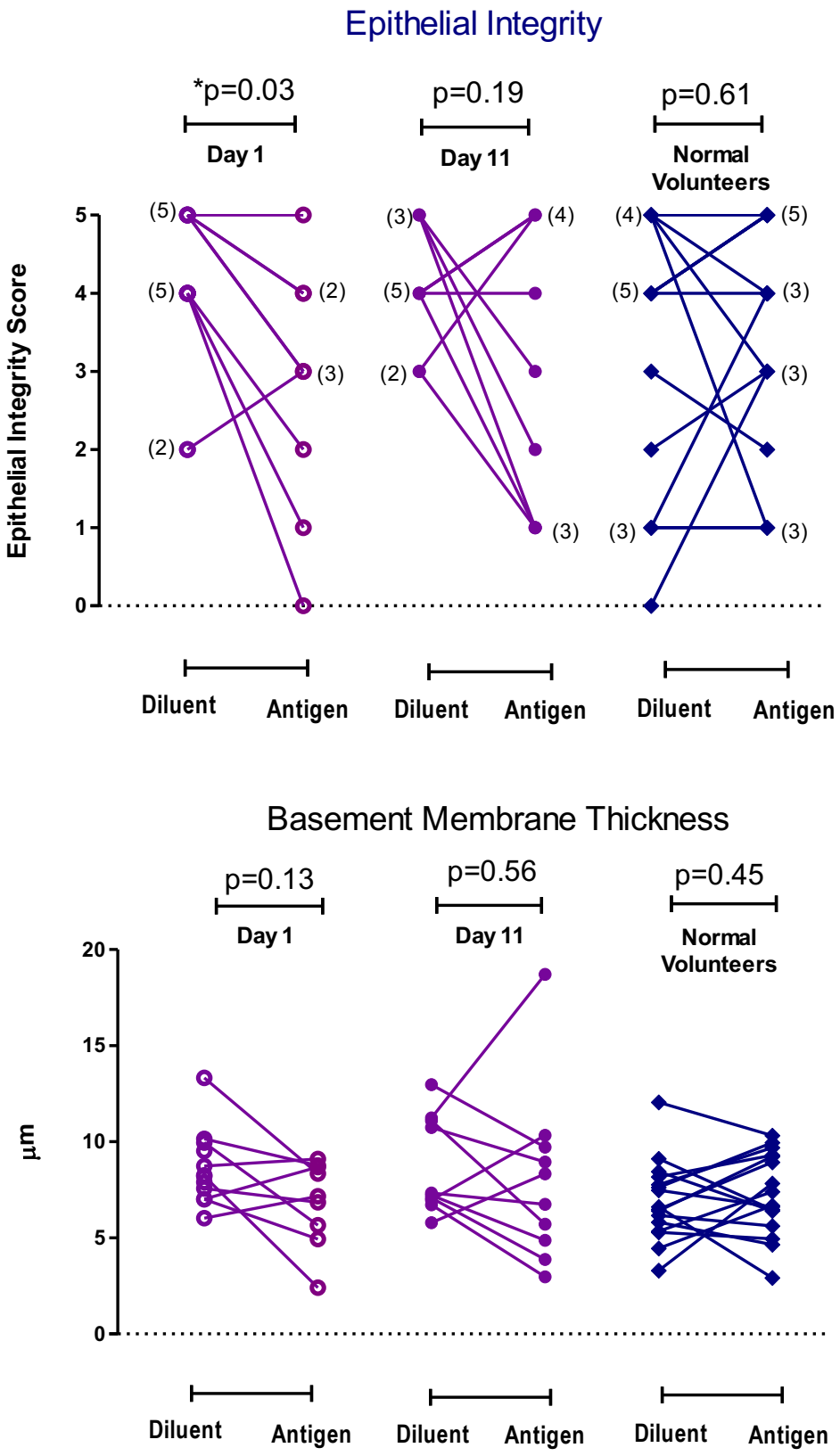


Figure 5.17: Epithelial Integrity Score and Basement Membrane thickness (µm). Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=16). Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

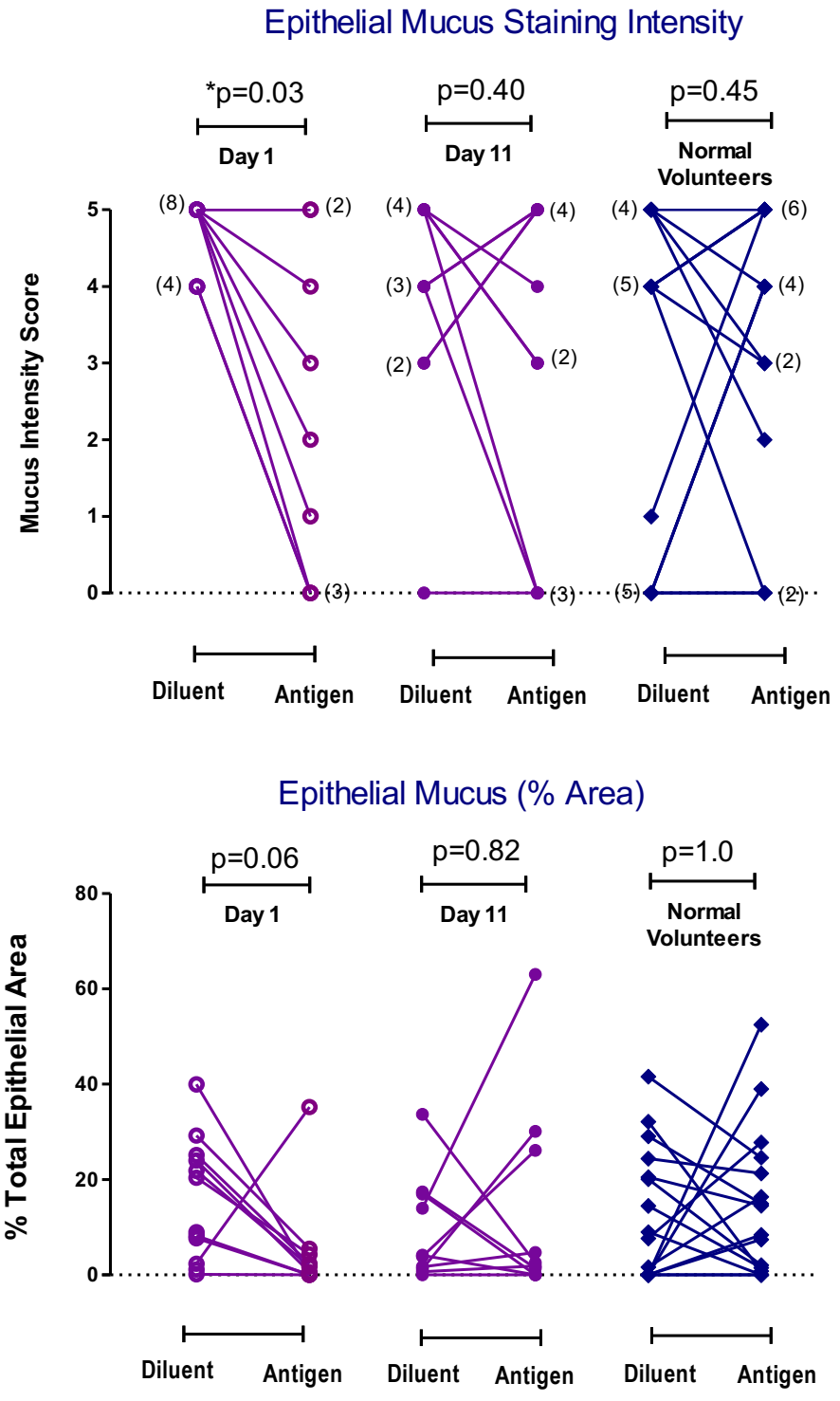


Figure 5.18: Intensity of epithelial mucus staining and Epithelial Mucus Content (% of total epithelial area. Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=16). Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

Lymphangiogenesis and lymphatic vessel markers were measured by the following four parameters: total D2-40+ve cells, total D2-40+ve vessels, percentage lymphatic vessel area, and absolute lymphatic vessel size. None of the four markers showed any difference at either day 1 or day 11 when comparing diluent versus allergen samples (figure 5.18a and b).

Alteration in submucosal lymphatics was not a feature of repetitive allergen challenge in allergic rhinitics.

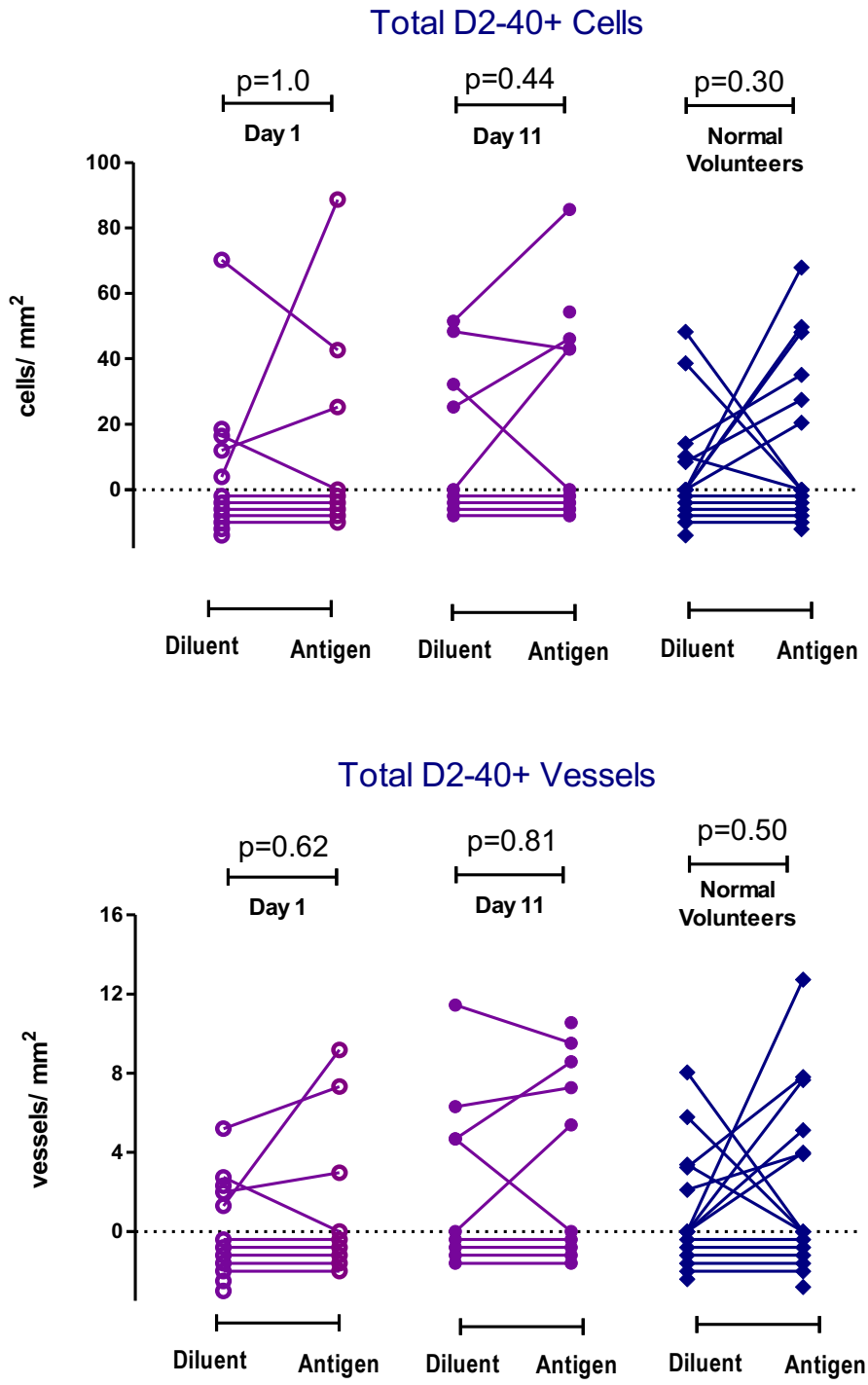


Figure 5.19a: Lymphangiogenesis: using D2-40 as lymphangiogenic marker. Counted as total number of D2-40 +ve cells and vessels/mm². Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=16). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

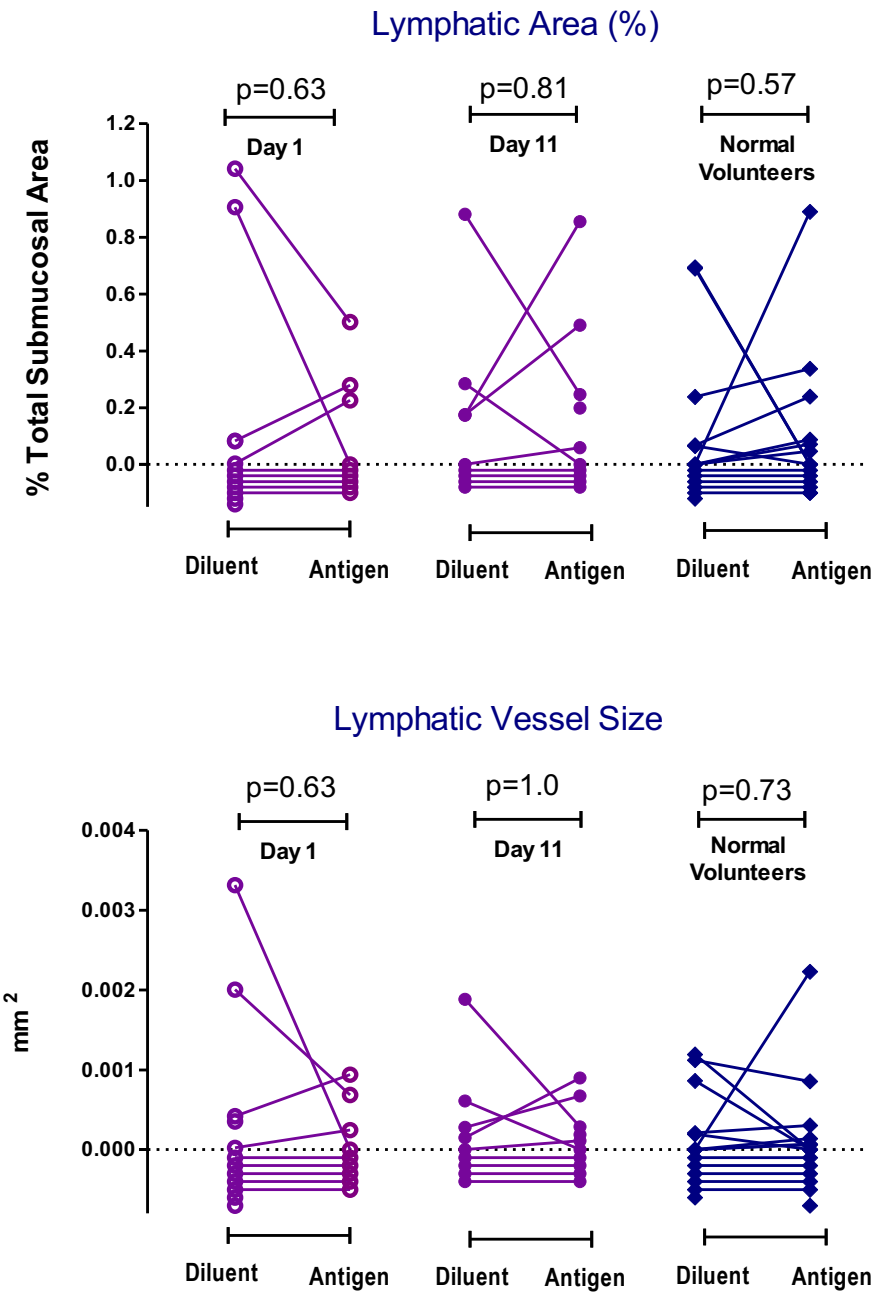


Figure 5.20b: Lymphangiogenesis: using D2-40 as lymphangiogenic marker. Measured as lymphatic area (% total submucosal area) and lymphatic vessel size mm². Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=16). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

Differences in submucosal glands were measured via four parameters: total glandular area, total intra-glandular area, and total gland tissue area. None of the above demonstrated a difference between diluent versus allergen challenge samples at day 1 or at day 11 (figure 5.19).

Alterations in submucosal glands was not a feature of allergic rhinitics in this repetitive allergen challenge protocol.

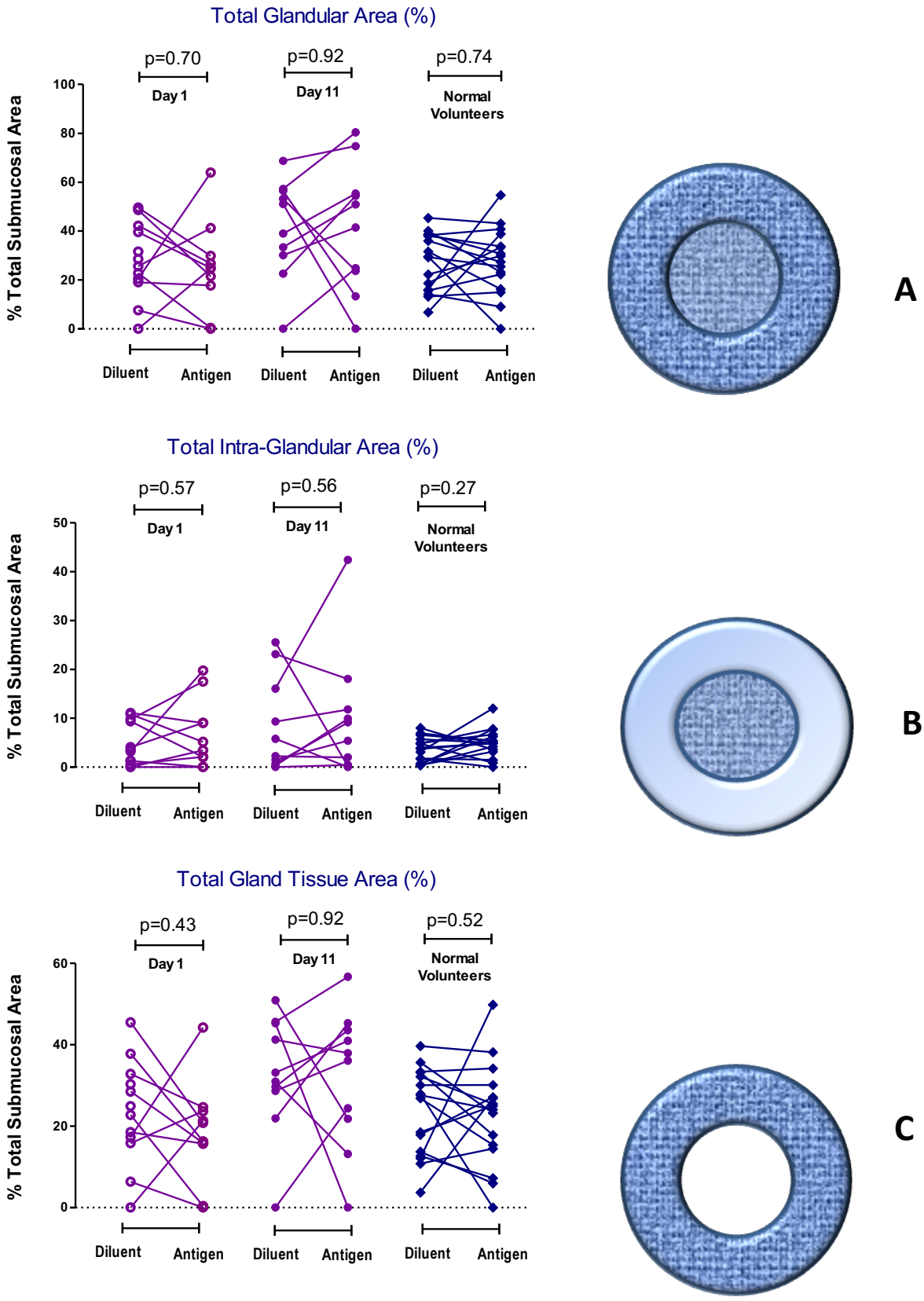
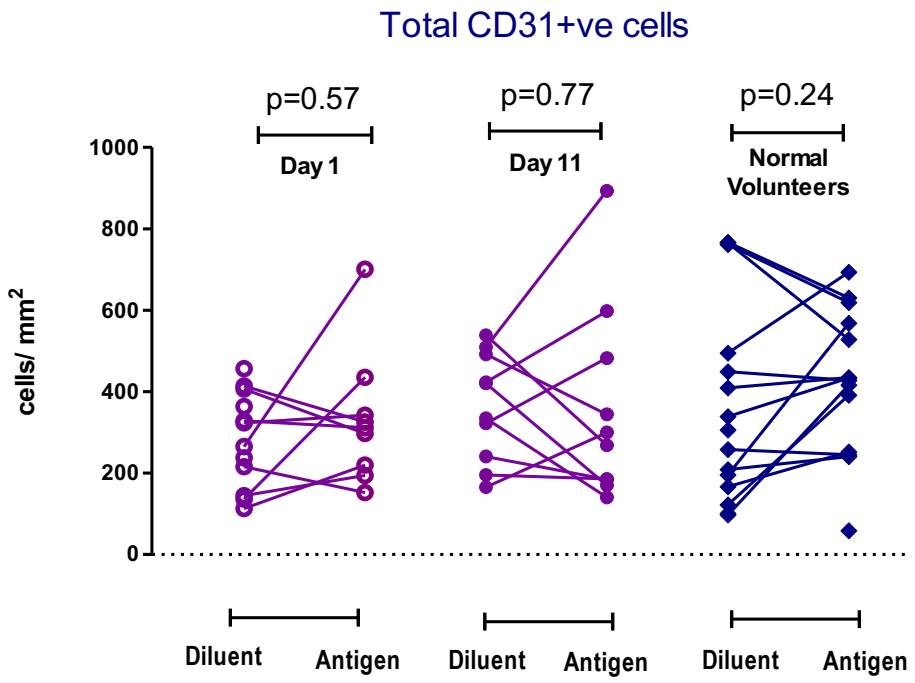


Figure 5.21:Submucosal Glandular Area: measured area is represented by textured blue background on schematic gland drawing, representing (A) gland tissue and secretory contents, (B) secretory content area only and, (C) glandular tissue only. Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=16). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

Vascular vessel and angiogenesis markers included: total CD31+ve cells, total CD31+ve vessels, total vascular area and absolute vascular vessel size. None demonstrated a statistically significant change at either day 1 or day 11 following allergen challenge when compared to diluent (figures 5.20a and b).

As for the Natural Allergen Exposure protocol, VEGF staining included four parameters: total number of VEGF positive cells, total number of VEGF positive vessels, percentage of VEGF positive submucosal vascular area (as compared to total submucosal vascular area) and VEGF positive vessel size (figures 5.21a and b). VEGF negative measures included: total number of VEGF negative vessels, percentage of VEGF negative submucosal vascular area (as compared to total submucosal vascular area) and VEGF negative vessel size (figure 5.22). There was no difference at either day 1 or day 11 in any of the above parameters in allergic rhinitics when comparing allergen to diluent.

Angiogenesis and established vascularity did not alter in this induced model of allergic rhinitis.



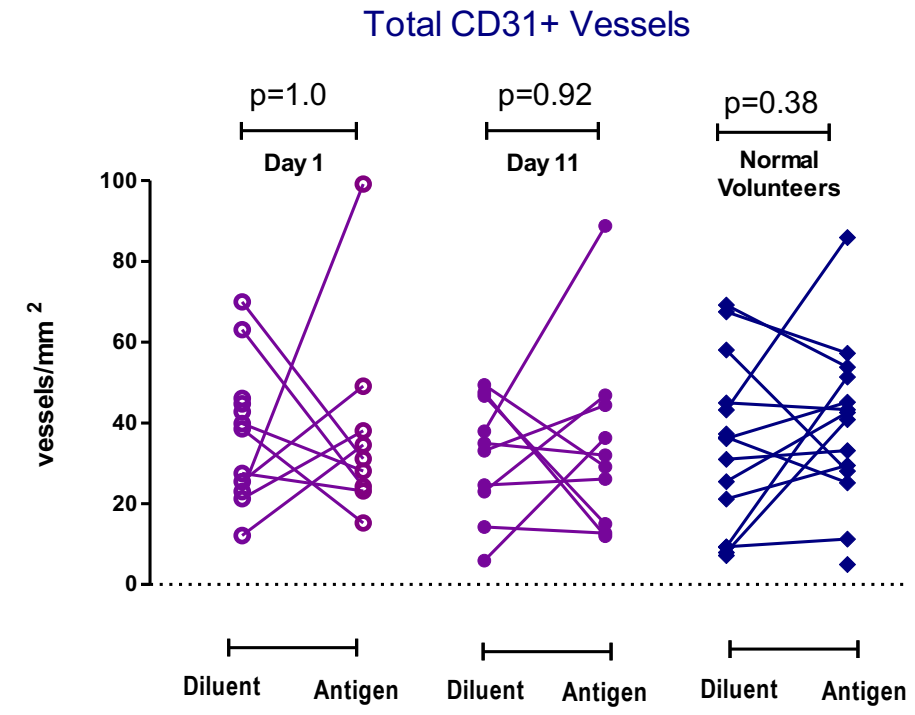


Figure 5.22a: Angiogenesis: using CD31 as angiogenic marker. Counted as total number of CD31+ve cells and vessels/mm^2. Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=16). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

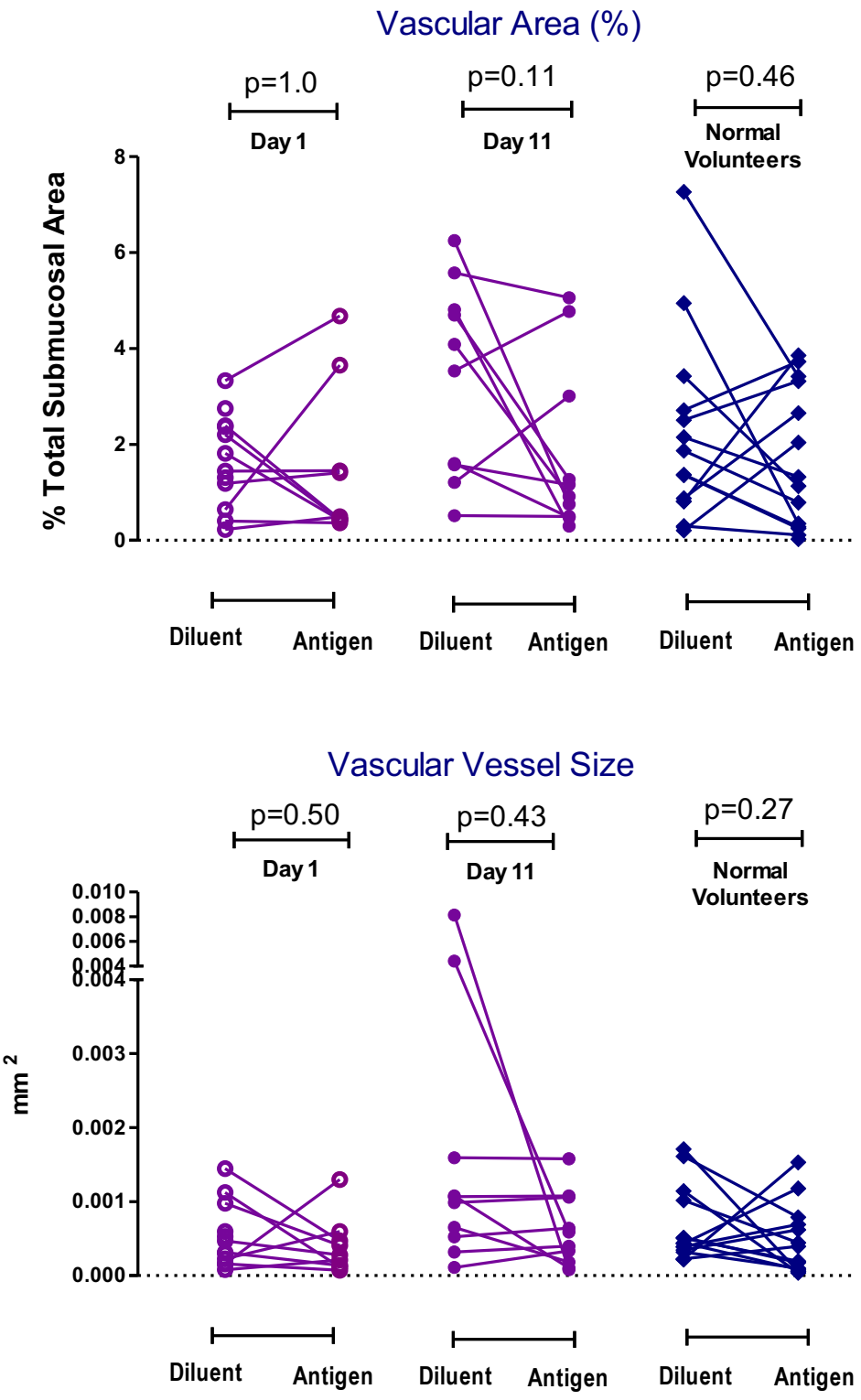


Figure 5.23b: Angiogenesis: using CD31 as angiogenic marker. CD31+ vascular area (% total submucosal area) and vascular vessel size mm². Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers (n=16). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

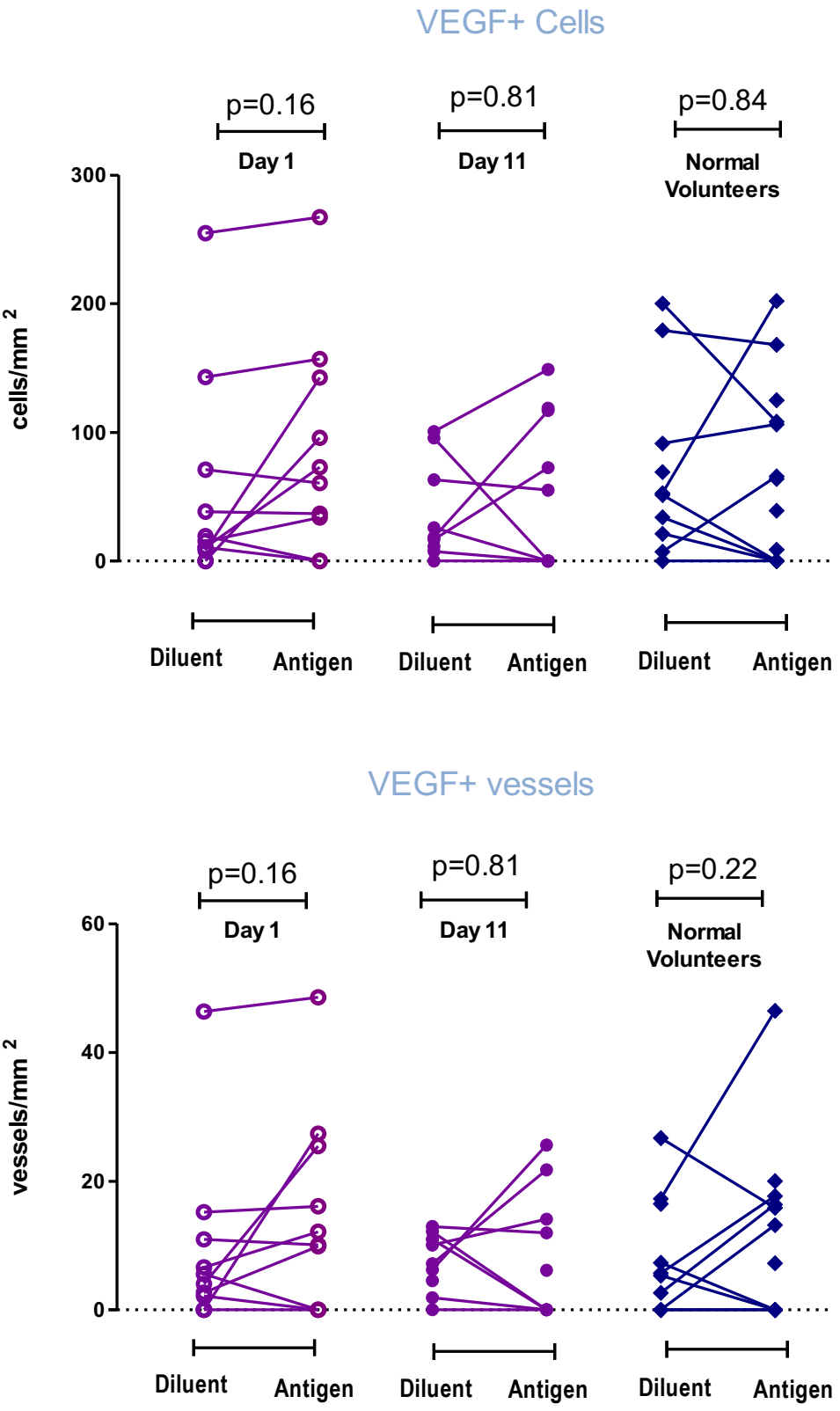


Figure 5.24a: VEGF positive submucosal signal. Counted as total number of VEGF +ve cells and vessels/mm². Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=16). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

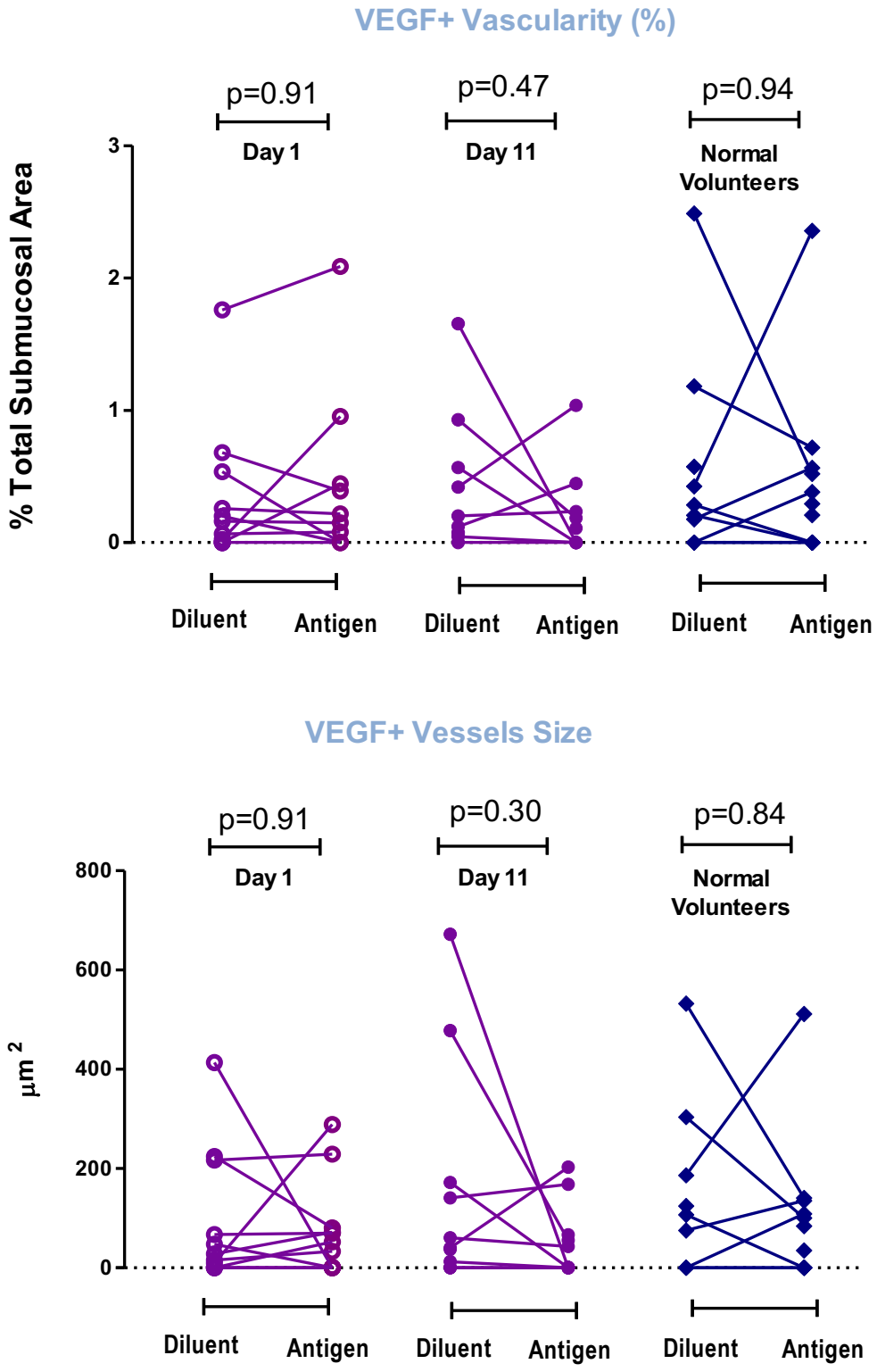


Figure 5.25b: VEGF positive submucosal signal- VEGF+ vascular area (% total submucosal area) and VEGF+ vascular vessel size µm². Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=12). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

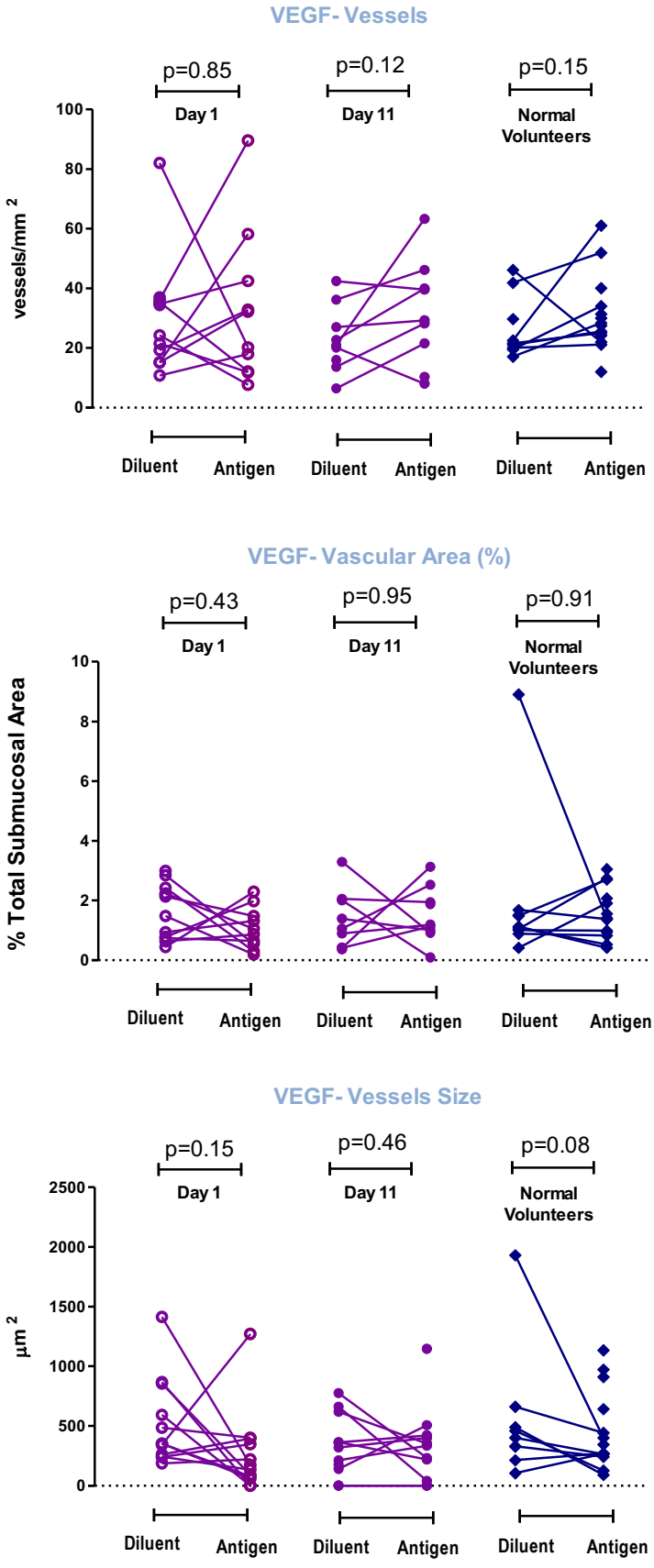


Figure 5.26: VEGF negative submucosal signal- vessel number, surface area, size. Wilcoxon signed rank test diluent and antigen challenge for Day 1 (n=10); Day 11 RAC (n=9) and normal volunteers (n=12). Significance was accepted as p<0.05.

Metalloproteases MMP7 and MMP9 showed no change at either day 1 or day 11 post allergen challenge when compared to diluent. Their inhibitor TIMP-1 did rise significantly at 24 hours but not 11 days post challenge (figure 5.23). This rise was also observed at 24 hours in normal non-atopic controls. MMP9: TIMP1 and MMP7:TIMP1 ratios did not change significantly at either day 1 or day 11 post allergen challenge when compared to diluent. The intensity of TIMP1 staining in submucous glands was not significant at either time point (figure 5.24).

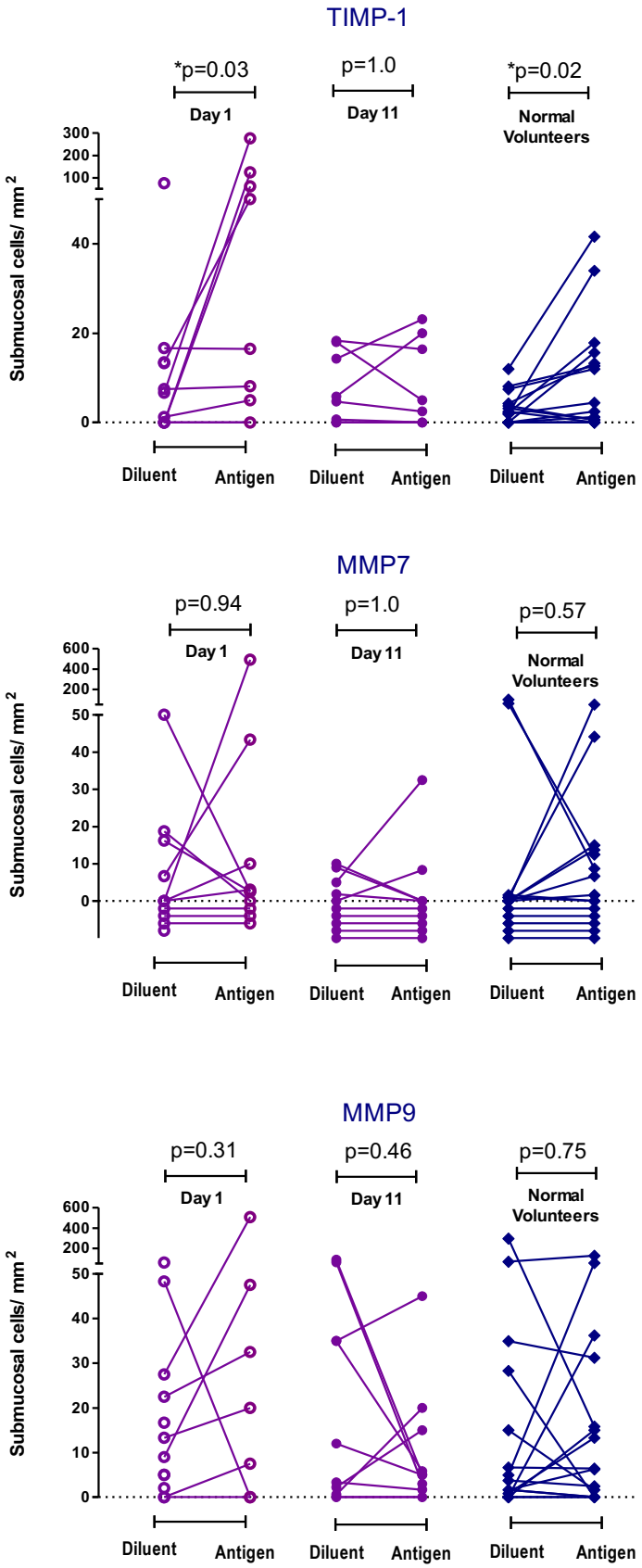


Figure 5.27: Matrix Metalloproteases 7 and 9 with their inhibitor TIMP-1/mm². Wilcoxon signed rank test diluent and antigen challenge for Day 1 (n=12); Day 11 RAC (n=10) and normal volunteers (n=16). Significance was accepted as p<0.05.

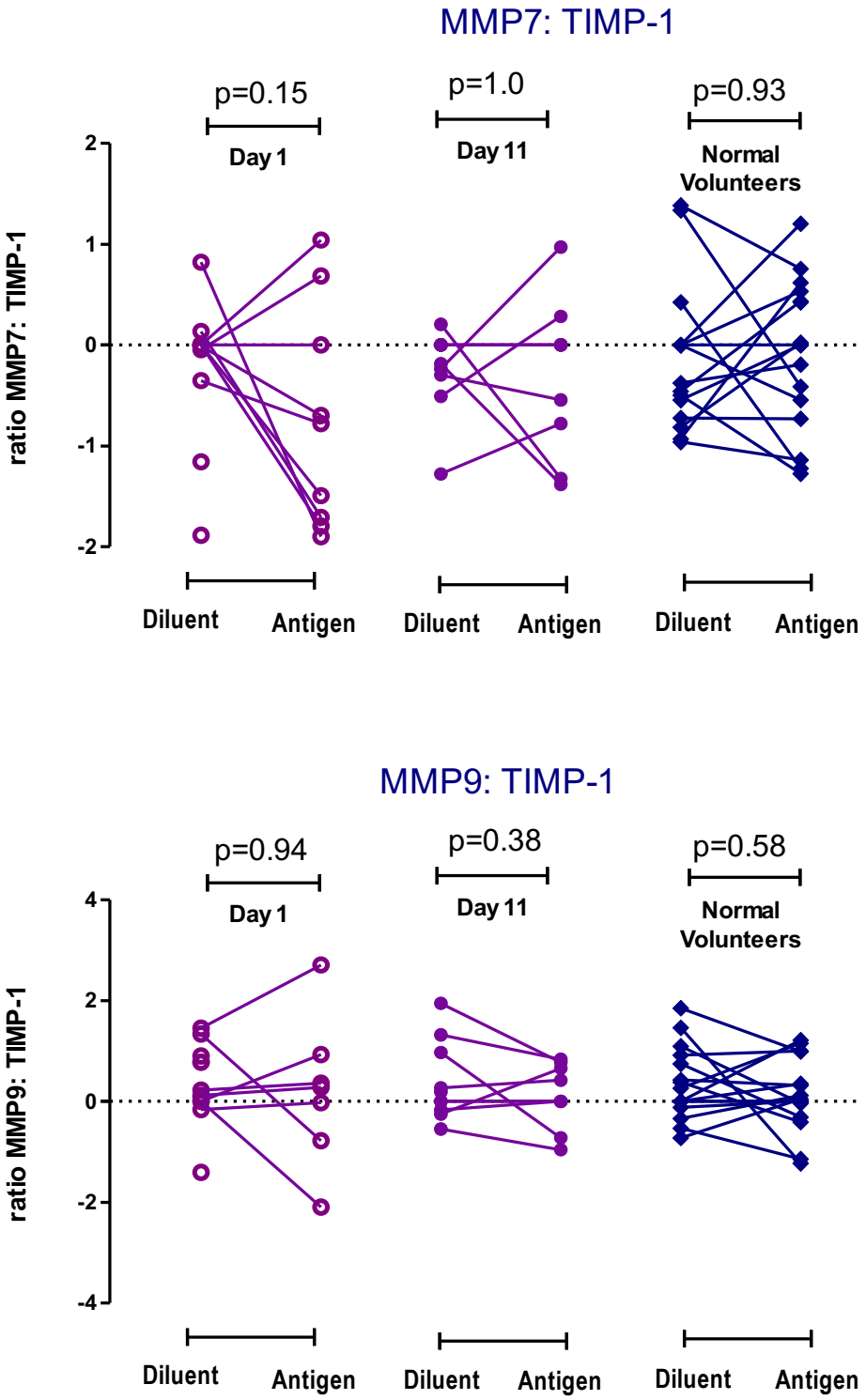


Figure 5.28: Matrix metalloproteases 7 and 9 ratio to TIMP-1. Wilcoxon signed rank test diluent and antigen challenge for Day 1 (n=12); Day 11 RAC (n=10) and normal volunteers (n=16). Significance was accepted as p<0.05.

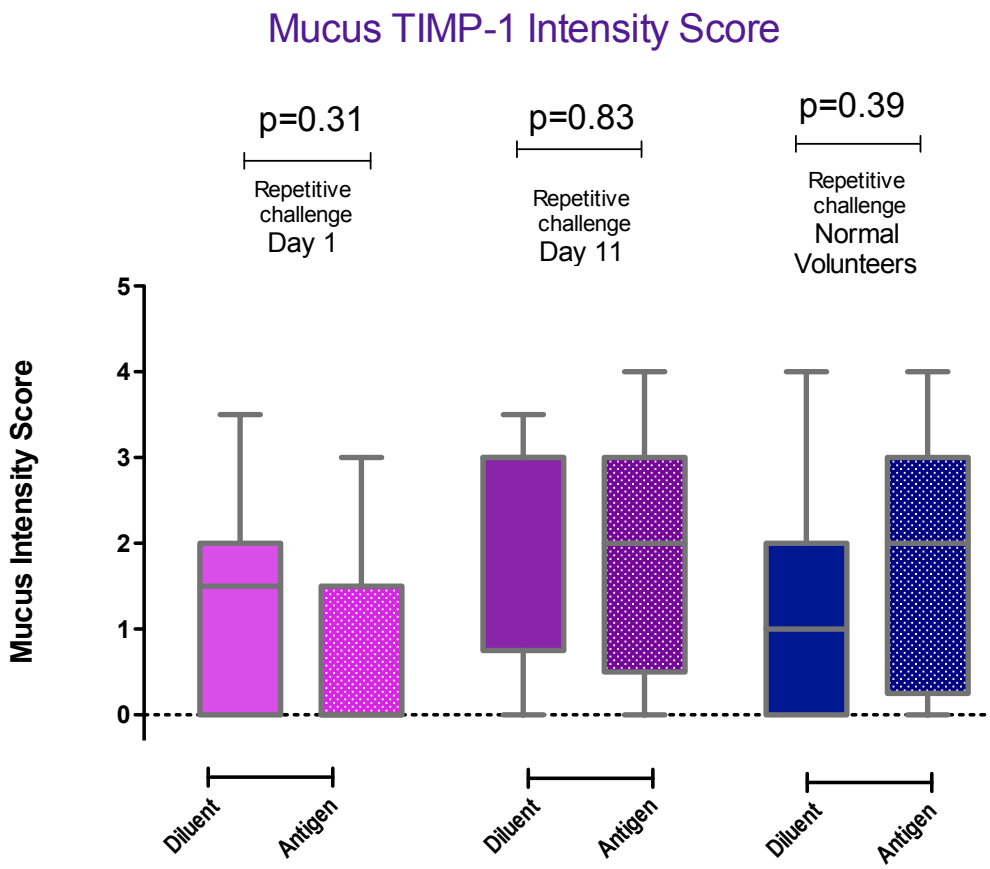


Figure 5.29: Tissue inhibitor of matrix metalloprotease TIMP-1 mucus intensity score. Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=16). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

Smooth Muscle Actin staining was unchanged at either 24 hours or 11 days post repetitive allergen challenge when compared to diluent. Total HSP-47 staining was increased at day 1 and day 11, when compared to diluent, as well as at 24 hours in normal controls. When localised to fibroblasts only, the increase was significant at only 24 hours post allergen versus diluent in both allergic rhinitics and normal non-atopic controls (figure 5.26).

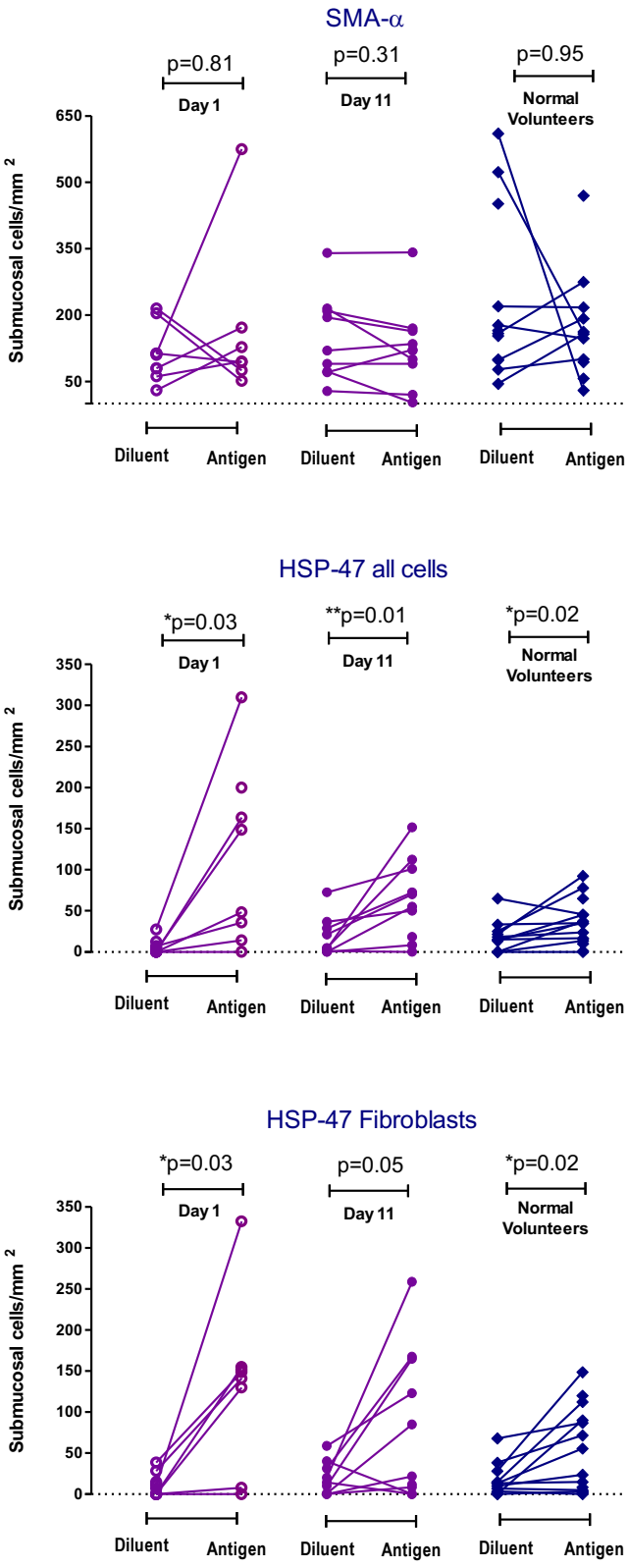


Figure 5.30: Smooth Muscle Actin and Heat Shock Protein-47 (measured in all cells and in a fibroblast cell subset). Within group comparison of diluent and allergen challenge Day 1 RAC (n=11); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=12). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

Whilst epithelial TGF-β was unchanged at both day 1 and day 11 diluent versus allergen challenge, submucosal TGF-β was significantly increased at day 11 but not day 1. Submucosal activin showed a significant increase at day 11 only (allergen versus diluent). ALK-4 was seen to be higher after 11 days although this was just short of significance with p=0.06. Submucosal pSMAD-2 demonstrated no change at either day 1 or day 11 post allergen challenge.

Increases in TGF-β, activin and ALK-4 were seen post allergen challenge in this induced model of allergic rhinitis.

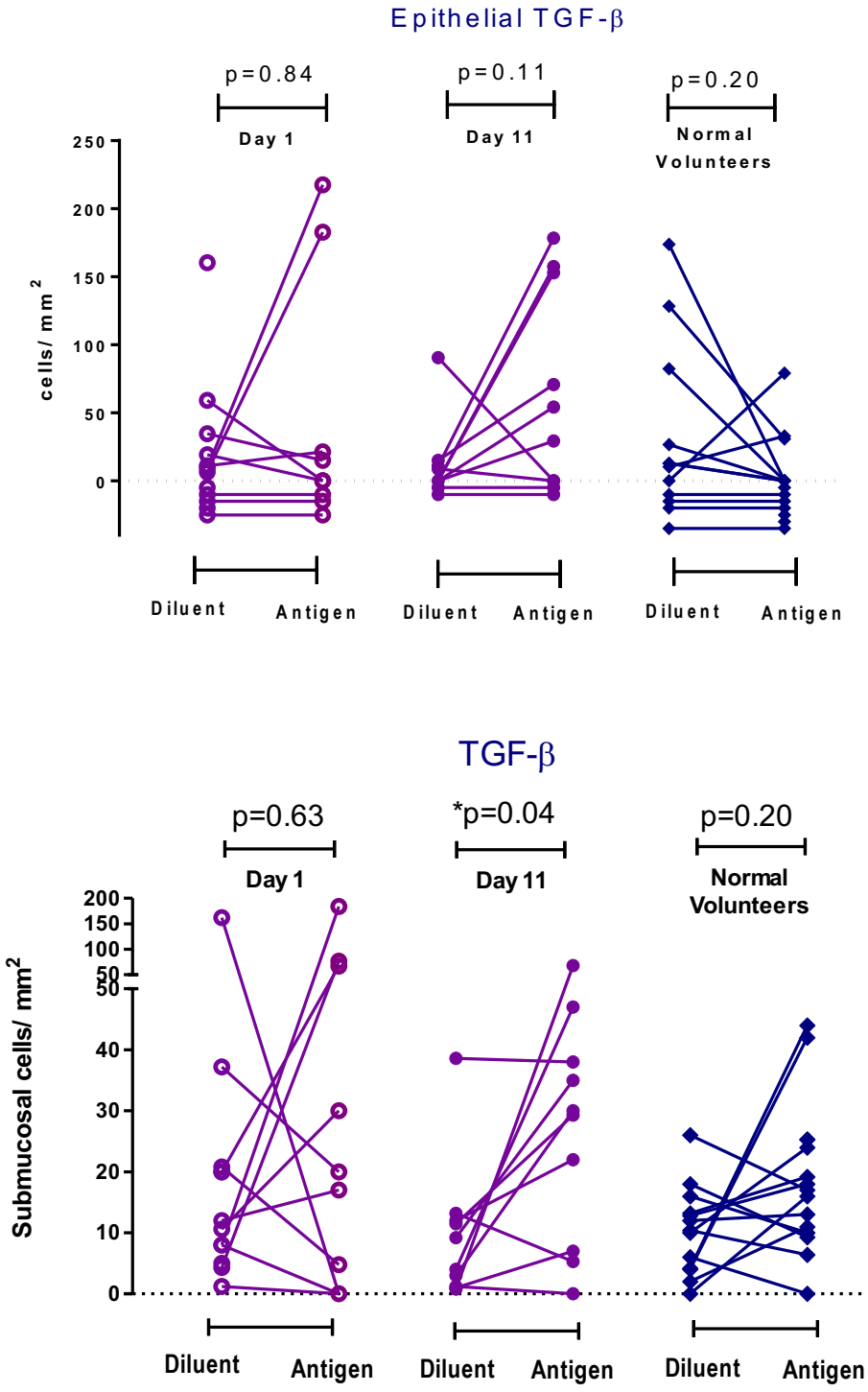


Figure 5.31: TGF-β: epithelial and submucosal cell counts /mm². Within group comparison of diluent and allergen challenge Day 1 RAC (n=12); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=15). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

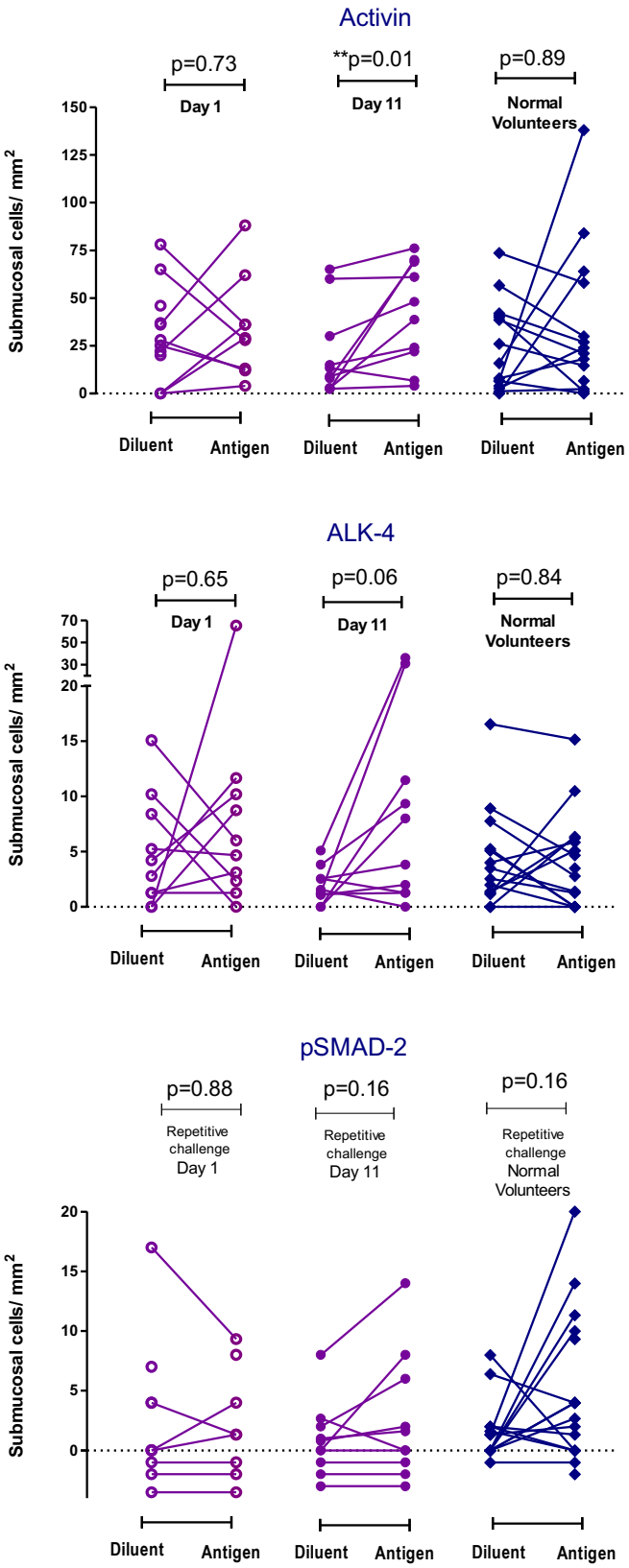


Figure 5.32: Submucosal activin, ALK-4 and pSMAD-2/mm². Within group comparison of diluent and allergen challenge Day 1 RAC (n=11); diluent and allergen challenge Day 11 RAC (n=10); diluent and allergen challenge (two weeks apart) normal volunteers(n=14). Paired data analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

Discussion

This chapter examined evidence for remodelling in natural and induced repetitive allergen challenge models of allergic rhinitis. Each marker of remodelling was taken in turn and, where appropriate, compared to lower airway disease (666).

Epithelium

Epithelial damage was demonstrated at 24 hours post allergen challenge in the RAC protocol with quick recovery and no evidence of epithelial loss by two weeks. Such loss was most likely due to the almost immediate, rapid and pronounced expulsion of goblet cell and epithelial gland contents as was evidenced by changes in epithelial mucus staining intensity, and loss of epithelial mucus content over the same time period. In allergic rhinitis goblet cells can increase in function without necessarily increasing in size or number (667). Without prolonged exposure to allergen, it is likely that epithelial damage is quickly repaired as no statistically significant difference is seen between epithelial integrity of allergic rhinitics versus normal controls (668). Repair of epithelial damage was complete by day 11 implying high epithelial turnover will compensate for paced, versus sudden, allergen challenge. This is unlikely to be dose dependent as single allergen or threshold models of allergic rhinitis will all use high enough doses to ensure clinical response, which will include extrusion of gland contents. It suggests a binary effect of gland function on epithelial integrity: at a certain allergen load, contents are dispersed with ensuing effect on epithelial integrity. Further allergen insults are not able to bestow additional change until both the epithelium and the glands have recovered, which as evidenced by the RAC data, they are able to do rapidly, and certainly under two weeks.

A number of signalling molecules are thought to contribute to epithelial repair, including epithelial growth factor receptor, EGFR (669) and TGF-β . Heparin binding EGF signals through EGFR to enhance epithelial repair in a time-dependent manner (670). Repetitive allergen challenge and seasonal exposure did not elicit a demonstrable rise in epithelial TGF-β although it was present in significantly higher levels submucosally after two weeks of RAC compared to levels found in the seasonal allergic rhinitics. Desquamative injured lung disease states such as pneumonitis obliterans, cystic fibrosis or ARDS are characterized by activation of reparative processes to shield the denuded basement membrane (671). It can be postulated that the nature and duration of such mechanical interventions is likely to shape the epithelial reparative response. With near constant exposure to noxious stimuli, rapid epithelial regeneration is a pre-requisite in the nose.

The dynamic nature of asthma endows a contractile/ expansile mechanical alteration to the lower airways. The absence of smooth muscle implies lack of such changes in the nose. Sudden expulsion

of epithelial gland contents gives rise to shearing type forces that are not maintained with persistent allergen exposure as evidenced by RAC protocol. The rise in TGF-β did not correlate with epithelial loss; the former was increased over time with sustained allergen load, the latter peaked immediately after single challenge and then waned indicating independent mechanisms of action. In the absence of epithelial TGF-β alteration in both polyp and turbinate tissue of nasal polyp patients, mechanical insults in the nose were either insufficient by themselves or were of inadequate in magnitude to elicit TGF-β mediated epithelium driven activation and, by extension, epithelium initiated remodelling.

Cross talk occurs between epithelial and sub epithelial fibroblasts via multiple channels including integrins (672). Prolonged denudation of the epithelial surface barrier engages fibroblast migration and the production of pro-fibrotic mediators (673). The attenuated fibroblast sheet seen in asthma, of high relevance during embryonic development and post fetal life remodelling, is absent in the nose although epithelial/ fibroblast cross talk does exist in the upper airway possibly via IL-13 triggered epithelial TGF-β release (674). EMTU activation and, by extension, the drive to remodel may be activated by an amalgamation of factors. A combination of inflammation, direct injury, and mechanical stretch will all play their variable part. Topographic alteration is relatively limited in the nose, mainly evidenced as tissue oedema and vascular engorgement in allergic rhinitis. The most noticeable change, with nasal obstruction as a proxy measurement, is seen in nasal polyposis where pseudocyst formation and extravasation significantly alter tissue volume and architecture over a prolonged period. Combined with tenacity of inflammatory infiltrate- which in contrast resolves in allergic rhinitis- nasal polyposis provides conditions in which epithelial/ fibroblast signalling may flourish. The relapsing/ remitting nature of allergic rhinitis make it unlikely to manifest these conditions.

Basement membrane

Alterations in basement membrane thickness may be due to increased tenascin deposition (in lamina densa), increased collagen expression (in lamina reticularis) or increased extracellular matrix component deposition within the basement membrane itself as secreted by submucosal parenchymal cells. Thickening of the basement membrane is a feature of remodelling in lower airway disease with variable interpretations as to its relevance in airway hyperreactivity. In both models under investigation here- natural allergen exposure, and repetitive allergen challenge- no change in basement membrane thickness was noted either in season or with single or multiple doses of allergen.

In lower airways, tissue eosinophilia is related to AHR with a concomitant rise in tenascin (221). In our NAE and RAC models, lack of tenascin deposition induced basement membrane thickening may represent either low level eosinophilia, or sufficient respite in between challenges to prevent

quantifiable accumulation. Whilst eosinophilic upregulation is evidenced and linked to clinical severity on allergen exposure, such a spike in cellular infiltrate is likely to be too transient and insufficient for maintenance of collagen deposition (599) over any appreciable time. This raises the possibility of both a threshold level of inflammation necessary for initiation of remodelling changes and a temporal sustenance of the same for adequate propagation of histopathological alteration of tissue architecture. TGF-β is known to upregulate tenascin expression in lower airways disease. The behaviour of tenascin demonstrates dynamic and potentially reversible response to changes in tissue microenvironment (675). Although tenascin may enhance fibroblast migration in wound healing, tenascin deposition in and of itself is unlikely to be of clinical significance (676). Anti-IL-5 therapy, whilst reducing subepithelial tenascin, does not ameliorate AHR, improve airway function or decrease clinical symptom severity (221). As such tenascin may be a proxy marker of subepithelial collagen deposition without necessary causation of persistent remodelling associated changes.

Sirius Red stains of basement membrane are well-established in studies of remodelling (580). Histological staining of reticular basement membrane thickness measurement does not allow for quantification of protein density, which requires immunostaining via immunofluorescence and confocal microscopy. Utilised in asthma, where remodelling is known to occur, differences in reticular basement membrane thickening and collagen deposition are only significant when asthmatics as a whole study group (as opposed to stratified by disease severity) are compared with normal controls. This finding was not linked to any change in submucosal collagen deposition, TGF-β or eosinophil counts (677). In both NAE and RAC protocols an initial histological stain was used to establish presence of increased basement membrane thickness prior to utilisation of more discerning measurements of collagen deposition, had it been applicable.

Basement membrane measurements did not alter with seasonal, single or repetitive allergen challenge. Conversely positive control samples in the form of nasal polyp tissue did demonstrate thinning of the basement membrane, with a greater degree of thinning in polyp rather than turbinate samples of nasal polyposis participants (see Chapter 7). Basement membranes display heterogeneity and disease specificity (678). Basement membrane thinning occurs in the context of contractile forces cooperating with MMPs to enhance new budding/ growth in airway models (679). Thinning of mesenchyme occurs during tubular structure development (680). Dynamic interplay between mechanical forces and remodelling cytokines such as TGF-β and fibroblast growth factor (FGF) will operate at times of changing morphology. During new lung bud formation, alterations of localised TGF-β signalling will thin basement membranes allowing for growth at either end of the thinned section, resulting in cleft formation between two new buds (681).

Basement membrane changes are heterogeneous, depending on the context of change in tissue architecture. Whilst increased TGF-β signalling is a feature of airway remodelling in asthma, concomitant thickening of the basement membrane (682) may not be universal feature of the remodelled airway *per se*, but rather a reflection of the persistent eosinophilic/ Th2 driven tissue environ of asthma itself. Established and ongoing deposition of extracellular matrix components and newly formed collagen contribute to this process. Conversely, an expansile tissue environment undergoing active growth may demonstrate thinning of the basement membrane to allow for invagination and new growth. A summation of effects may be necessary to chronically alter basement membrane thickness. Such adaptation, whilst present in lower airways, is unlikely to be singularly responsible for disease severity. Reticular basement membrane thickening alone does not contribute to airway hyperreactivity as seen in eosinophil bronchitis (683), indicating that it may not be needed or present in instances of prolonged clinical symptoms. In allergic rhinitis, which lacks a smooth muscle layer, contractile forces and persistently high levels of eosinophil infiltrate, the basement membrane may not alter. This study reflects such a hypothesis.

Submucous glands

In both upper and lower airways, submucous glands contribute to mucus and liquid production-secretions which are then propelled along ciliated ducts into the nose or airway lumen. The anatomy of the upper and lower airway diverge with submucous gland nestled between smooth muscle and cartilage in the lung but sitting in the submucosal layer in the nose (293). Submucosal PAS staining was used to characterise gland pathology. Airway mucus contains mucin (684), a glycoprotein with the carbohydrate portion staining well with PAS (685).

Volume of submucous glands can increase several fold in pathological states such as cystic fibrosis (686) (687). The degree of hyperplasia or hypertrophy contributing to this has not been clearly defined. In NAE, no significant difference was noted with seasonal AR samples. However, analysis of in- versus out of seasonal biopsies demonstrated evidence of increased submucosal glandular area with gland tissue itself (as opposed to intra-glandular contents) contributing to this rise (Figure 5.37) suggesting that these glands have dispersed their contents. This slight increase in gland volume does not differentiate between states of hyperplasia or hypertrophy. Comparison of RAC samples at day 11 versus non-atopic normal controls confirm this finding ($p=0.06$).

Mucus production is under cellular, cytokine and neuronal control. IL-13 will endow submucous glands with increased production with responsiveness to this and other Th2 cytokines varying between proximal and distal airways in a murine model (688). Further impact of the parasympathetic system

via acetylcholine triggers signalling via structural cell secreted IL-6, IL-8, and MCP-1 acting on gland cells directly to enhance mucus production. Mediation is reliant on M3 receptor, which is not a conduit for airway inflammation as knock-out M3 models have persistence of Th2 driven inflammation although do not recapitulate remodelling phenotype (689). It may be postulated that the increase of submucous gland tissue (via either hypertrophy or hyperplasia) is a reversible event under both neuronal and cytokine control. The relative contribution of each is yet to be established.

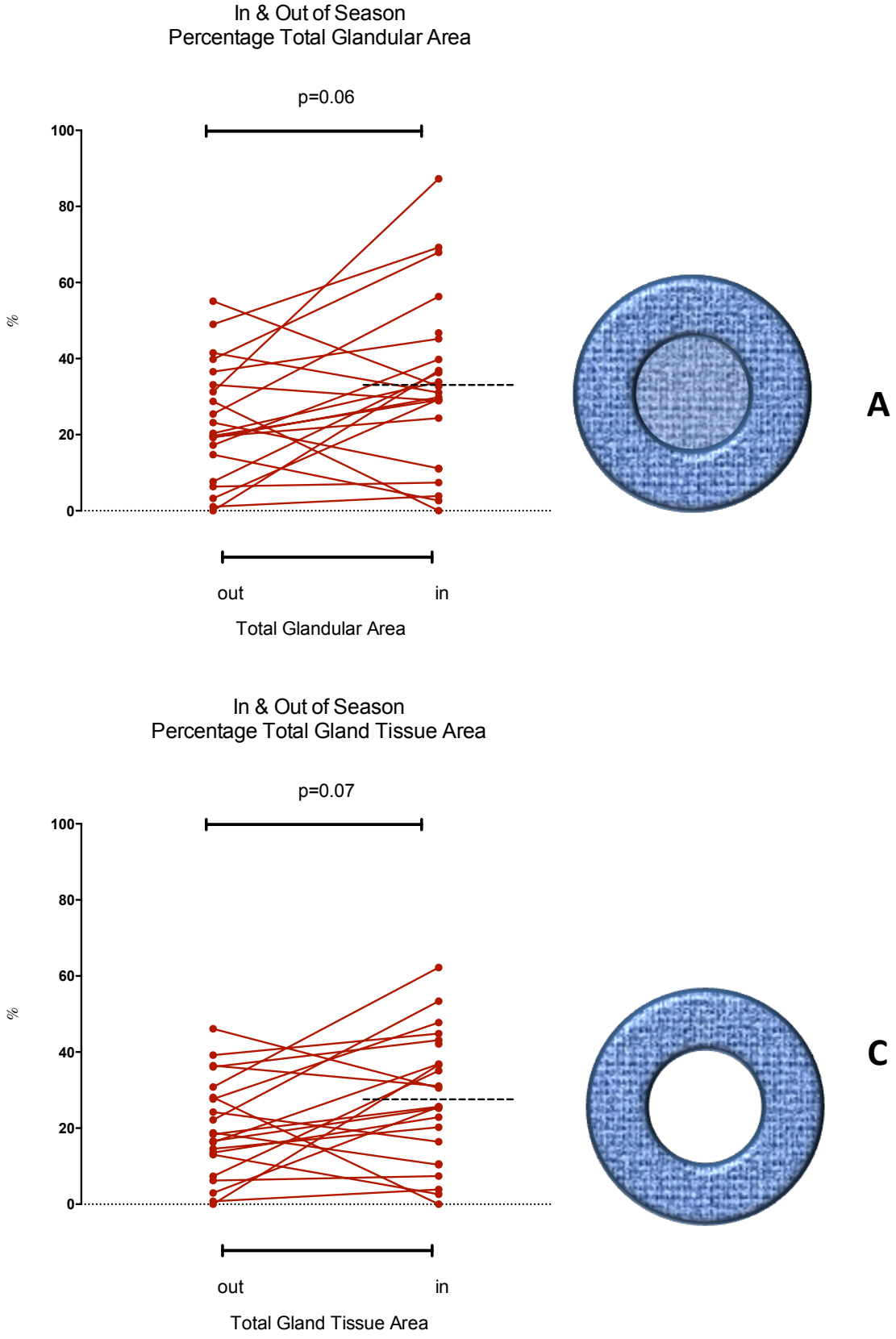


Figure 5.33: : Submucosal Glandular Area: measured area is represented by textured blue background on schematic gland drawing, representing (A) gland tissue and secretory contents, (B) secretory content area only and, (C) glandular tissue only. Increases in submucosal glands with the pollen season. Paired data was analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

Vascular and Lymphatic Changes

CD31 for angiogenesis and vascular markers (690), and D2-40 for lymphatic vessel enumeration have been shown to be appropriate immunohistochemistry targets in normal tissues and vascular disorders, including cancer (691). VEGF as a potential target in the study of remodelling has its origins in observations of the late phase reaction in skin, where increased blood flow with concomitant oedema contributes to clinical symptoms (214). VEGF can enhance Th2 responses, increase collagen production with reticular basement membrane thickening and airway smooth muscle hyperplasia, it is associated with production and activation of TGF-β, and induces airway oedema and angiogenesis (563). The angiogenic properties of VEGF may vary depending on tissue type, with relatively few angiogenic properties in respiratory tissues (692). Within the same organ, VEGF may show particular expression patterns, such as alterations between different gland acini (693). This study examined VEGF in the submucosa without segregation of expression. In both natural and induced models of allergic rhinitis, no alteration in VEGF levels were observed.

A multitude of other pro-vascular growth factors are also potentially involved in neovascularisation. VEGF and FGF-2 genes are expressed by eosinophils in asthmatic airways (694). Amelioration of such sources does not completely abrogate neovascularisation. Neutralisation of VEGF from eosinophils and mast cells reduces angiogenesis in a chick model by 55% and 30% respectively. When antibodies to both VEGF and FGF-2 were administered, the angiogenic response reduced by 70% suggesting significant but not exclusive influence of these pro-angiogenic factors (695). In asthma, higher VEGF and VEGFR1/2 expression inversely correlated with airway function (696). FGF-2 in BAL is higher in atopic asthmatic patients and increased further post-acute allergen challenge indicating IgE and mast cell dependent mechanisms influence, but are not solely responsible for, angiogenic development (697). The lack of VEGF upregulation in NAE and RAC models of allergic rhinitis fits with lack of neo-angiogenesis in response to allergen exposure or challenge.

In murine asthma model overexpression of VEGF (in $VEGF_{165}$ transgenic mice) following allergen exposure results in overexpression of IL-13, enhanced mucus production, airway smooth muscle hyperplasia and augmented collagen deposition. Furthermore, this murine model demonstrated increased numbers of activated pulmonary dendritic cells (563). INF-γ inhibited baseline as well as IL-4, IL-5 and IL-13 triggered VEGF production suggesting a bidirectional feedback loop between T cell and mast cell responses in angiogenic pathways. Whilst histamine enhances VEGF expression, tryptase proteolytically releases extracellular matrix bound VEGF and FGF-2 (698). Different isoforms of VEGF are bound by heparin sulphate proteoglycans to variable degrees making a dynamic and mutable

reservoir of VEGF available by proteolytic cleavage (699). This study demonstrated that constitutive levels of VEGF maintain vascular integrity in the allergic nose.

Non-eosinophilic cellular sources of VEGF-A in the airways include macrophages (532). M1/ M2 macrophage polarisation is evidenced by differing phenotypic and functional behaviour, demonstrating evidence of remodelling independent of Th2 cytokines in M2 rich milieu (700). Via VEGF-A, macrophages, of unknown polarity, are capable of inducing inflammatory neovascularisation by angio- and lymphangiogenesis (561). Such macrophage behaviour might be associated with a particular subtype and will show variability in expression depending on type of airway disease, for example, alveolar macrophages release TGF-β and fibronectin in greater amounts in chronic bronchitis versus asthma (701). Increased numbers of submucosal macrophages, as was seen in polyp samples, might be necessary to maintain a pro-angiogenic influence.

In the nose VEGF mRNA and lavage content may be higher in allergic rhinitics but this was not demonstrably coupled to a significant immunohistochemical difference (693). Nasal lavage may demonstrate early and short lived VEGF alterations with allergic stimulation without alteration in total IgE response demonstrating quick resolution (702). In murine transgenic models, VEGF overexpression needs to be persistent to effect enhanced collagen deposition, partly via TGF-β dependent mechanisms (563). In the same study, VEGF induced remodelling was abrogated in the absence of IL-13. VEGFA levels do not influence endothelial progenitor cell influx into respiratory submucosa. Recruitment of bone marrow derived endothelial cells into allergen stimulated airways is likely to be an early event as increases in micro density unfold with chronic allergen challenge (564). A naturally occurring anti-angiogenic growth factor, endostatin, will prevent features of airway remodelling in a mouse model (703). Furthermore, allergen load in the form of house dust mite correlates poorly to parenchymal VEGFA (704). In murine models, endothelial progenitor cell recruitment to the lungs post HDM challenge was independent of VEGFA (704). In instances where vascular remodelling does occur, such as in the turbinates of nasal polyposis patients, no alteration of VEGF need occurs for a pro-angiogenic environment to exist.

In allergic rhinitis, sputum VEFGA is higher only in those who also have bronchial hyperreactivity demonstrating the specific role of VEGFA in lower airways disease (705). Only 20% of the people in our study were clinically mild asthmatics who may be unlikely to demonstrate a positive VEGFA signal at submucosal level. IL-8 secreted by monocytes, macrophages and mast cells act as a pluripotent angiogenic cytokine. It enhances proliferation of endothelial cells, induces their chemotaxis and survival, and increases MMP mRNA in endothelial cells (545). In both NAE and RAC protocols, IL-8 was

not increased at baseline or on allergen challenge, whether natural or induced. Other cytokines such as IL-17, produced by Th17 cells, are capable of robust angiogenic influence in the absence of VEGF (546). Although not measured in these studies, it provides alternative means of altering tissue vasculature sidestepping alterations in VEGF.

In asthma, levels of VEGFA correlate with levels of MMP9 (706) indicating VEGF levels vary in response to acute alteration is tissue topography as opposed to influencing ongoing change. VEGF enhances MMP-1 expression as well as leukocyte adhesion molecules (707). In a murine model of repetitive allergen challenge, VEGF, absolute MMP9, and absolute TIMP-1 but not MMP9/TIMP-1 ratio was elevated in OVA treated mice, an effect obliterated by anti-angiogenic treatment (708). In both NAE and RAC models, VEGF, and MMPs both in ratio and in absolute terms, did not change. This suggests an absence of extracellular matrix remodelling in the nose and concurs with the evidence that allergen challenge does not enhance vascular or lymphatic tissue locally. Bronchial constriction in the lungs is equivalent to vascular engorgement and nasal blockage in the nose (113). Increased vascularity and lymphatics were only seen in turbinate samples of nasal polyposis patients, a finding not coupled to enhancement in VEGF levels indicating alternative pathways of enhancement of new vessel formation in turbinates of nasal polyposis.

VEGF expression by fibroblasts and epithelial cells is increased in response to TGF-β, whilst such enhanced secretion by eosinophils was in response to IL-5 and GM-CSF (222). IL-5 and TGF-β only demonstrated a transient rise, except in the case of persistent allergen exposure. As such, these cytokines, either solely or in combination, are unlikely to be sufficient to drive increased VEGF, via blood recruitment, endogenous release of stores, or de-novo production. In nasal polyposis where there exists a primary eosinophilic drive, coupled to polyp derived TGF-β secretion, VEGF+ signal is maintained despite decreased vascularity of polyp tissue. Such differences between nasal polyposis and allergic rhinitic samples do hint at fundamentally different pathophysiology's causing each disease.

Whilst evidence in lower airway disease suggests increased levels of VEGF at baseline, evidence for increase with allergen is conflicting. Demonstrations of no significant increase in the nose may intimate release of preformed stores in the lower airways as opposed to *de novo* synthesis, and this is in a context of known neovascularisation in asthma. In allergic rhinitis, VEGF levels as well as established blood and lymphatic vessel formation, exhibit no alteration following natural or induced allergen exposure. This would suggest paucity of pro-angiogenic signalling either alone or combined with sufficient anti-angiogenic signalling to prevent new blood and lymphatic vessel formation. Neo-vascularisation and lymphangiogenesis was therefore not a feature of allergic rhinitis.

Matrix Metalloproteases and their inhibitors

Alteration of extracellular matrix architecture is reliant on the delicate balance between inhibitory signals via TIMPs and proteases capable of breaking down ECM protein bonds (709). Matrilysin/ MMP7 influences airway epithelial repair post injury (492), whilst gelatinase/ MMP9 aids the establishment of neovascularization during arterial remodelling (502). MMP7 is present in fibrovascular growths, aids angiogenesis and fibrovascular proliferation (710). Severe lung injury with loss of epithelial integrity activates and upregulates epithelially expressed MMP7 to cleave E-cadherin (a calcium dependent transmembrane glycoprotein regulating cell-to-cell adhesion) and thereby allows epithelial mobilisation to protect newly made bare basement membrane (671).

Linking into the context of epithelial repair, monocytes, macrophages and mast cell secreted IL-8 may act as a pluripotent angiogenic cytokine. It enhances proliferation of endothelial cells, induces their chemotaxis and survival, and increases MMP mRNA in endothelial cells (545). As previously mentioned, a murine model of repetitive allergen challenge saw an increase in VEGF, absolute MMP9, and absolute TIMP-1 but not MMP9/TIMP-1 ratio in OVA treated mice; an effect eradicated by anti-angiogenic treatment (708). In both natural allergen exposure (NAE) and repetitive allergen challenge (RAC) model, IL-8 was unchanged in allergic rhinitics. Nasal polyp participants have increased IL-8 underpinning on-going angiogenic influence in this disease. Compared to the normal control group increases in matrix metalloproteases alteration was identified by an increase in absolute TIMP1 in both polyp and turbinate samples with concomitant increase in MMP7 seen in polyp samples only and a decrease in MMP9 in turbinate samples respectively. The ratios of MM9: TIMP1 and MMP7: TIMP1 both rise in nasal turbinate samples. This underscores the presence of continued dysregulation of ECM architecture, with underlying pro- angiogenic processes, and implies that this alteration manifests differently between polyp and turbinate samples of polyposis patients. Alteration of biomechanical and tensile forces on the extracellular matrix will modify MMP secretion (711). No such alteration was seen in natural and induced models of allergic rhinitis, where paucity of new blood and lymphatic vessel formation coexist with absence of extracellular matrix alteration.

MMPs are not constitutively expressed and may be secreted in soluble form. Leukocytes and endothelial cells are potential sources of MMP-7 during inflammation (712). Inflammatory cells in the context of airway remodelling also provide an early source of MM9 and TIMP-1 (713) with macrophages of particular import. The initial response to allergen challenge causes an immediate rise in TIMP-1 which fulfills a homeostatic regulatory function and coincides with a rise in macrophage numbers. With mild, and possibly, remitting allergen stimulation an early increase in TIMP-1 is sufficient to downregulate any increase in MMP. When coupled to lack of epithelial damage, any rise

in MMP level is likely to be slight and brought under control with relative ease. As a result, in natural allergen exposure protocol, TIMP increases in both normal and allergic rhinitics with allergen challenge, but in the absence of sustained and sufficient allergen load, TIMP returns to baseline and does so quickly having fulfilled its regulatory role including limiting MMP activity. With regular unremitting allergen stimulation as seen in RAC protocol, two weeks of allergen exposure will cause an initial rise in TIMP as seen at 24 hours, with subsequent return to baseline of TIMP and downregulation of MMPs, re-establishing the homeostatic state. It can be postulated that sufficient regulatory mechanisms are activated to prevent a prolonged increase in TIMP-1 with no marked rise in MMPs also abrogating the need for sustained increase. Decreasing TIMP levels are coupled to declining allergen load as seen in natural allergen exposure.

In support of this, MMP9 and TIMP1-possibly of eosinophilic origin- are both seen to rise post house dust mite allergen at 6 hours but wane rapidly (526). There is further evidence of such biphasic expression of MMPs with MMP9 first increasing before falling in CRS. Furthermore levels of MMP9 correlate with osteitis, reinforcing the link between degree of cellular infiltrate at the onset of inflammation (524). Increases in TIMP 24 hours after allergen challenge in allergics as well as normal non-atopic individuals underscores its homeostatic role via a regulatory pathway that is activated and effective in allergic airway disease. Attempts at such reparative processes are reflected in the coupling of TIMP-1 with HSP-47. Following the initial rise in both TIMP-1 and HSP-47 as is seen at 24 hours post allergen, both levels fall away with relative respite in allergen load, as witnessed by seasonal biopsies. Comparison of in versus out of season allergic rhinitic biopsies demonstrated a fall in both markers with the season. Following the initial rise post allergen, attempts at preventing alteration of tissue architecture by production of extra collagen are successful in abrogating excessive new ECM production. Evidence of similar restitution of function are seen in perennial allergic rhinitis tissue homogenates, where no alteration of MMP and TIMP-1 mRNA was found (714). Reparative remodelling processes continue in nasal polyposis patients with increased TIMP1 coupled to an increase in HSP47 and SMA in polyp and turbinate samples respectively.

Collagen and fibroblast transformation

In asthma, HSP47 is seen to rise acutely following allergen challenge accompanied by procollagen I, although only the latter demonstrates maintenance of this significant increase at a week post challenge. The significance of this in lower airways disease with reactive airway smooth muscle is the implication that collagen producers are more likely to exhibit airway hyperreactivity (628). AHR is related to smooth muscle mass, which is absent in the nose. Collagen production and myofibroblast

transformation in the nose, if present, will occur by mechanisms other than smooth muscle involvement.

New myofibroblasts may be products of transformation *in situ*, or may come from haemopoietic precursors which are evidenced by the presence of stem cell antigen CD34 (715). As there are no ASM in the nose, myofibroblasts must come from resident fibroblasts or be recruited from haemopoietic progenitors, which downregulate CD34 and upregulate SMA on transformation once resident in tissue. Several different structural cells may transform into myofibroblasts with tissue resident, or bone marrow derived fibroblasts being the most likely. Mammalian cells in general (679), particularly myofibroblasts, alter their phenomenology depending on disease state for example cancer and fibrosis (716).

Type, duration and frequency of mechanical stress, with further dependence on prevailing matrix composition, determines a wide variety of fibroblast responses. These include proliferation (with prolonged, non-remitting stretch force), enhancement of collagen synthesis (in presence of favourable ECM conditions) or apoptosis (intermittent stretch forces) (717). Nasal polyp fibroblasts differ from bronchial asthmatic fibroblasts in their cytokine secreting response to mechanical strain (718). Whilst collagen production is seen in all tissues subjected to allergic assault, the rise is much greater in those predisposed to an eosinophilic Th2 type environment. Myofibroblast transformation is likely to be a late effect of remodelling; a relapsing/ remitting, short burst of allergic load or a low but persistent load may not produce the tissue milieu needed for phenotypic change. Furthermore, ASM resident fibroblasts are likely to be phenotypically different from other airway fibroblasts.

Fibroblast transformation, new myofibroblast generation and the subsequent behaviour of such cells is influenced by the cellular and cytokine milieu as well as mechanical forces experienced by these tissues. Airway fibroblasts in the context of lower airway remodelling may have a duality of purpose: to secrete inflammatory mediators and to enhance ECM remodelling, contributing to the clinical picture of the asthmatic late response (719). Fibroblast secreted inflammatory chemotactic factors alter with type of cytokine milieu (720). Inflammatory cells such as eosinophils, linked in to a TGF-β rich environment are necessary for initiation of such myofibroblastic change, with structural cells assuming responsibility for longer term maintenance of these alterations (721). With the initial rise in eosinophils, and a Th2 dominated scenery post allergen challenge, the RAC protocol saw a rise in HSP-47 after Day 1 and Day11 allergen challenge, linked in with a rise in TGF-β at two weeks. Seasonal exposure demonstrated a decrease in TGF-β, which was linked to a concomitant drop in HSP-47 in seasonal samples further suggestive of a dose dependent process.

Persistence of TGF-β signalling is linked to increased subepithelial fibrosis via enhanced and constant collagen I and III expression, a finding that is not ameliorated with steroid treatment (722). TGF-β and activin both aid myofibroblast transformation. Fibroblasts need mechanical strain in addition to TGF-β to transform into myofibroblasts with IL-13 aiding this process. TGF-β itself works synergistically with Th2 cytokines to enhance eotaxin expression in lower airway fibroblasts (723). This reflects a bidirectional regulatory loop involved in the initiation of TGF-β expression, which in turn enhances further allergic inflammation. Long term maintenance of TGF-β induced fibrosis is the remit of structural cells, which by extension will not be ameliorated by steroid treatment.

The RAC protocol demonstrated increases in HSP-47 acutely after one day and with persistent challenge after two weeks. The increase in HSP-47 following allergen challenge in non-atopic controls would suggest a non-allergen specific response with no such rise evidenced in seasonal samples. Low levels of HSP-47 are found in unchallenged tissue and levels revert to normal unless stimulus in the form of allergic inflammation and/ or mechanical shear, is unrelenting. Mechanical strain results in increased collagen synthesis in human airway fibroblasts but myofibroblasts undergo apoptosis or revert to undifferentiated state if not stimulated (380). Changes in tensile forces on the extracellular matrix will further enhance fibril assembly (724). An allergic infiltrate is unable to solely maintain a rise in collagen synthesis, with mechanical tissue alterations a pre-requisite for ongoing change (725). Such persistence of allergic cellular inflammation with concomitant favourable cytokine environment, linked to ongoing alterations in tissue topography are present in nasal polyposis but absent in allergic rhinitis. As such, the transient increase in HSP-47 is not maintained and, with no increase in smooth muscle actin, such changes do not beget full myofibroblastic transformation despite short lived enhanced attempts at collagen synthesis. These findings are replicated in the skin where remodelling markers of tenascin, myofibroblasts, pro collagen positive cells all return to baseline by 72 hrs post allergen injection (214).

A weak inverse correlation exists between SMA and TIMP-1 in induced allergen samples indicating persistent allergen challenge induces regulatory pathways which, for the most part, can revert to baseline in the absence of further allergen exposure. TIMP, HSP-47 and SMA all decrease as the need for collagen synthesis is brought under control. The altered tissue topography seen in nasal polyposis enhanced by TGF-β mediated collagen remodelling results from persistent mechanical strain and inflammatory drive. Of interest is the thinning of the basement membrane in nasal polyps, a finding not replicated in allergic rhinitis, where initiation of collagen synthesis does not translate into persistent myofibroblast transformation. This further supports the different pathways contributing to tissue changes seen in polyps and allergic rhinitics.

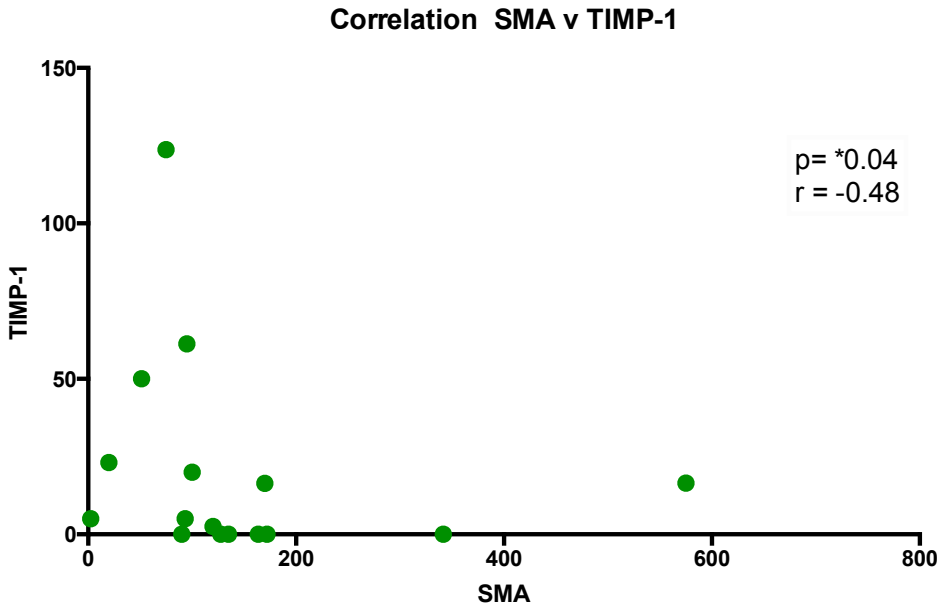


Figure 5.34: Correlation of SMA and TIMP-1 in allergic rhinitics in repetitive allergen challenge protocol. Spearman rank coefficient was used to calculate strength of correlation. A p value less than 0.05 was considered significant.

TGF-β and family

TGF-β and its family members provide a kaleidoscope of regulatory functions in injured airways. Increased allergen load causes an increase in both TGF-β and activin (see Chapter 6) but this is quickly abrogated when the level of ongoing allergen exposure declines. A rapid, biphasic rise in activin is seen post LPS injection with elevation at 1 and 4 hours followed by a return to baseline (478) (726). In contrast, follistatin demonstrates a tempered rise that is sustained for 24 hours. Such secretory contours are reproduced in very diverse tissues, including placenta (727). Post inflammatory stimulus, activin mRNA levels are highest in the liver with protein level highest in the bone marrow. Following LPS administration, activin levels decrease 35% in the bone marrow and increase 5 fold in the lung, indicating post transcriptional, protein level regulation versus new transcription (728). Activin may inhibit T cell responses directly or via increase in T reg cell function (729) (730).

TGF- β is produced by inflammatory cells and structural airway cells (fibroblasts, epithelial, endothelial and smooth muscle) but the main sources are eosinophils (731) and macrophages (701). Initially TGF-β will rise rapidly as matrix stores are released but the rate of production of new TGF-β may stay constant. Expression of TGF-β is in harmony with submucosal macrophages, the main source of TGF-β (732) (733). Alterations of TGF-β are coupled to changes in macrophage cell counts as was evidenced in seasonal symptomatic samples where both were reduced compared to normal controls (NAE protocol) and increased post repetitive allergen challenge (RAC protocol). In the absence of eosinophils and macrophages to produce TGF-β, and mast cells, CD4 cells, and neutrophils to secrete activin, regulatory mechanisms possibly through T-regs, may come into play to further negate remodelling processes. This is reflected in lower airways disease and reproduced in studies using acute and chronic provocation in allergic asthma, where after an initial rise, epithelial activin and TGF-β both fall (734) (485).

Epithelial TGF-β tends to rise with persistent allergen challenge but not significantly and since there is no epithelial damage there is unlikely to be EMTU activation in the short term. A similar rise was evidenced in submucosal TGF-β and activin, both of which rose with repetitive allergen challenge. Although below statistical significance, intracellular pSMAD2 was also increased after two weeks. Persistence of allergen insult without amelioration or periods of relative quiescence are likely to upregulate TGF-β and activin expression, reflecting the picture seen in lower airway disease (389). Activin was seen to rise and return to baseline relatively quickly with baseline levels higher than normal but without an increase post challenge in the lower airways (735). Seen in the context of variable allergen load, with amelioration of granulocyte counts and the rescinding of an acute allergic inflammatory landscape, TGF-β also falls, with the return of activin to its baseline levels. Regulatory

mechanisms may overcorrect this homeostatic process with TGF-β being less than normal in seasonal samples possibly to alter pro-fibrotic responses whilst propagating an IgA switching B cell response (736).

Continued ALK4 elevation in seasonal allergic rhinitics might result from previously elevated activin levels, which increased with persistent and prolonged allergic inflammatory response. Failure to internalise and degrade pericellular activin may lead to enhanced and persistent signalling which could potentially deplete pSMAD2 that are translocated and held in the nucleus rapidly post activation. Alternatively, or in conjunction, increases in pre-receptor activin agonism either by competition at receptor level with inhibin, or binding and internalization with follistatin, may upregulate ALK-4 expression. TGF-β can signal through ALK5 or ALK1, where ALK5 signalling links its intracellular pathway with pSMAD2/3, and ALK1 exerts through pSMAD 1/5/8. In the presence of activin, TGF-β may preferentially signal through ALK5, with pSMAD2 providing an intracellular clue as to the level of ALK5 mediated TGF-β signalling. It can be postulated that in the setting of relapsing/ remitting inflammation, TGF-β may be the overriding influence of SMAD2 in natural allergen exposure. Cellular levels for TGF-β and pSMAD2/3 level correlate in both nasal polyposis (737) and murine models of remodelling in asthma (738). This coupling seems to be, at least in part, due to TGF-β and to some extent activin A regulated fibroblast SMAD2/3 expression (479). Evidence for altered ALK4 expression is seen in decreased epithelial immunostaining in murine model of allergic asthma; a finding abrogated by follistatin administration (485). Conversely, mild atopic asthmatics may demonstrate increased ALK4 in both epithelial cells and submucosal fibroblasts (735) although evidence to the contrary also exists (739). It is therefore likely that disease state, progression, timepoint post challenge and the prevailing inflammatory milieu all coalesce to affect alteration in cellular ALK4 expression.

In this setting pSMAD2 will decrease in harmony with TGF-β and is not compensated for by the transient rise in activin seen with allergic stimulus. Baseline activin was no different between allergic rhinitis and normals and this may be due to periods of quiescence in AR which may allow for other regulatory pathways possibly T cell driven, to come to the fore. Such regulatory mechanisms could explain the relatively modest symptoms of house dust mite patients who on the whole are intermittently and mildly symptomatic. With periods of rest from allergen load, allergic inflammation is not sustained enough to drive TGF-β mediated remodelling. These findings agree with those in asthma: namely decreased pSMAD2 levels in mild disease (735), reproduced here in symptomatic allergic rhinitis, coupled with increase from this baseline on antigen stimulation (740) or more severe disease states (741), as evidenced in this study by allergen stimulation during repetitive allergen exposure.

This study demonstrated activation of TGF-β and activin pathways with sufficient and sustained allergen load. These findings are supported by what is seen in nasal polyposis (see Chapter 7) where polyps are the 'engine' that drive remodelling. Without sustained allergic inflammation, TGF-β and activin drop back to baseline, and indeed may even overcorrect and be found at levels lower than found in normal non-atopic controls. Regulatory effector points are scattered throughout the TGF/activin signalling pathway with receptor and transcription factor level control exercising considerable regulation over final outcome. TGF-β and activin induce specific Treg subpopulations (454) (739). Reduction of TGF-β family receptors in the asthmatic or rhinitic airway may suggest a failure of this regulatory feedback loop in such patients (739) (742).

There is almost no SMAD2 in normal lower airways; a finding replicated in our study in the nose. As a result, SMAD3 may be the predominant signalling channel in fibroblasts (743). Dermal fibroblasts only produce SMA in response to SMAD3. Uniquely quantifying SMAD3 signalling is difficult due to the lack of a discrete monoclonal antibody. Although SMAD3 antibodies are available, they all have considerable cross reactivity with other ubiquitin-proteasome SMADs due to similar phosphorylation sites. SMADs, which are degraded by pathway, may provide a more rapid response to alteration of the activin pathways than ALK4. Similarly, SMAD levels may wane quicker with amelioration of enhanced activin signalling with longer lag times at receptor level. TGF-β receptors may increase in mast cells of perennial allergic rhinitics without concomitant increase in SMAD 3/6/7 genes (388). These findings are replicated in this study with no significant rise in SMAD2, despite increases in both activin and TGF-β, on repeated allergen challenge.

Dichotomous roles of TGF-β and activin are reflected in divergent secretory profiles during the inflammatory response and in knockout model phenotypes. Whilst activin secretion is biphasic and wanes rapidly at 4-6 hours, the TGF-β rise is more gradual with highest secretory levels at 24 hours. Whilst activin is released in its active conformation, TGF-β is released initially in a latent form and needs further activation via ECM mechanical alteration (744) (477). TGF-β is essential for survival as knock-out mice die of overwhelming autoimmune infections (745). Underscoring the importance of integrin and ECM binding of TGF-β, integrin KO mice demonstrate a similar phenotype (746) (747). In our models, periods of quiescence from continuous high allergen load undermine the drive towards sustained remodelling and fail to alter ECM architecture. This finding is further supported by absence of vascular remodelling, where endothelial cells could provide a further potential source of TGF-β. The negative triumvirate of findings: the abrogation of inflammatory cell response, and the absence of both vascular, and ECM remodelling results in a reduced TGF-β response in our model of natural allergen exposure.

Most of TGF-β activation studies are *in-vitro* experiments as *in-vivo* measurements of the active molecule are difficult (400). In this study, immunohistochemical analysis of TGF-β provided clear staining in the submucosa, therefore measurement at protein level was pursued instead of mRNA study to examine post translational levels. Epithelial damage transcribes into increased TGF-β signalling. The absence of such a rise was reflected in the lack of loss of epithelial integrity with natural or induced allergen load. Baseline activin staining was similar between allergic rhinitics and normal non-atopic controls, reflecting a lack of activin upregulation. Such paucity of activin driven 'priming' may demonstrate lack of pro-fibrotic tendency post injury when compared to normals, a state reflected in lower airways (735). Mirroring this state of harmony, the relative homeostatic effect of TGF-β is likely to provide allergic rhinitics with sufficient regulatory control as epithelial TGF-β is not significantly different between these two study groups.

In murine model of remodelling, up to three months of continuous challenge is required before remodelling changes manifest with such changes being more marked in lower than upper airways (748). Nasal inflammation whether caused by allergen or nasal polyposis provides an environment for enhanced TGF-β signalling. Eosinophils, the major cell type responsible for mucosal TGF-β, are increased post allergen as well as in polyposis. Continuous, albeit non-allergic, eosinophilia in polyps provides an uninterrupted supply of TGF-β whilst respite from allergen load, and therefore eosinophilic inflammation in allergic rhinitis diminishes TGF-β over time. Other cell types, such as CD4+ T cells may work in harmony to provide a further boost to TGF-β signalling. Activin and TGF-β are capable of coordinating the post inflammatory response (749). Increased activin is capable of mediating enhanced T cell regulation (730). Further enhancement of the TGF-β/ activin regulatory pathway occurs via a cytokine feedback loop whereby stimulation by IL-12 releases furin by T regs, in turn releasing bound TGF-β from the surface of DCs (750). Conversely, a decrease in inflammatory cellular sources of activin such as neutrophils may indicate aberrant regulatory responses post allergic upper airway injury.

Sustained exposure to allergic instigators initiates and subsequently drives altered TGF-β/ activin signalling. Respite from such exposure permits variable resolution of instigators of remodelling delivering a diverse histopathological picture in allergic rhinitis. Such findings are supported by studies in mild asthma and reflect the disease process in lower airways (735).

Summary of chapter

Nasal congestion is the symptomatic equivalent of lower airway obstruction. In the upper airway, such obstruction is rapidly reversible. It does not leave, and is not caused by, a remodelling 'footprint'. It is an acute, fully reversible event requiring sufficient allergen load to exhibit symptomatic deterioration. This threshold dose, giving rise to significant allergic inflammation is, by itself, insufficient to be coupled to persistent dynamic change.

Whilst allergic inflammation was evidenced in both natural and induced models of allergic rhinitis, no alteration in vascular, lymphatic, epithelial or basement membrane remodelling was seen. Sudden expulsion of glandular contents impacted on epithelial integrity almost immediately post first challenge, but chronic exposure was coupled to sufficient reparative processes to counteract such change. Smooth muscle actin demonstrated weak inverse correlation with TIMP-1 with sustained and persistently significant allergen load which was insufficient to cause an alteration in nasal tissue architecture. Transient, non-specific increase in TIMP-1 is quickly abrogated without alteration in tissue matrix metalloproteases.

Epithelial TGF-β did not change with natural or induced models of allergen exposure. Immediately post induced allergen challenge, submucosal TGF-β was seen to rise but in the absence of persistently high allergen load, TGF-β levels drop. This finding was coupled to a decrease in ALK-4 in seasonal samples, indicating possible preferential signalling mechanisms in the context of upper airway allergy. TGF-β, activin and ALK-4 do show significant rise with persistently high allergen dose as seen with repetitive allergen challenge, indicating that TGF-β family pathways are activated in upper airways inflammation, but responses are dose and duration dependent.

Overall, TGF-β pathways are activated in allergic upper airways inflammation but are not sustained and do not result in nasal remodelling.

Chapter 6 : Comparison of Allergic Inflammation and Remodelling in Natural versus Induced Models of Allergic Rhinitis

Introduction

In this chapter, the previously described parameters of inflammation and remodelling are compared between in-season nasal samples from Timothy grass allergic patients in the natural allergen challenge protocol, and nasal samples from allergic rhinitis participants following 10 days of Timothy grass challenge as per the repetitive allergen challenge protocol. This comparison provides evidence for the feasibility of using the RAC model in place of seasonal exposure for disease characterisation and interventional drug studies .

Inflammation

Comparison of in-season seasonal allergic rhinitics (iS) with RAC day 11 allergen challenge samples (RAC A2), nasal secretion measurements demonstrated no difference in Th2, Th1 or Treg cytokine markers (figure 6.1).

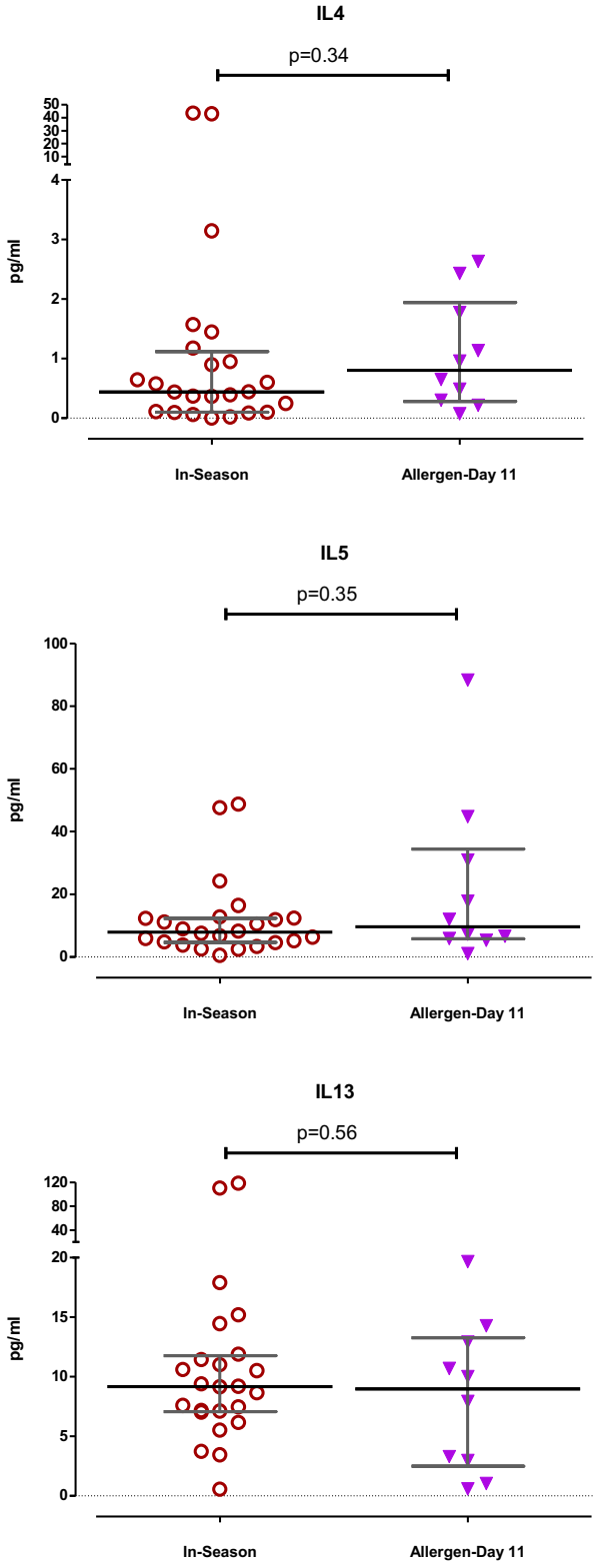


Figure 6.1: Nasal Secretions: Th2 cytokine measurements in pg/ml. Comparison of in-season seasonal allergic rhinitics (n=24) and RAC day 11 allergen challenge samples (n=10). Graphs show median with interquartile range and comparisons are performed using Mann-Whitney test. P<0.05 was taken as significant.

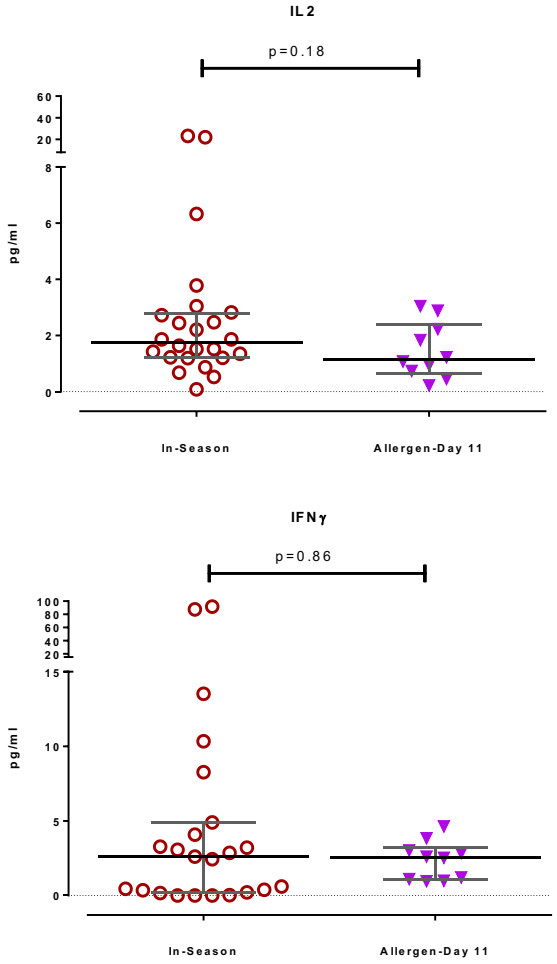


Figure 6.2: Nasal Secretions: Th1 cytokine measurements in pg/ml. Comparison of in-season seasonal allergic rhinitics (n=24) and RAC day 11 allergen challenge samples (n=10) Graphs show median with interquartile range and comparisons are performed using Mann-Whitney test. P<0.05 was taken as significant.

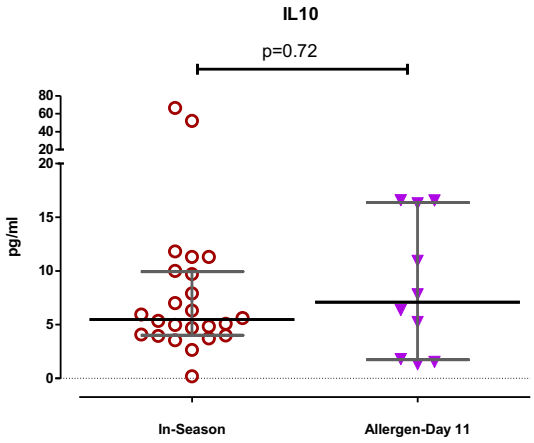


Figure 6.3: Nasal Secretions: T regulatory cytokine measurements in pg/ml. Comparison of in-season seasonal allergic rhinitics (n=24) and RAC day 11 allergen challenge samples (n=10). Graphs show median with interquartile range and comparisons are performed using Mann-Whitney test. P<0.05 was taken as significant.

Epithelial and submucosal eosinophil and neutrophil cell counts were significantly higher in RAC day 11 than in-season allergic rhinitic samples. No difference was demonstrated between RAC day 11 and seasonal epithelial or submucosal mast cells (figure 6.4, 6.5). The number of submucosal macrophages, but not basophils, were elevated in RAC A2 samples (figure 6.6). Submucosal CD3 and CD4 positive lymphocytes were similarly higher at day 11 in RAC model but not in-season allergic rhinitics (figure 6.7).

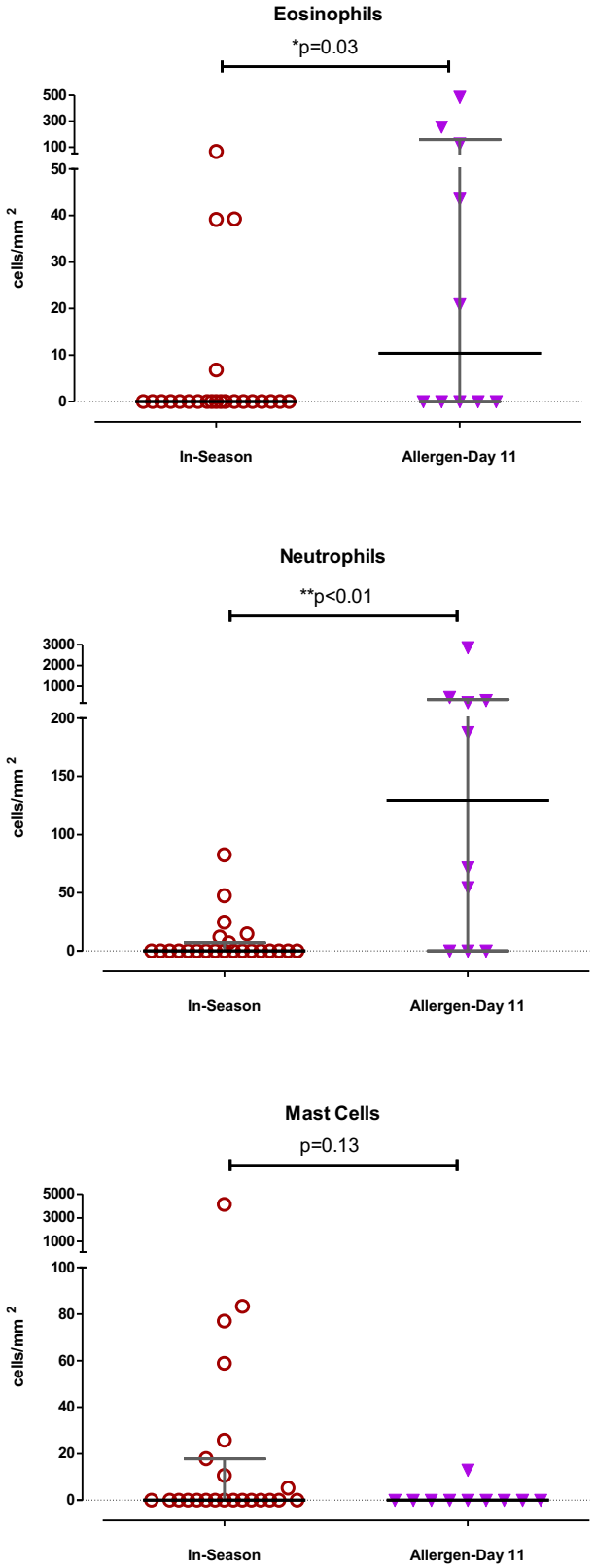


Figure 6.4: Epithelial Inflammatory Cell Counts/mm²: Comparison of in-season seasonal allergic rhinitics (n=23) and RAC day 11 allergen challenge samples (n=10). Eosinophils (BMK13), neutrophils (anti-neutrophil elastase) and mast cells (AA1) stained using the APAAP technique. Graphs show median with interquartile range and comparisons are performed using Mann-Whitney test. P<0.05 was taken as significant.

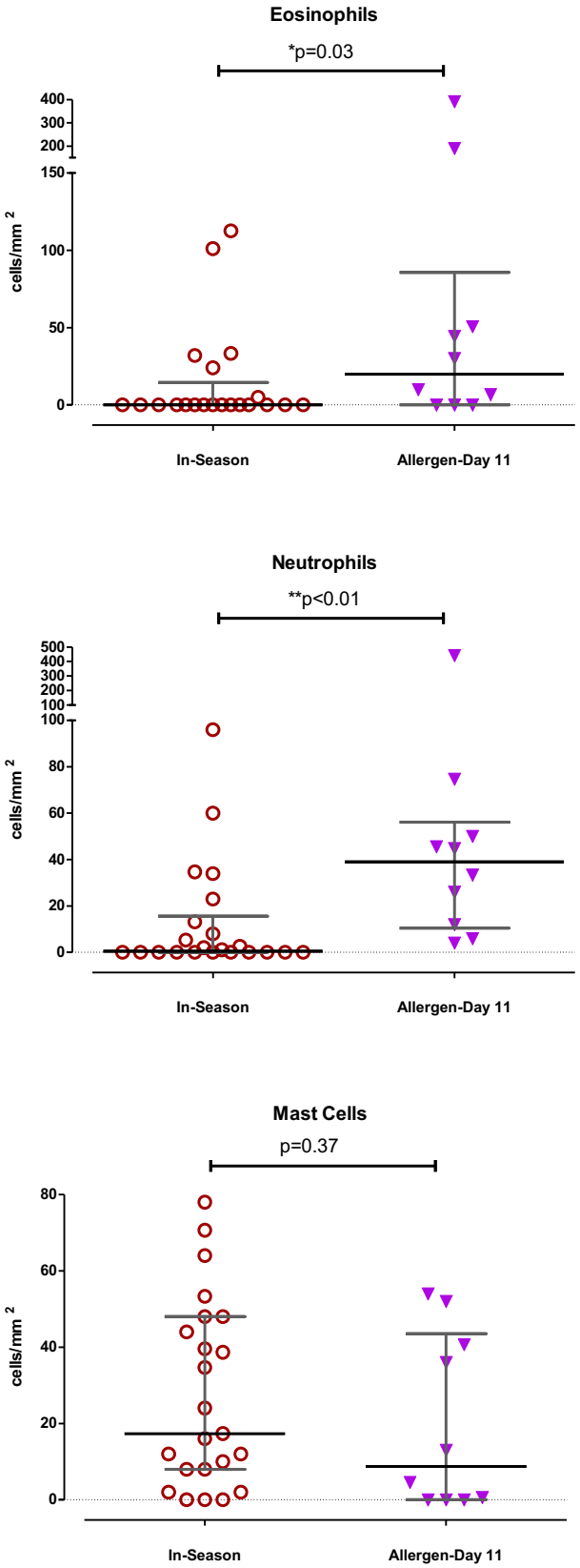


Figure 6.5: Submucosal Inflammatory Cell Counts I /mm²: Comparison of in-season seasonal allergic rhinitics (n=23) and RAC day 11 allergen challenge samples (n=10). Eosinophils (BMK13), anti-neutrophil elastase and Mast cells (AA1) APAAP stained. Median with interquartile range. Mann-Whitney test. P<0.05 was taken as significant.

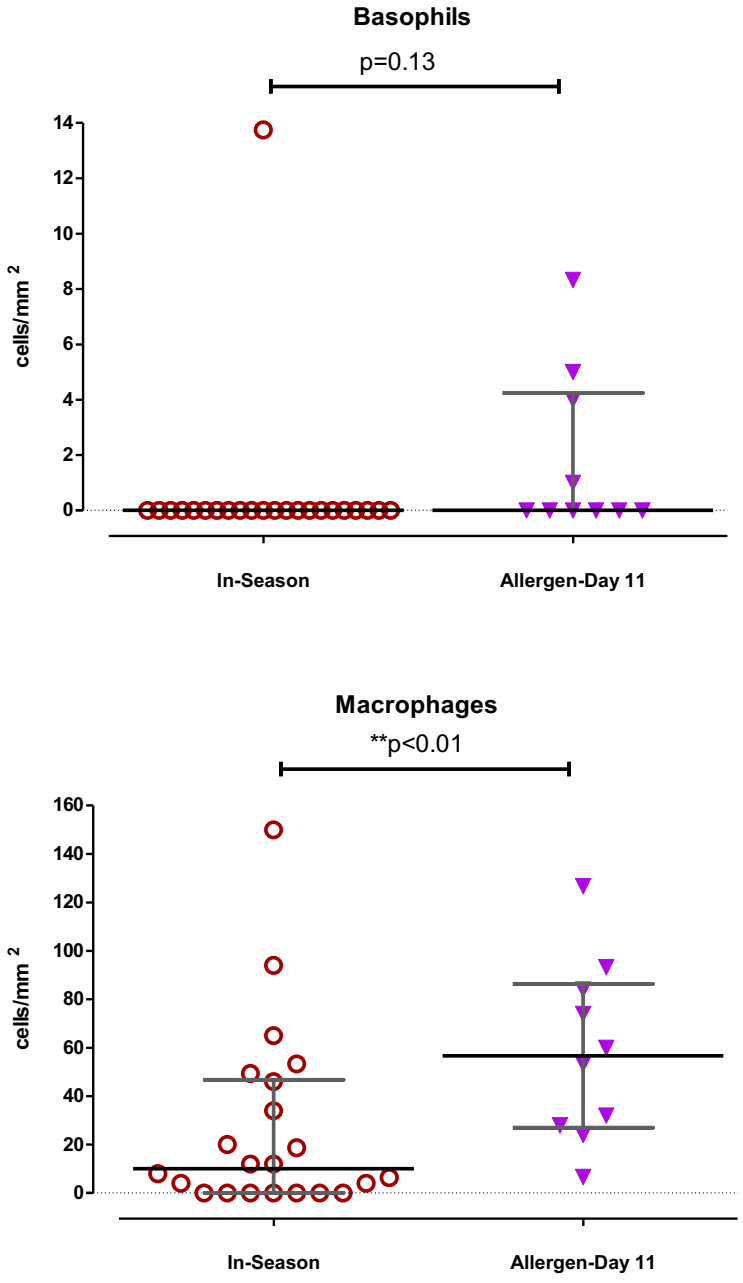


Figure 6.6: Submucosal Inflammatory Cell Counts II /mm^2: Comparison of in-season seasonal allergic rhinitics (n=23) and RAC day 11 allergen challenge samples (n=10). Basophils (2D7) and macrophages (CD68) APAAP stained. Median with interquartile range. Mann-Whitney test. P<0.05 was taken as significant.

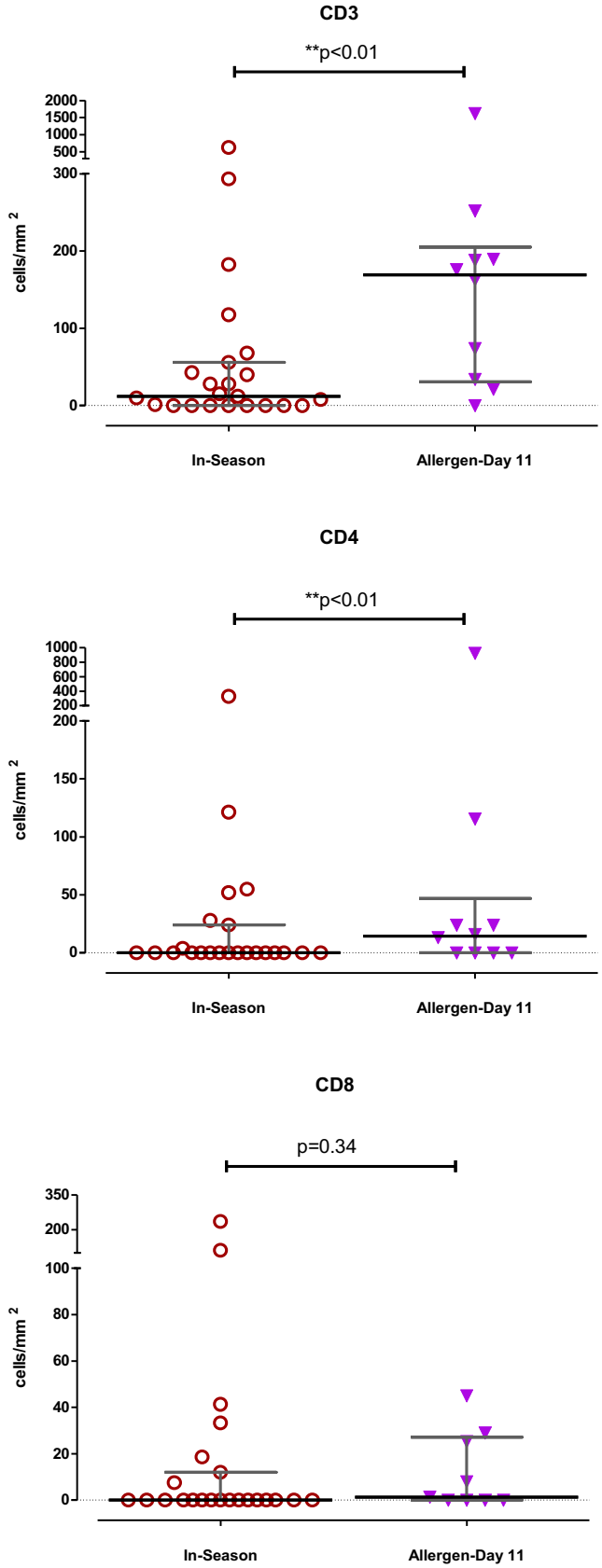


Figure 6.7: Submucosal T Cell Counts/mm^2: Comparison of in-season seasonal allergic rhinitics (n=23) and RAC day 11 allergen challenge samples (n=10). CD3, CD4 and CD8 APAAP stained. Median with interquartile range. Mann-Whitney test. P<0.05 was taken as significant.: Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05

Remodelling

Epithelial integrity and basement membrane thickness was not statistically different between RAC day11 and in-season allergic rhinitic samples (figure 6.8). Epithelial mucus content measured by epithelial area occupied by goblet cells (%), and mucus staining intensity did not change significantly between the two inflammatory models (figure 6.9).

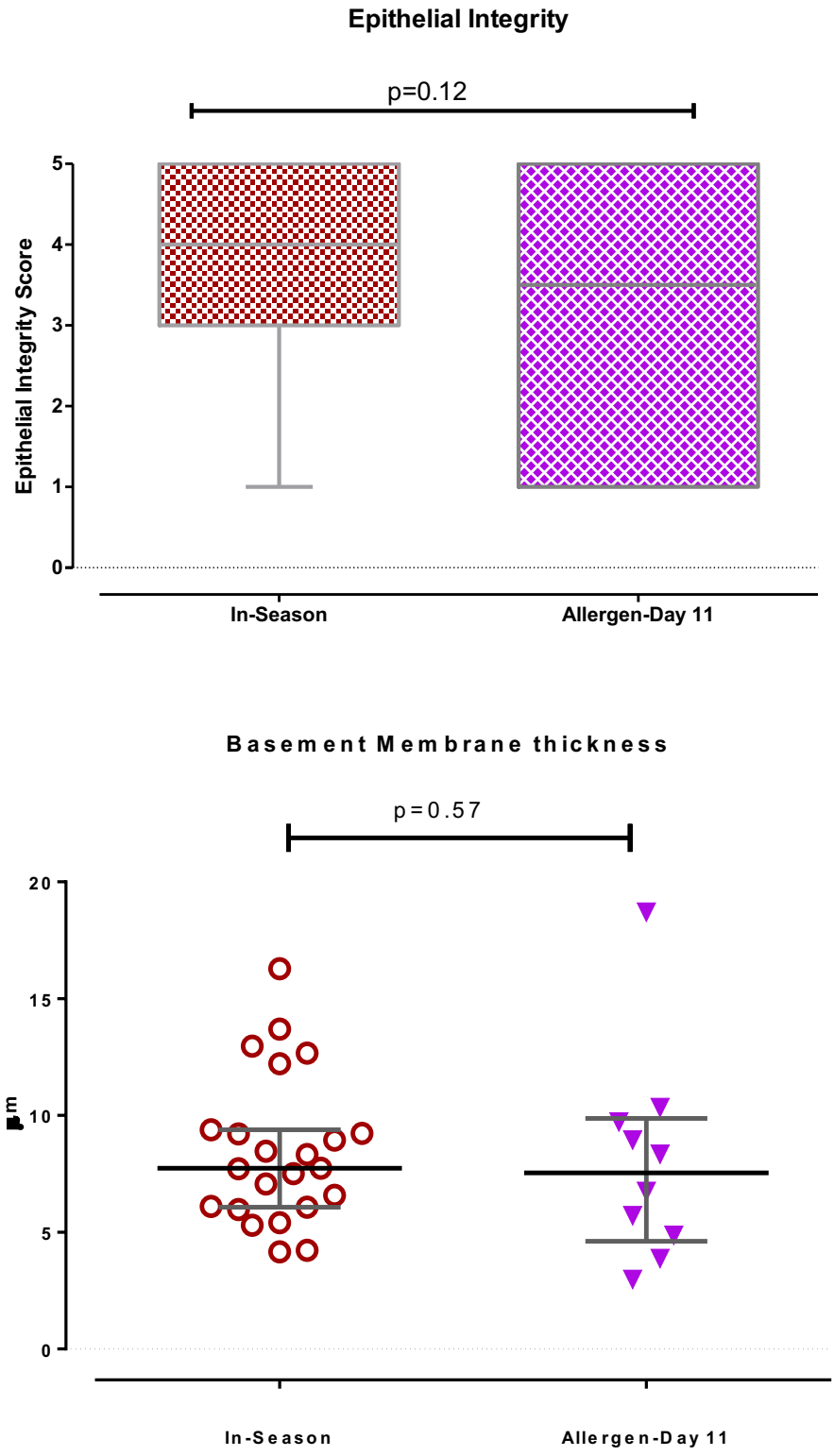


Figure 6.8: Epithelial Integrity Score and Basement Membrane thickness (µm): Comparison of in-season seasonal allergic rhinitics (n=23) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05

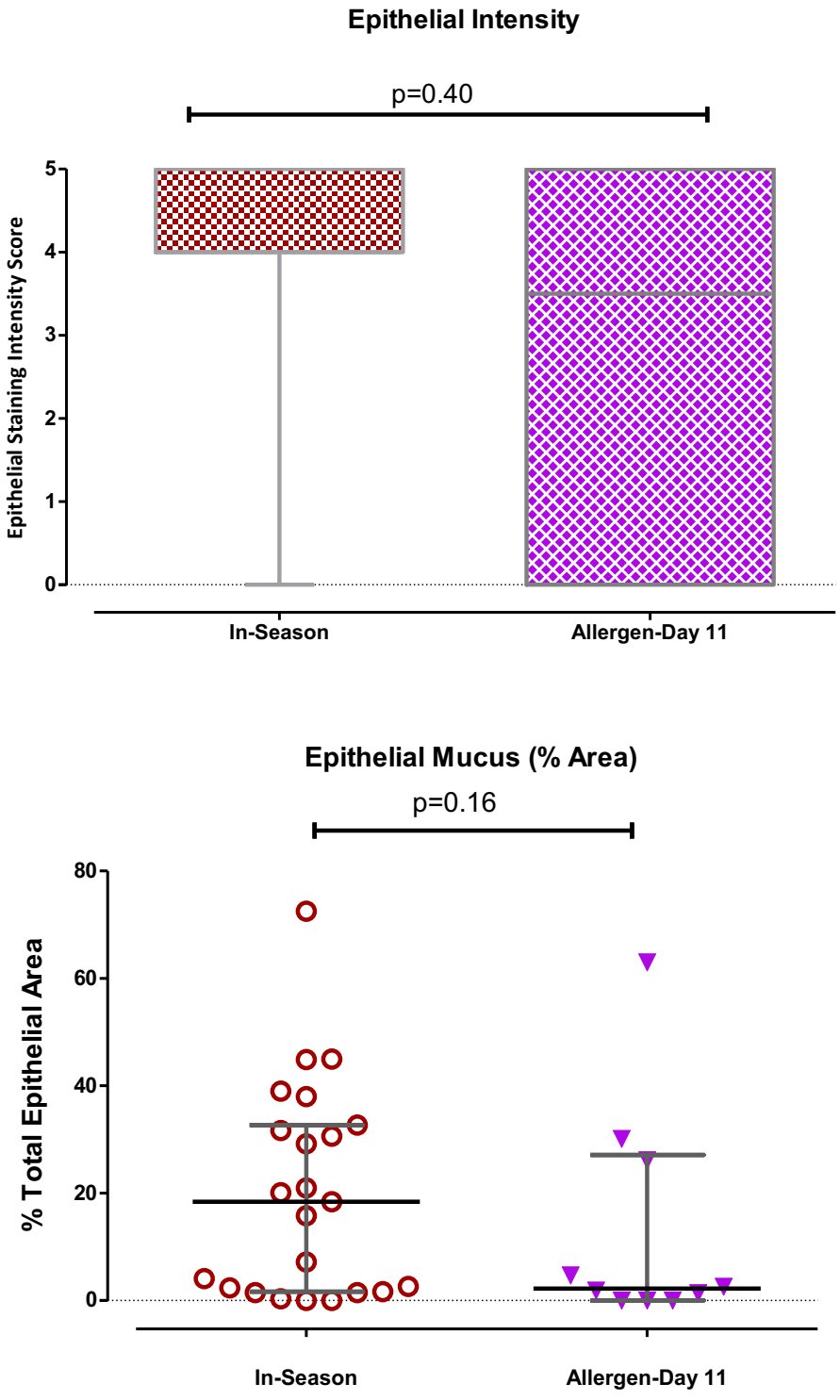
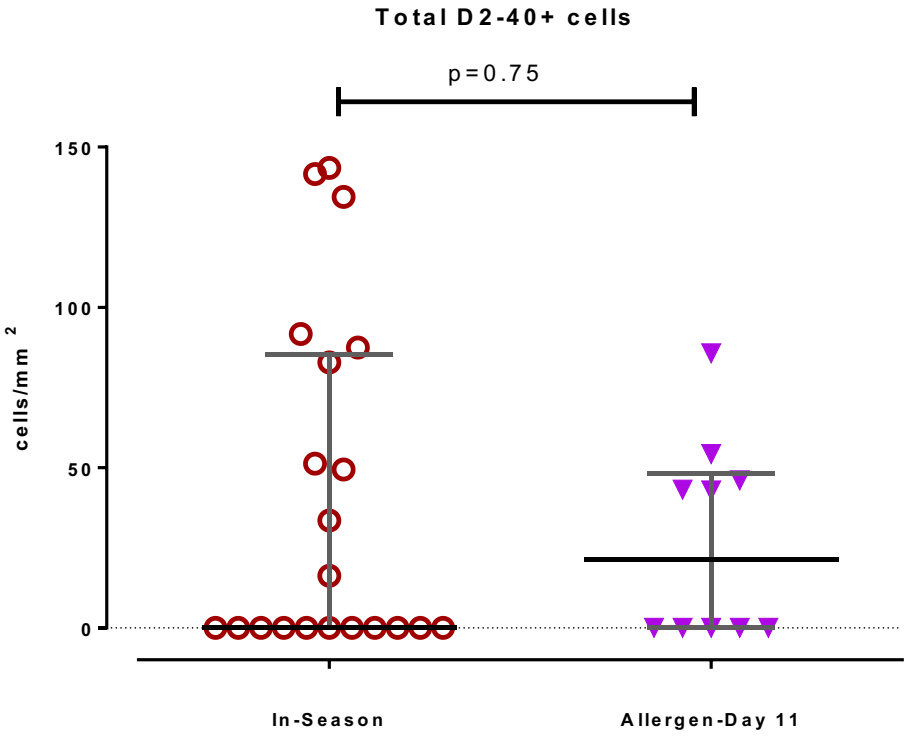


Figure 6.9: Intensity of epithelial mucus staining and Epithelial Mucus Content (% of total epithelial area) . Comparison of in-season seasonal allergic rhinitics (n=23) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05

The four parameters of lymphangiogenesis and established lymphatic vessels (total D2-40+ve cells, total D2-40+ve vessels, percentage submucosal area occupied by lymphatics and absolute lymphatic vessels size) were not significantly different between RAC day 11 and iS allergic rhinitics (figure 6.10). Three parameters for submucosal glands (total glandular area, total intra-glandular area, and total gland tissue area) (figure 6.11) and four markers of angiogenesis and established blood vessels (total CD31 +ve cells, total CD31+ve vessels, percentage submucosal area occupied by blood vessels, and absolute blood vessel size) were also the same between the RAC A2 and iS (figures 6.12).



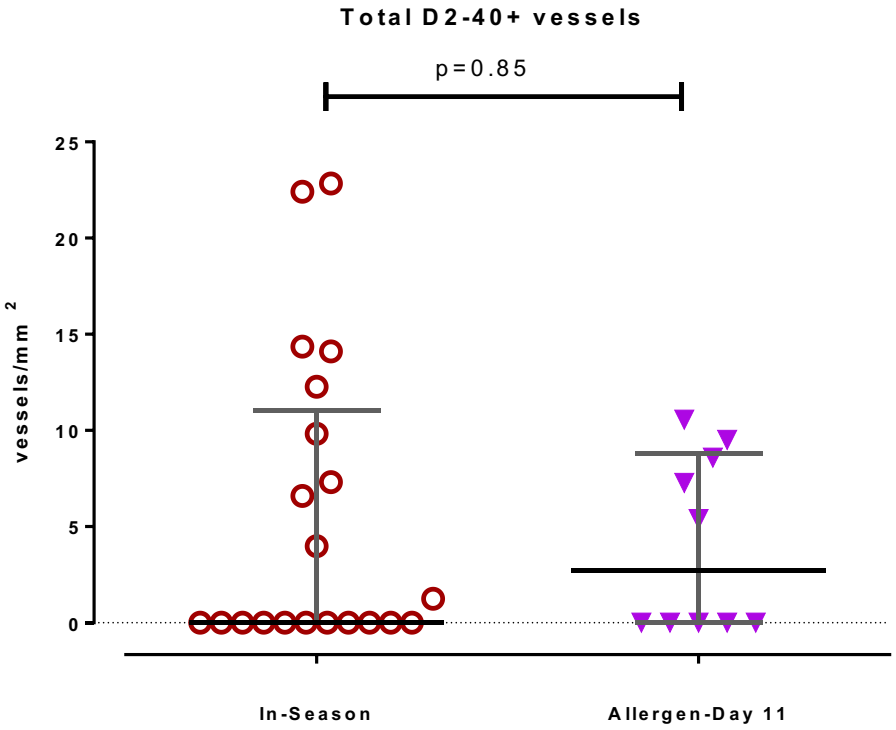


Figure 6.10a: Lymphangiogenesis: using D2-40 as lymphangiogenic marker. Counted as total number of D2-40 +ve cells and vessels /mm². Comparison of in-season seasonal allergic rhinitics (n=21) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05

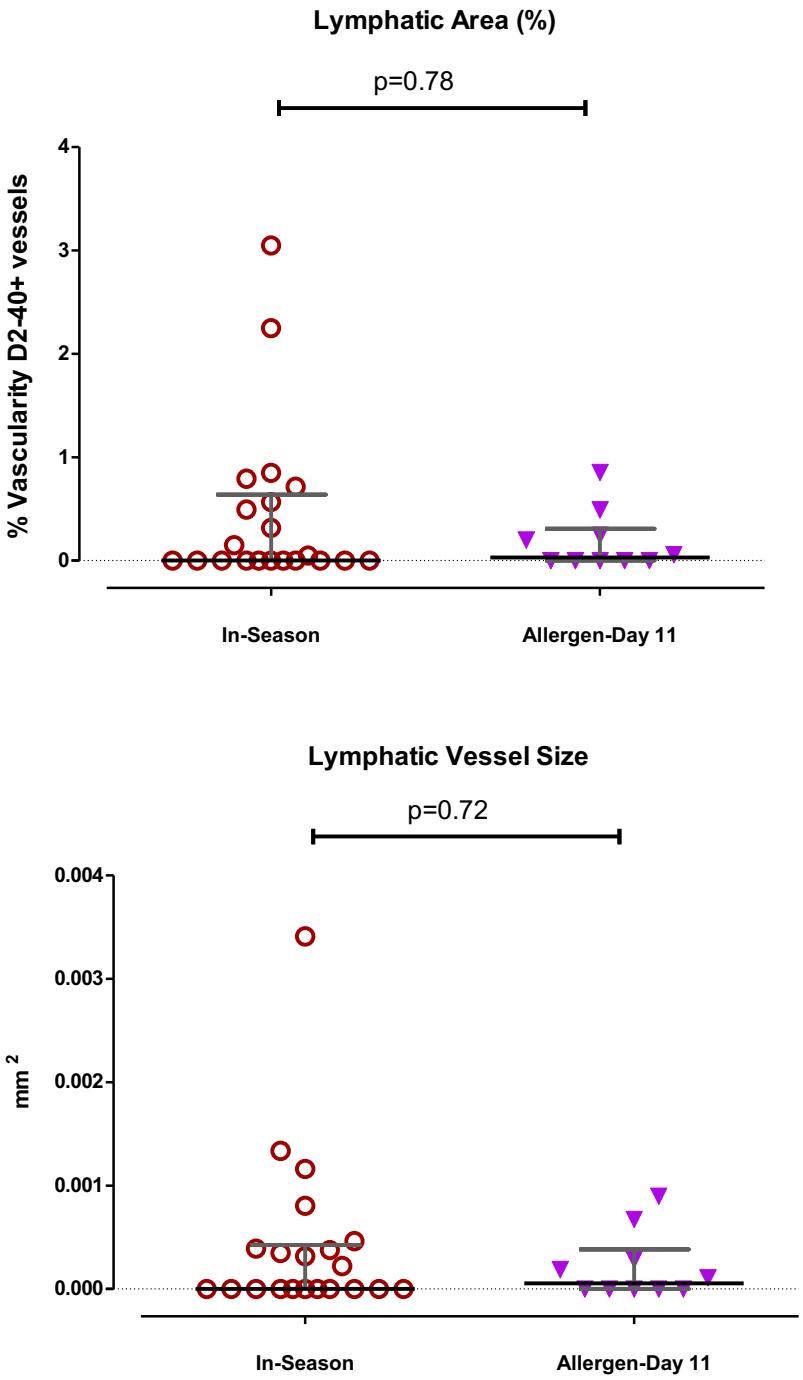


Figure 6.1011b: Lymphangiogenesis: using D2-40 as lymphangiogenic marker. Lymphatic area (% total submucosal area) and lymphatic vessel size (mm^2). Comparison of in-season seasonal allergic rhinitics (n=21) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as $p<0.05$

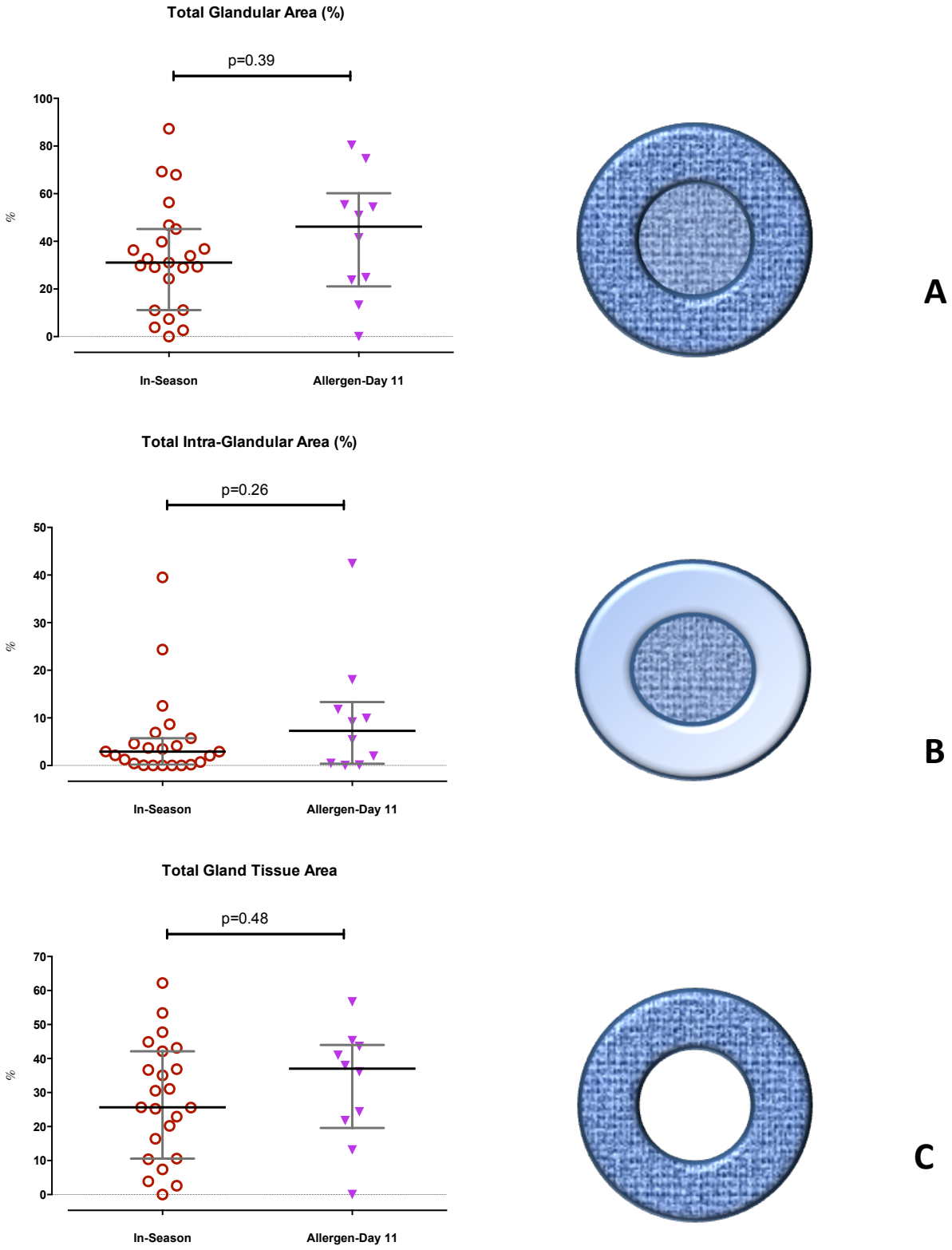
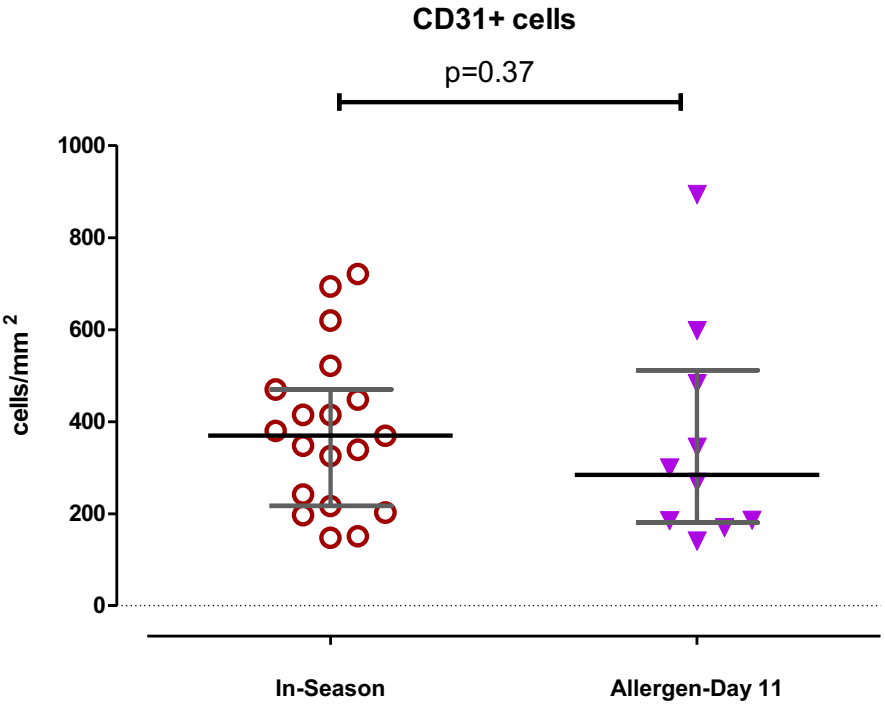


Figure 6.12: Submucosal Glandular Area: measured area is represented by textured blue background on schematic gland drawing, representing (A) gland tissue and secretory contents, (B) secretory content area only and, (C) glandular tissue only. Comparison of in-season seasonal allergic rhinitics (n=23) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.



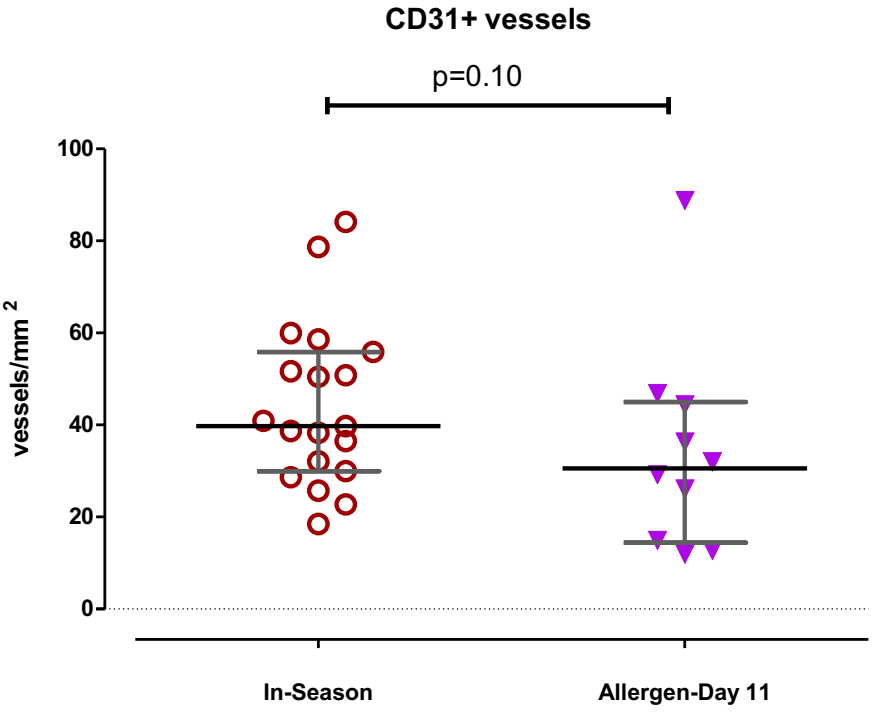


Figure 6.12a: Angiogenesis: using CD31 as angiogenic marker. Counted as total number of CD31+ve cells and vessels /mm². Comparison of in-season seasonal allergic rhinitics (n=19) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

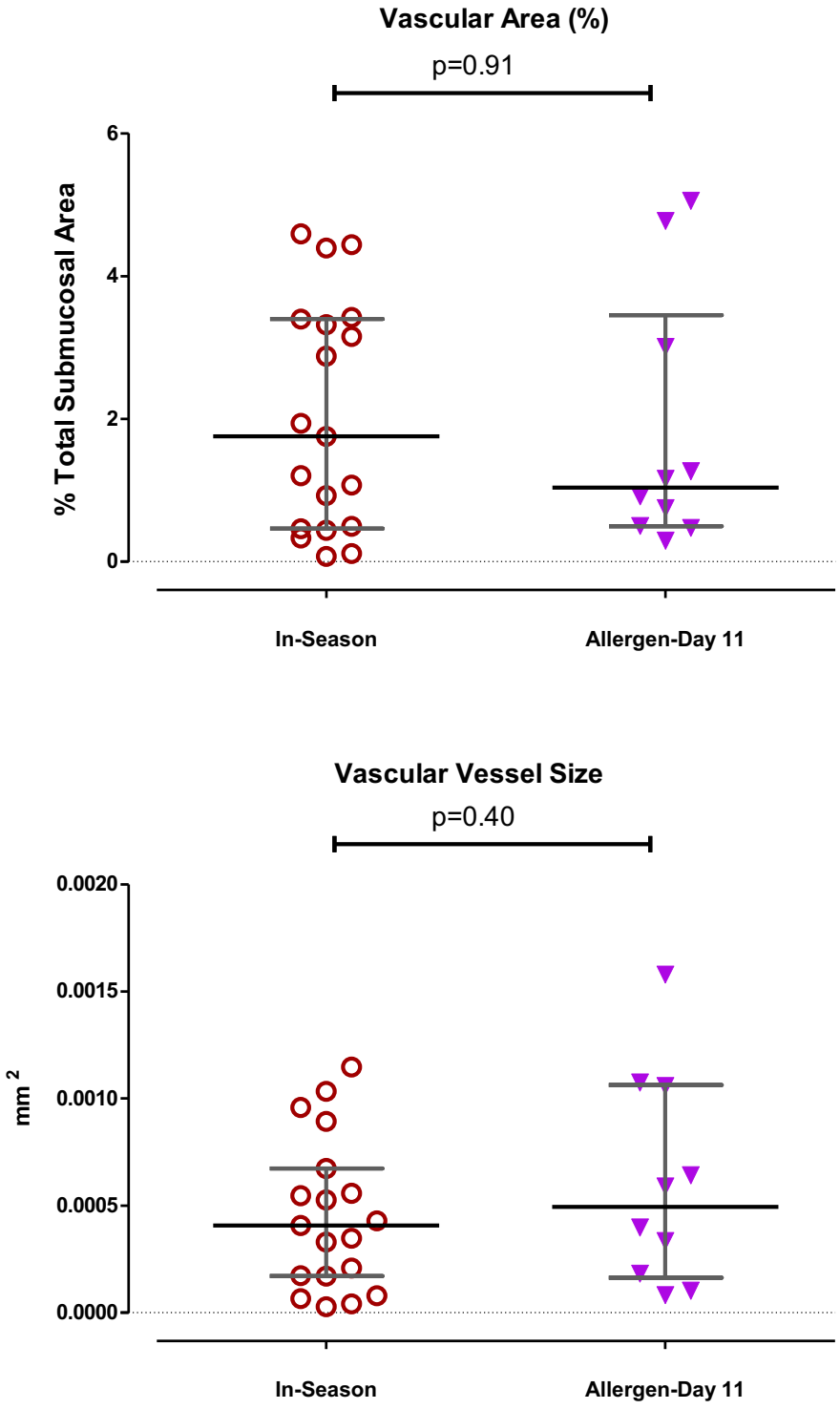


Figure 6.12b: Angiogenesis: using CD31 as angiogenic marker. Counted as vascular area (% total submucosal area) and vascular vessel size (mm^2). Comparison of in-season seasonal allergic rhinitics (n=19) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as $p<0.05$.

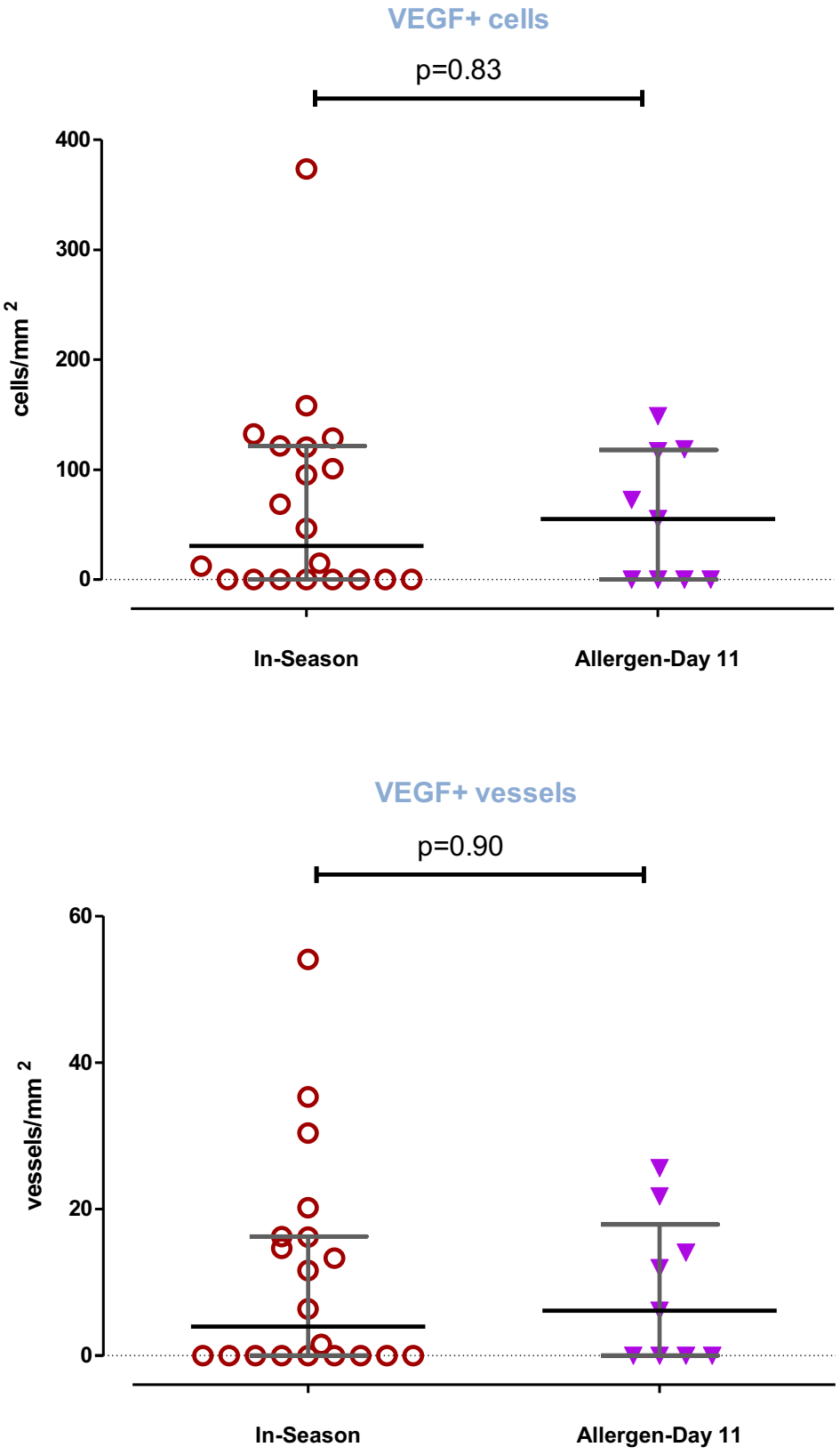


Figure 6.13a: VEGF positive submucosal signal- number of VEGF+ cells and vessels/mm². Comparison of in-season seasonal allergic rhinitics (n=20) and RAC day 11 allergen challenge samples (n=9). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

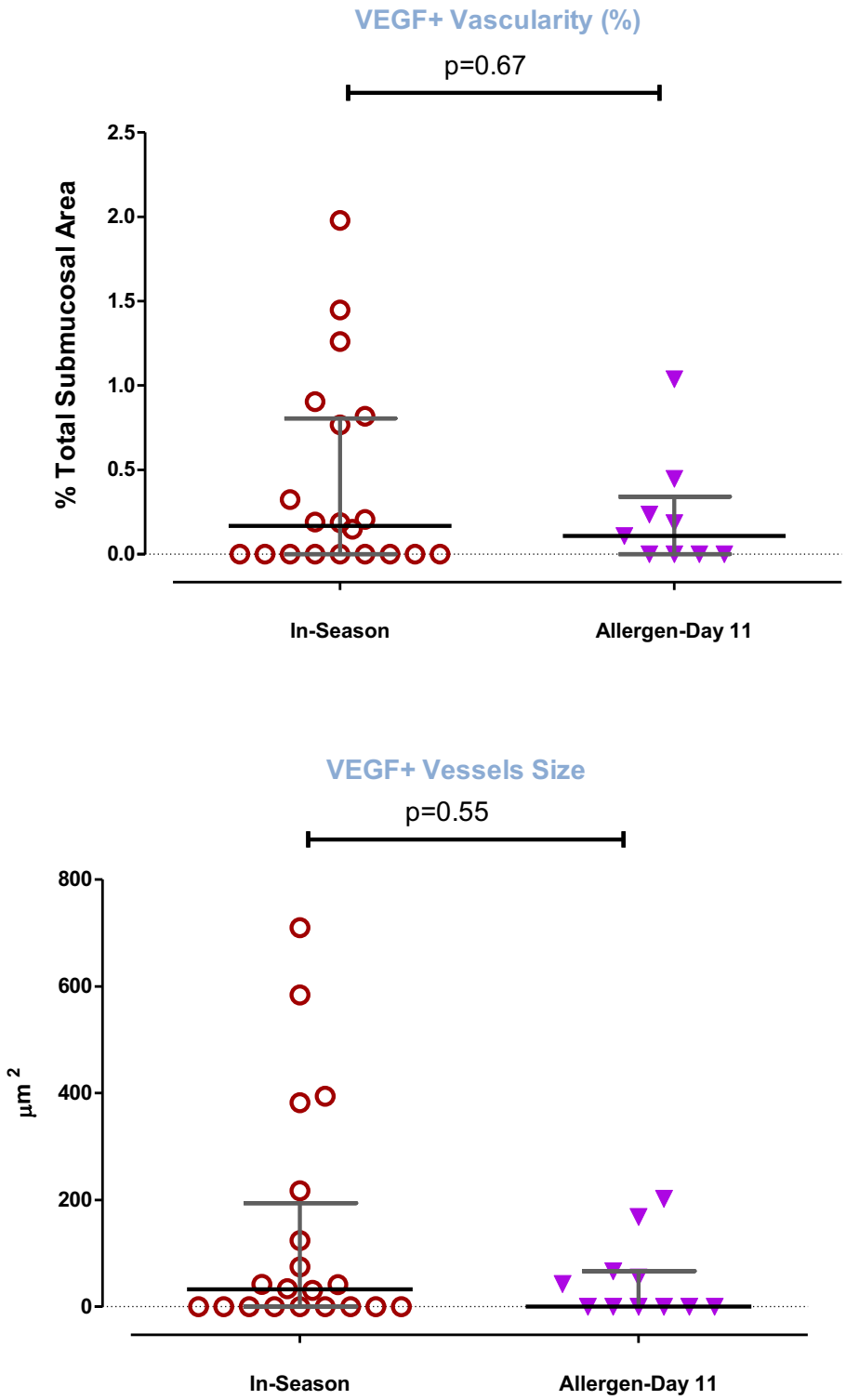


Figure 6.13b: VEGF positive submucosal signal- VEGF+ vascular area (% total submucosal area) and VEGF+ vascular vessel size (μm^2). Comparison of in-season seasonal allergic rhinitics (n=20) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

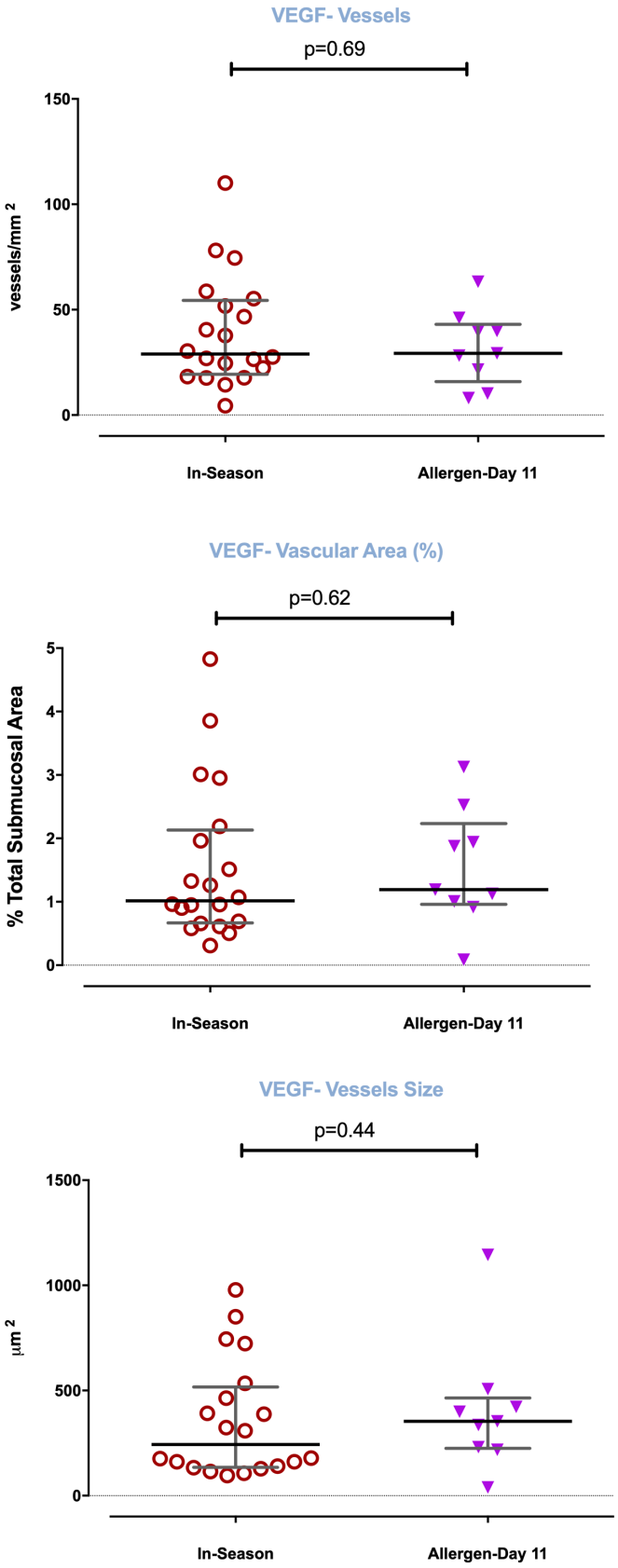


Figure 6.14: VEGF negative submucosal signal- vessel number/mm², surface area %, vessel size µm2. Comparison of in-season seasonal allergic rhinitics (n=20) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

VEGF staining was measured by four parameters: total number of VEGF positive cells, total number of VEGF positive vessels, percentage of VEGF positive submucosal vascular area (as compared to total submucosal vascular area) and VEGF positive vessel size. VEGF negative measures included: total number of VEGF negative vessels, percentage of VEGF negative submucosal vascular area (as compared to total submucosal vascular area) and VEGF negative vessel size. There was no significant difference between RAC A2 and iS allergic rhinitis for any of the above VEGF positive or VEGF negative markers for increased submucosal vascularity (figures 6.13a, b and 6.14).

Matrix metalloproteases MMP7 and MMP9, and their inhibitor TIMP-1 showed no significant difference between the two models (RAC A2 and iS). There was significantly more HSP-47 in RAC day 11 samples, whether measured as total number of cells or colocalised to fibroblasts (figure 6.15-6.17). Smooth muscle actin-α was not statistically different between the two models of allergen challenge/ exposure (figure 6.18).

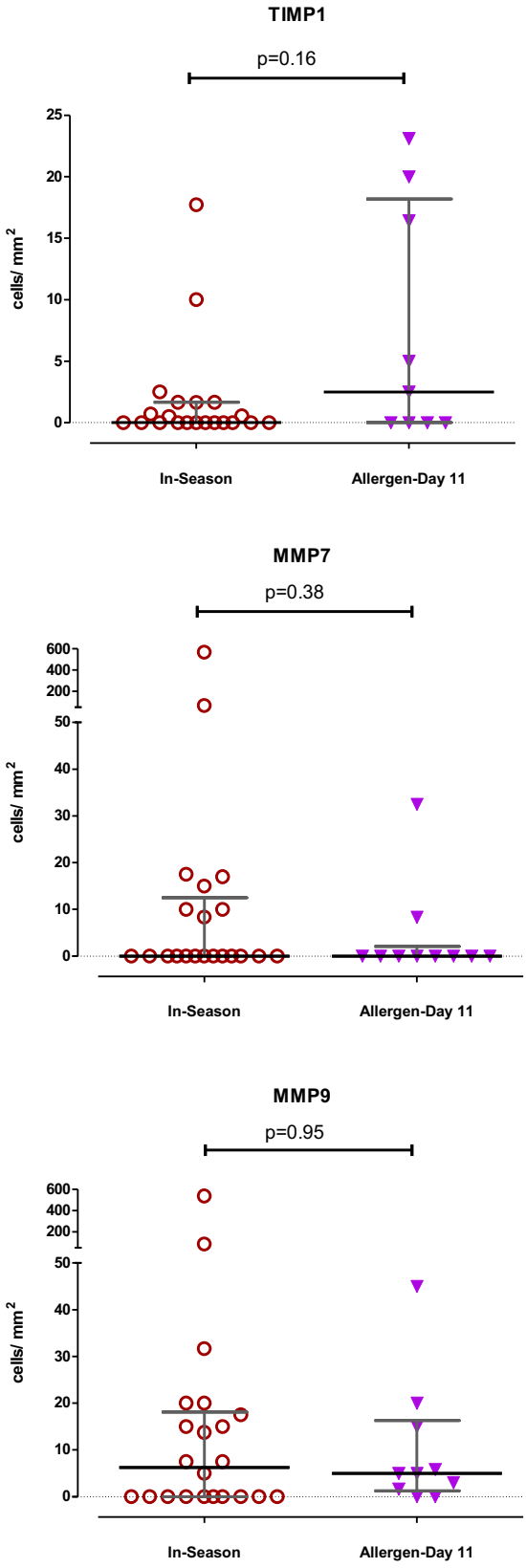


Figure 6.13: Matrix metalloproteases 7 and 9 with their inhibitor TIMP-1. Comparison of in-season seasonal allergic rhinitics (n=22) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

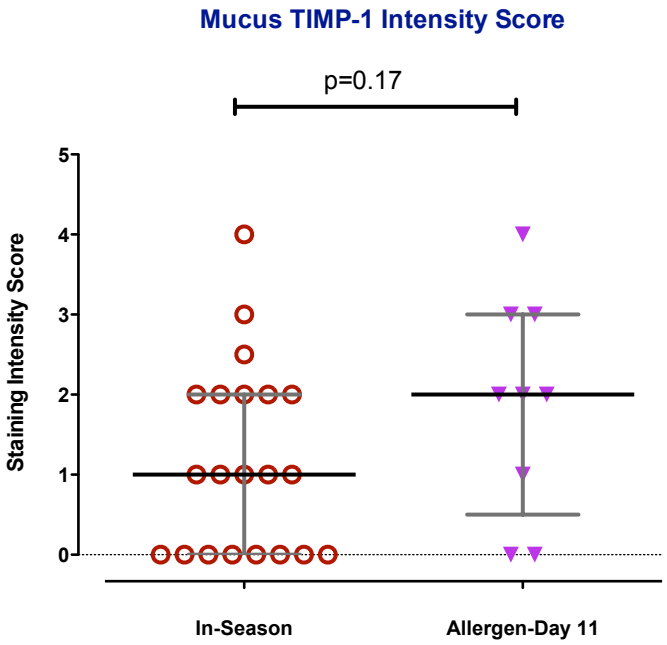


Figure 6.1614: Tissue inhibitor of matrix metalloprotease TIMP-1 mucus intensity score. Comparison of in-season seasonal allergic rhinitics (n=21) and RAC day 11 allergen challenge samples (n=9). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

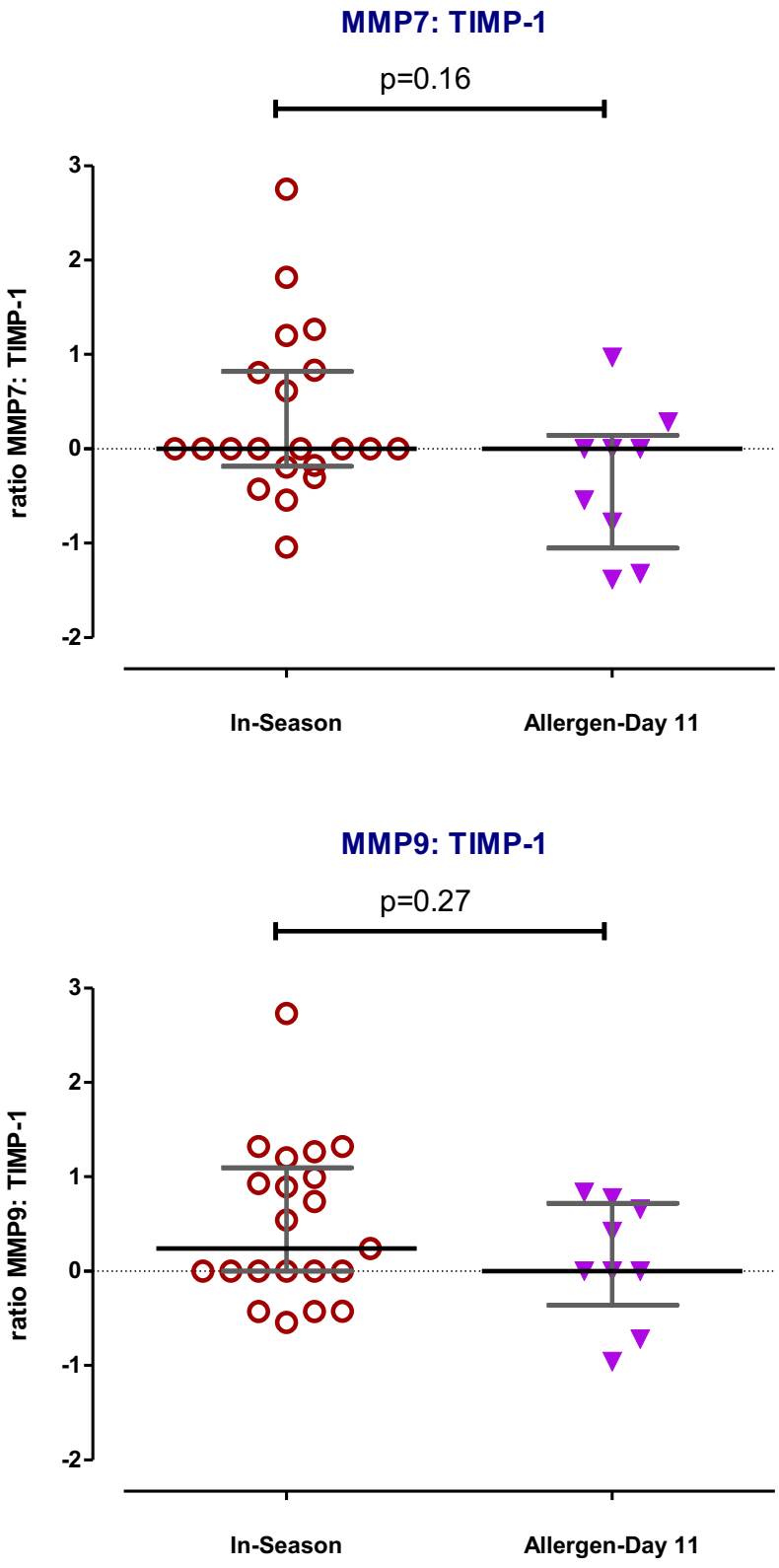


Figure 6.15: Matrix metalloproteases 7 and 9 ratio to TIMP-1. Comparison of in-season seasonal allergic rhinitics (n=21) and RAC day 11 allergen challenge samples (n=9). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

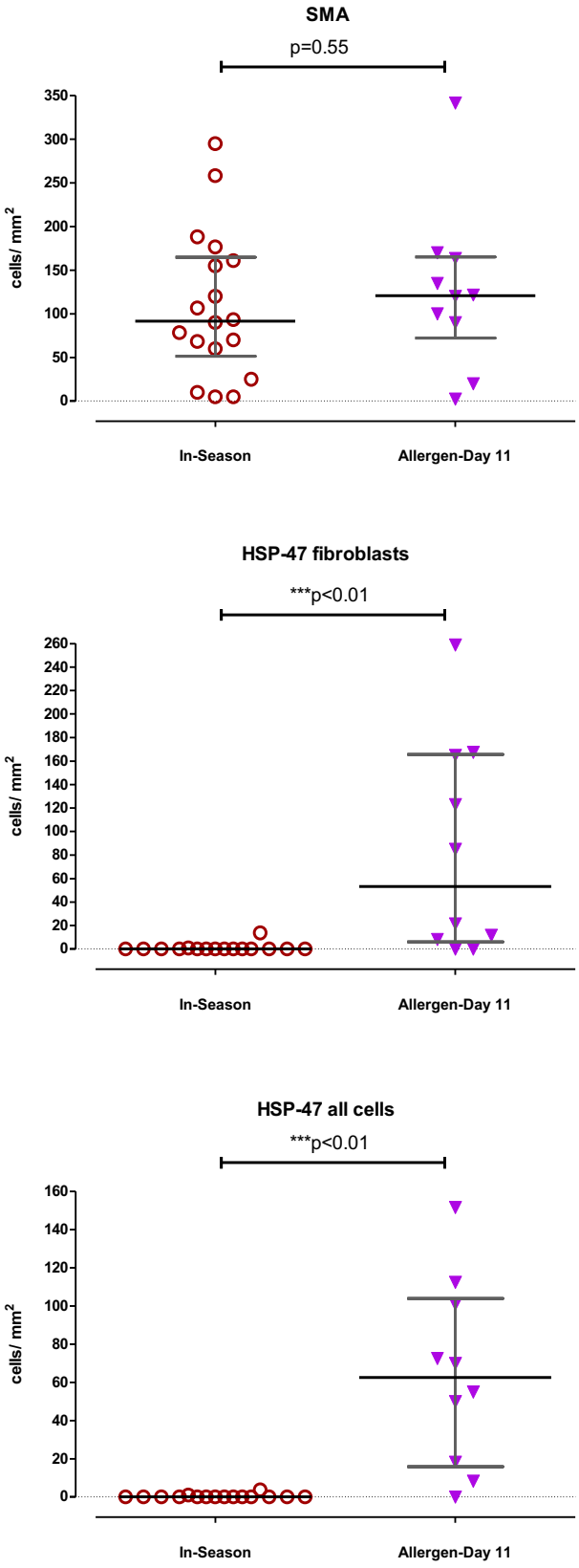
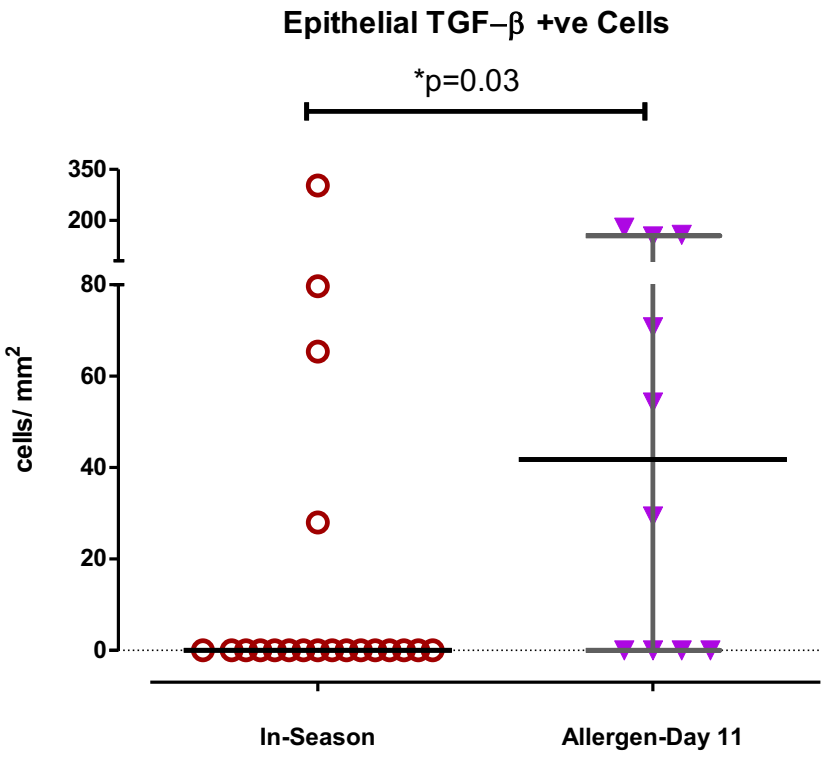


Figure 6.16: Smooth Muscle Actin and Heat Shock Protein-47 (all cells and in a fibroblast cell subset). Comparison of in-season seasonal allergic rhinitics (n=18) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

Epithelial and submucosal TGF-β was significantly higher in the RAC day 11 samples than in-seasonal allergic rhinitics (figure 6.19). Submucosal activin, ALK-4 but not pSMAD-2 were also more abundant in the repetitive challenge model at day 11 than with normal seasonal exposure (figure 6.20).



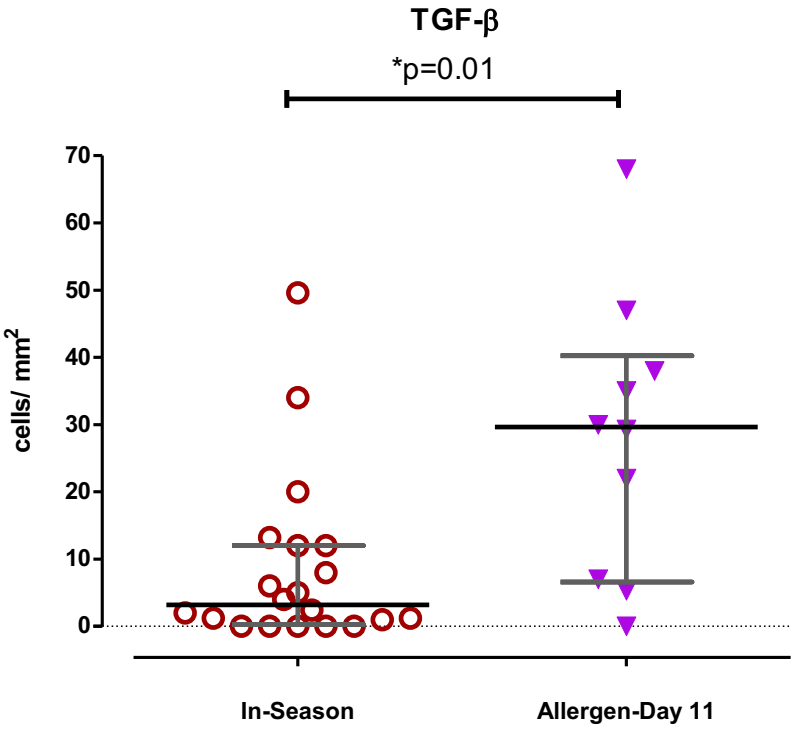


Figure 6.17: TGF-β: epithelial and submucosal cell counts/mm². Comparison of in-season seasonal allergic rhinitics (n=19) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

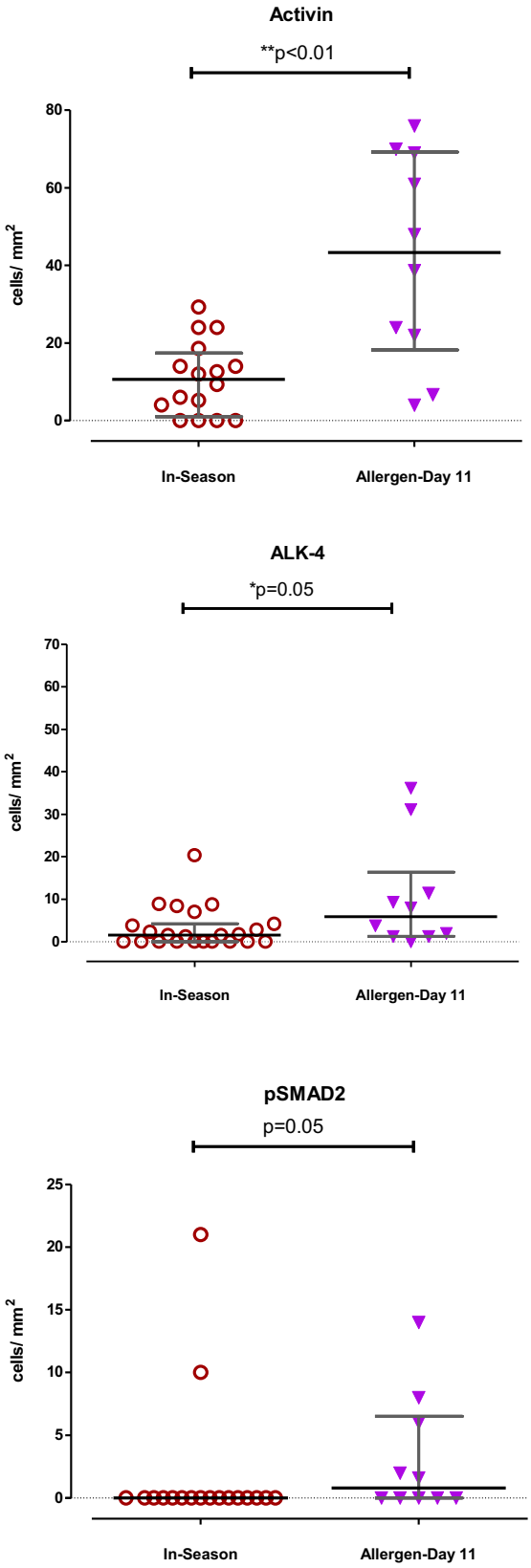


Figure 6.18: Submucosal activin, ALK-4 and pSMAD-2 cell counts/mm². Comparison of in-season seasonal allergic rhinitics (Activin n=16; ALK-4 n=23; pSMAD2 n=18) and RAC day 11 allergen challenge samples (n=10). Median with interquartile range. Non-paired data was analysed using Mann-Whitney test. Significance was accepted as p<0.05.

Discussion

Comparison of natural versus prolonged repetitive allergen challenge demonstrated no significant difference between Th1, Th2 or Treg cytokine profiles, indicating robust Th2 response in both NAE and RAC protocols. Persistent high dose allergen challenge (RAC A2) resulted in enhanced CD4, and by extension CD3, cell counts when compared to natural exposure. Both epithelial and submucosal inflammatory cell counts displayed heightened responses with eosinophils, neutrophils, and macrophages significantly more numerous after RAC than NAE protocols. Mast cells were less detectable, most likely due to enhanced degranulation, after ten days of repetitive allergen challenge. In general, repetitive allergen challenge is demonstrably a sound model to reliably replicate allergen exposure with robust cytokine and inflammatory cellular responses.

Albeit shy of statistical significance, epithelial mucus (% area and staining intensity) was reduced in the RAC A2 samples with no significant impact on epithelial integrity from prolonged allergen exposure. Submucosally, no difference was shown between in season and RAC sample changes in vascular (VEGF) and lymphangiogenic markers, with no concomitant alteration in blood or lymphatic vessels (CD31 and D2-40). Marginal alterations in MMP pathways were evidenced with prolonged allergen exposure, MMP9 and MMP7 tended to be less after 2 weeks of RAC compared to natural seasonal exposure. With no statistical significance in ratio of MMP7/9 and TIMP1, it suggests alteration in remodelling ECM pathways with sufficient regulatory pathway activation to maintain unaltered tissue topography.

Repetitive allergen challenge causes a significant rise in epithelial TGF-β indicating that the allergic rhinitic epithelium is at least partially primed for EMTU activation. With flash increase in HSP-47, it is likely that crosstalk between the epithelium and the mesenchyme will influence *de novo* collagen synthesis in response to adequate allergic insult. The enhanced inflammatory response seen in RAC after ten days is likely to contribute to more robust TGF-β/ activin pathway activation. Increases in epithelial TGF-β in RAC demonstrate that although not a feature of natural seasonal exposure, TGF-β mediated EMTU activation can occur in the upper airway. The low number of TGF-β positive cells following natural allergen exposure is likely to reflect the relapsing/ remitting nature of this condition with insufficiently prolonged high doses of allergen to affect a detectable response. Similarly, there was significantly more submucosal activin, ALK-4, and pSMAD2 following RAC A2 compared to iS showing that at a persistently symptomatic dose such pathways are activated and result in alteration at protein level. A persistently enhanced allergic cellular infiltrate will provide sources of both TGF-β and activin to sustain activated pathways.

Amelioration of such upregulation of TGF-β and activin during natural allergen exposure, hints at continuous regulatory mechanisms which prevent persistence of signalling. This contrasts with both lower airways disease where a constant inflammatory milieu is present, and nasal polyposis where, albeit not due to an allergic trigger, such inflammatory cells do persist. In both clinical states, activation of TGF-β family signalling is present and persistent. Such discrepancy with upper airway disease underscores the differing pathophysiology of nasal polyps and allergic rhinitis.

Summary of chapter

The repetitive allergen challenge protocol described here is a valid model of seasonal allergic rhinitis. This RAC exposure demonstrated a comparable cytokine response to that seen with in-seasonal allergic rhinitics as well as an enhanced inflammatory cell infiltrate. Coupled to these significant allergic responses were activated TGF-β and activin pathways with evidence of EMTU activation via epithelial TGF-β upregulation. Despite these, no significant remodelling was seen in the two study groups, underscoring the absence of remodelling in allergic rhinitis. The RAC protocol is a valid and applicable tool for purposes of examining the allergic response and for investigating potential therapeutic interventions.

Chapter 7 : Remodelling in Chronic Rhinosinusitis with Nasal Polyposis

Introduction

Chronic rhinosinusitis with nasal polyposis is a chronic pro-inflammatory condition with long term histological sequelae. Polyp samples rich in inflammatory cellular infiltrate provide an ideal positive comparison to allergic rhinitis tissue responding to stimulation. Turbinate samples from CRSwNP participants allowed quantification of remodelling changes in a chronic nasal model of inflammation.

Previous studies focusing on binary behaviour of polyps hint at dichotomy of TGF-β, fibrosis and allergic inflammation, where CRSsNP are pro-fibrotic, TGF-β high and CRSwNP demonstrate low TGF-β enhanced regulatory activity with its concomitant signalling molecules (751). These observations rely on variable biopsy sites and comparison groups (451) not only looking at TGF-β signalling but other markers of remodelling including MMPs (752). Such variability of comparison groups makes for difficult interpretation of the changes affecting nasal polyposis patients; a task further complicated by the different nature of polyp and turbinate histology from the same patient.

This chapter examined both inflammatory and remodelling alterations in CRSwNP samples in nasal polyp and turbinate tissue from the same individual. Comparison was made to non-atopic normal controls to paint an accurate picture of changes affecting this condition.

Results

Both polyps (NPp) and turbinate (NPt) samples are rich in inflammatory cellular infiltrate. Comparisons were made between nasal polyposis samples and normal controls. NPp epithelia had significantly more eosinophils, neutrophils and mast cells whilst NPt epithelium only had more eosinophils. Submucosally, NPp samples possessed higher eosinophil, neutrophil, basophil and macrophage cell numbers with NPt tissue having significantly more eosinophils, neutrophils and basophils (figures 7.1 to 7.3) (photomicrograph 7.1 and 7.2). NPp but not NPt samples demonstrated higher total CD3 and CD4 counts in the submucosal tissues (figure 7.4).

Comparison of polyp and turbinate samples showed significantly more CD4 in polyp tissue with enhanced thinning of the basement membrane (figure 7.6). There was a paucity of submucosal gland tissue in polyps versus turbinate tissue (figure 7.5), along with a decrease in all vascular and all but one (mean vessel size) lymphatic markers. HSP-47 was significantly more abundant in polyps whilst there was significantly more smooth muscle actin in turbinate samples (figure 7.8) (photomicrograph 7.5). Activin but not TGF-β, pSMAD or ALK-4 (figure 7.14 and 7.15), demonstrated significantly enhanced expression in polyps versus turbinates of polyposis participants.

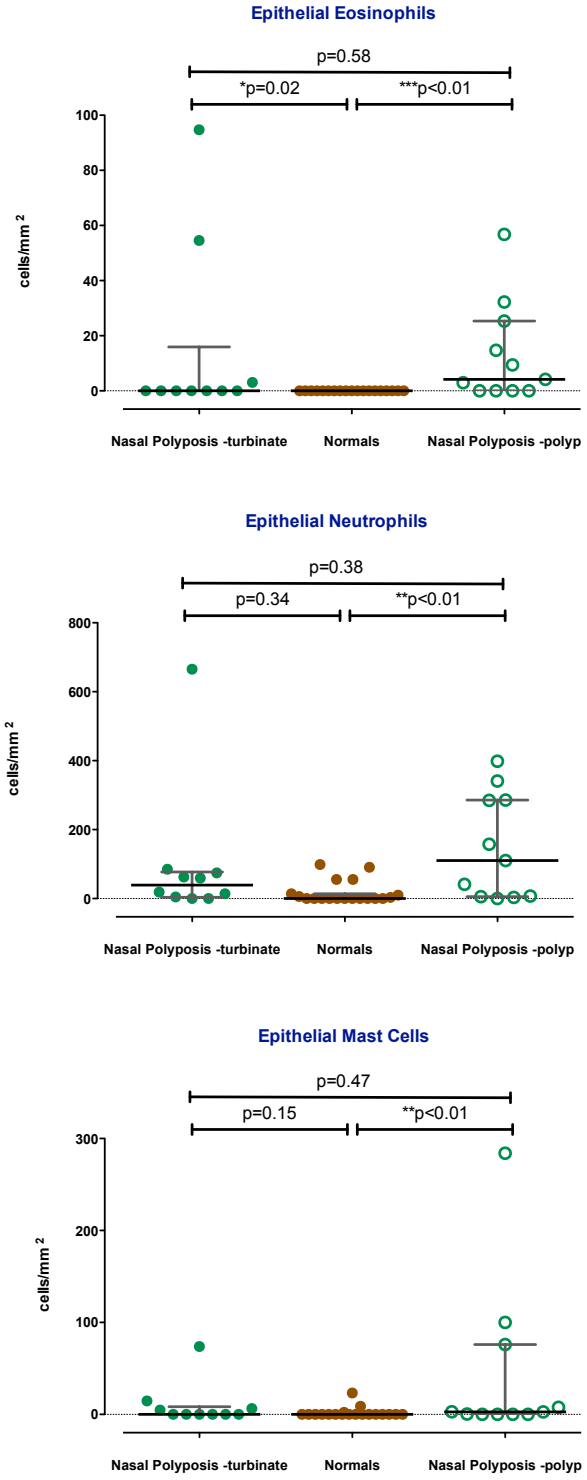


Figure 7.1: Epithelial Inflammatory Cell Counts/mm²: Paired nasal polyposis-turbinate and nasal polyposis-polyp samples, n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.

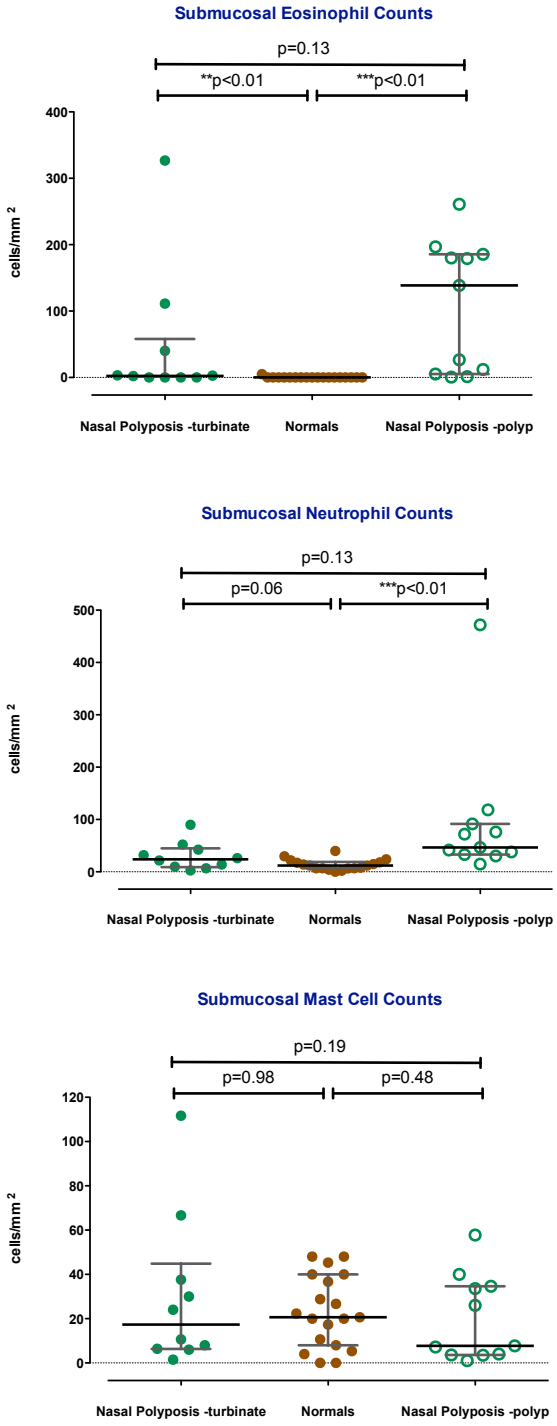
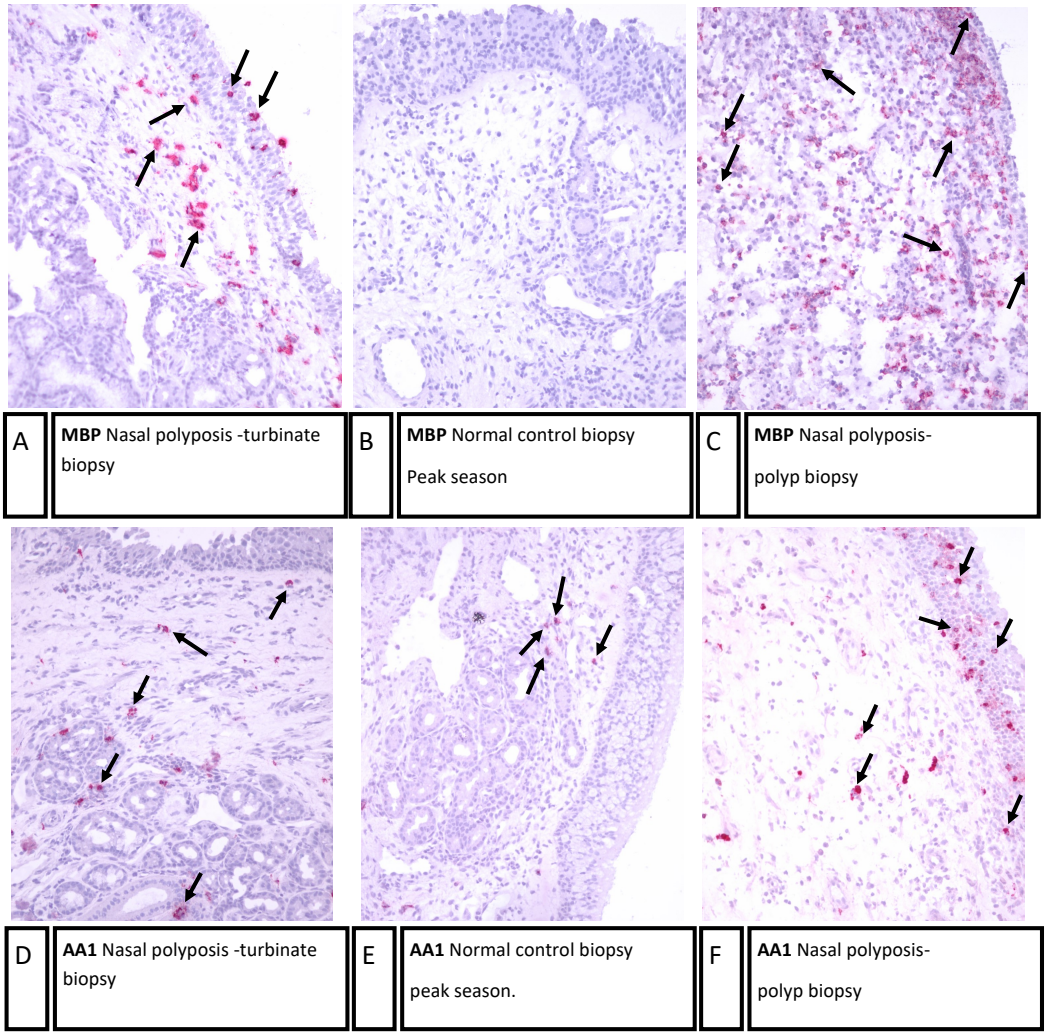


Figure 7.2: Submucosal Inflammatory Cell Counts I: Counts/mm²: Paired nasal polyposis-turbinate and nasal polyposis-polyp samples, n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.



Photomicrograph 7.1: MBP eosinophil staining using the monoclonal antibody BMK13 (A-C) and mast cell tryptase staining using monoclonal antibody AA1 (D-F) with the APAAP technique. Eosinophils and mast cells visible as discrete red cells (black arrows) with haematoxylin (blue) nuclear counterstain. Eosinophils were significantly increased in epithelium and submucosa of nasal polyposis turbinate tissue (A) and nasal polyposis polyp tissue (C) compared to turbinate tissue from normal control group (B). Epithelial mast cells were only a significant feature in nasal polyposis polyps (F) whereas submucosal mast cells were found in nasal polyposis turbinates (D), polyps (F) and normal control turbinates (E). Photographed using x20 lens.

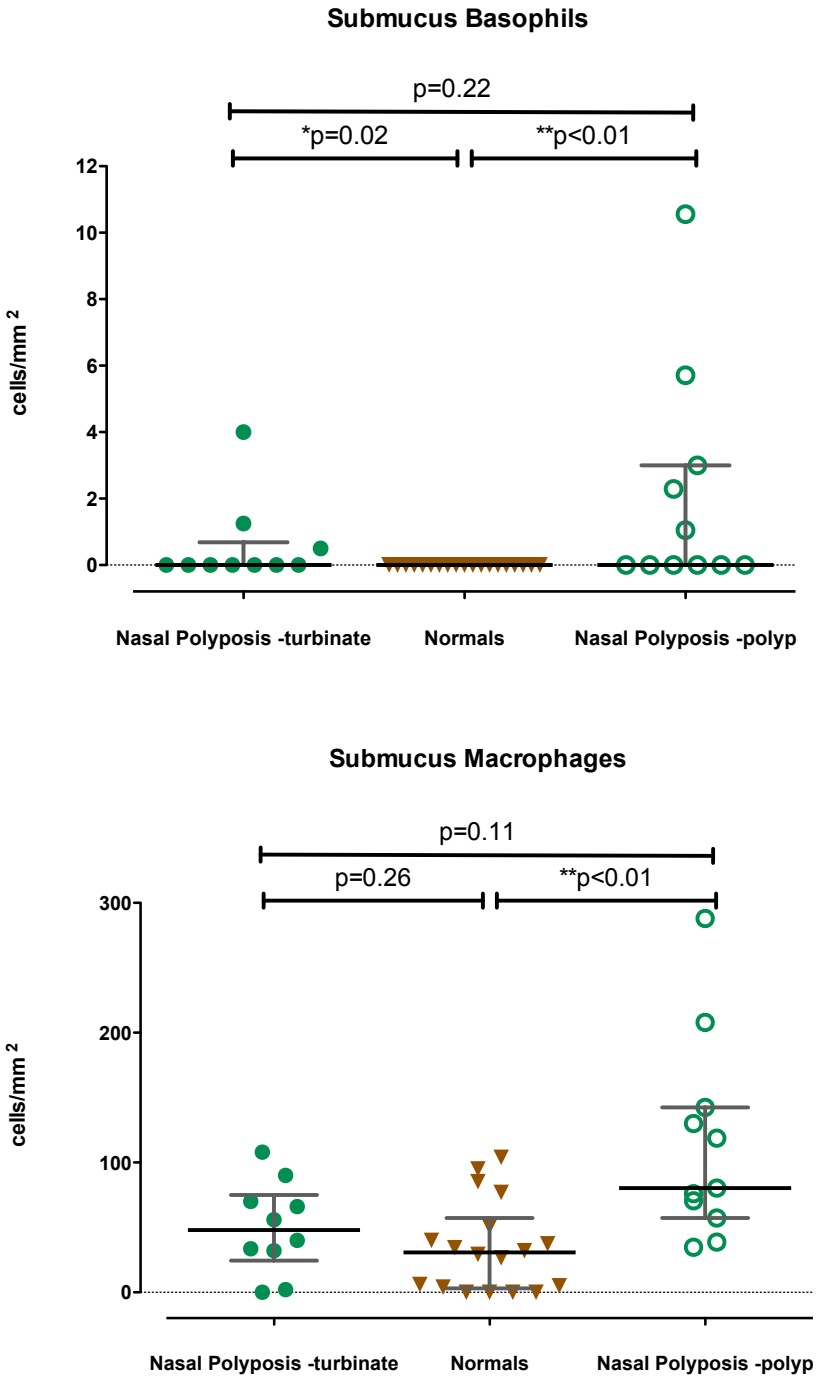
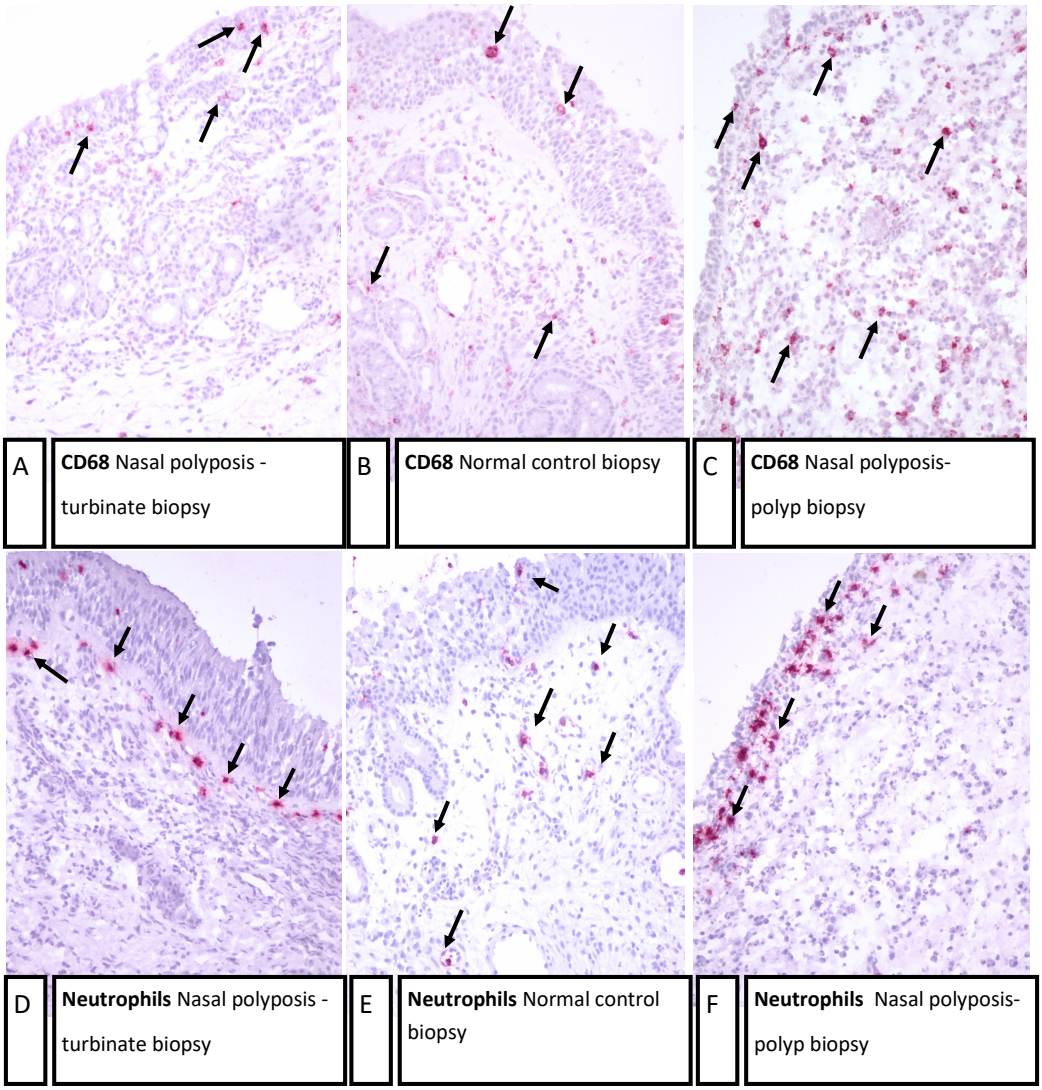


Figure 7.3: Submucosal Inflammatory Cell Counts II: Counts/mm²: Paired nasal polyposis-turbinate and nasal polyposis-polyp samples, n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.



Photomicrograph 7.2: Macrophage staining using the monoclonal antibody CD68 (A-C) and neutrophil staining using monoclonal antibody against elastase (D-F) with the APAAP technique. Macrophages and neutrophils visible as discrete red cells (black arrows) with haematoxylin (blue) nuclear counterstain. Similar numbers of macrophages and neutrophils were evident in submucosa of normal controls (B,E) and turbinate biopsies from polyposis subjects (A,D) however both cell types were found in increased numbers in nasal polyps (C,F). Photographed using x20 lens.

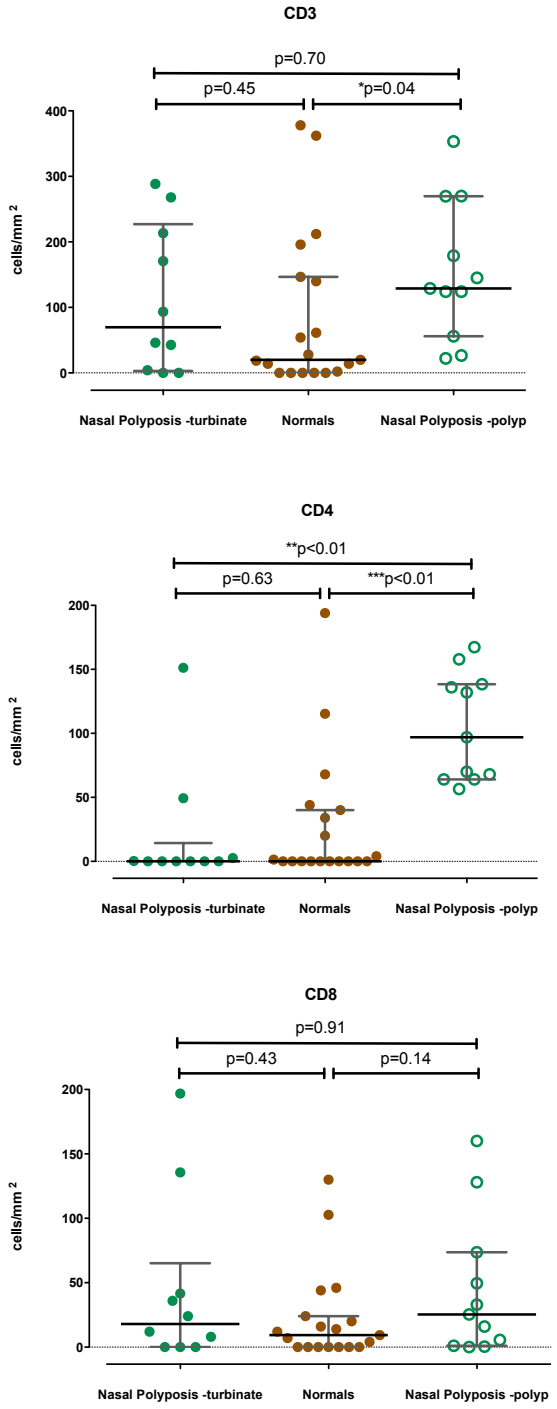


Figure 7.4: Submucosal T Cell Counts/mm²: Paired nasal polyposis-turbinate and nasal polyposis-polyp samples, n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.

Polyp samples had a significantly thinned basement membrane with no alteration in epithelial integrity (figures 7.5 and 7.6) (photomicrograph 7.4). Known to exhibit pseudocyst formation, this study confirmed absence of structured glandular tissue in polyp submucosa. It also revealed a significant paucity of blood vessels with reduced percentage vascular area ($p=0.04$) and number of CD31 positive cells ($p=0.06$) when compared to normal controls. Comparison with turbinate samples showed significantly less vascularity for all four markers (number of CD31+ cells, percentage vascularity, number of vessels and absolute vessel size) (figure 7.12). The vessels that were present in polyps were VEGF positive. VEGF staining in polyps showed no difference when compared to normal controls or to turbinate samples, but VEGF negative vessels were considerably reduced in polyps versus normal controls (figure 7.13). Lymphatics are present in polyps and exhibited no difference in all four lymphangiogenesis markers. Activation of matrix metalloproteases saw a rise in MMP7 ($p=0.09$) and TIMP-1 (**$p<0.01$) (figures 7.9 and 7.11) (photomicrograph 7.6), with no resultant change in MMP 7/9 to TIMP-1 ratio (figure 7.10). There was no difference in intensity of TIMP-1 staining in submucosal glands. HSP-47 expression was enhanced in nasal polyps both in total number as well as those expressed by fibroblasts only (**$p<0.01$) (figure 7.8) (photomicrograph 7.5). There was no concomitant alteration of polyp smooth muscle actin expression. Submucosal TGF-β was (**$p<0.01$), but epithelial TGF-β was not increased in NPp versus normal controls (figure 7.14). ALK-4 ($p=0.02$) was also increased submucosally without associated changes in activin or pSMAD2 (figure 7.15).

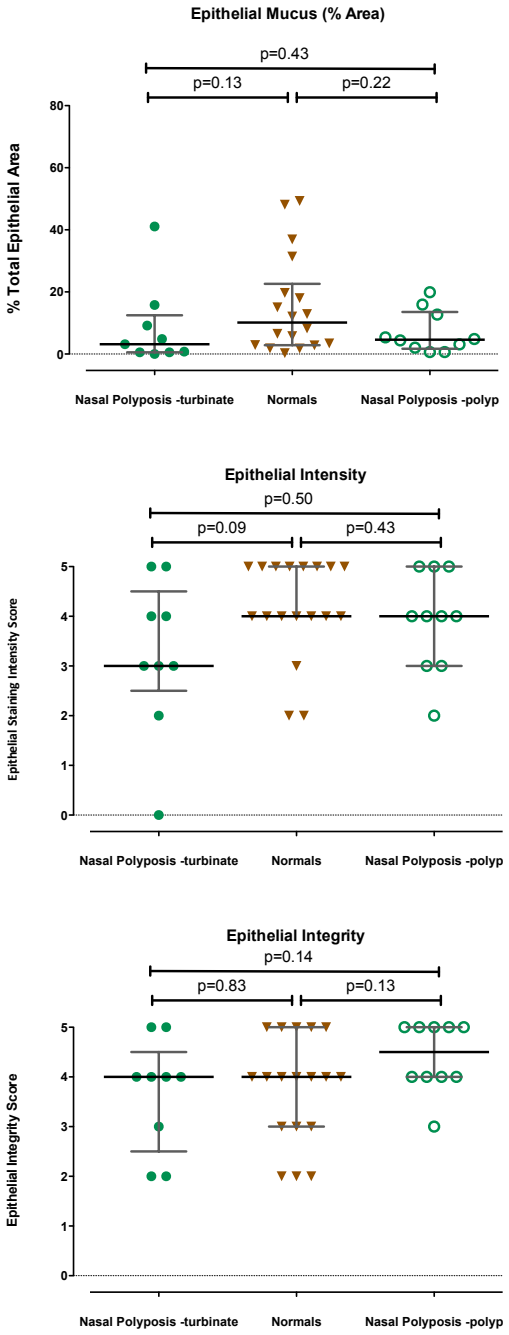


Figure 7.5: Epithelial integrity, mucus staining intensity and percentage mucus staining area/mm^2: Paired nasal polyposis-turbinate and nasal polyposis-polyp samples, n=9, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.

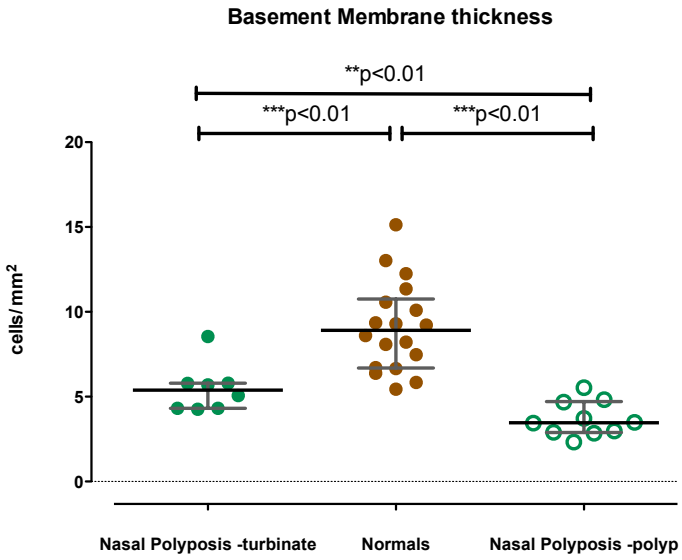


Figure 7.6: Basement membrane thickness: /mm^2: Paired nasal polyposis-turbinate n=8 and nasal polyposis-polyp samples n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.

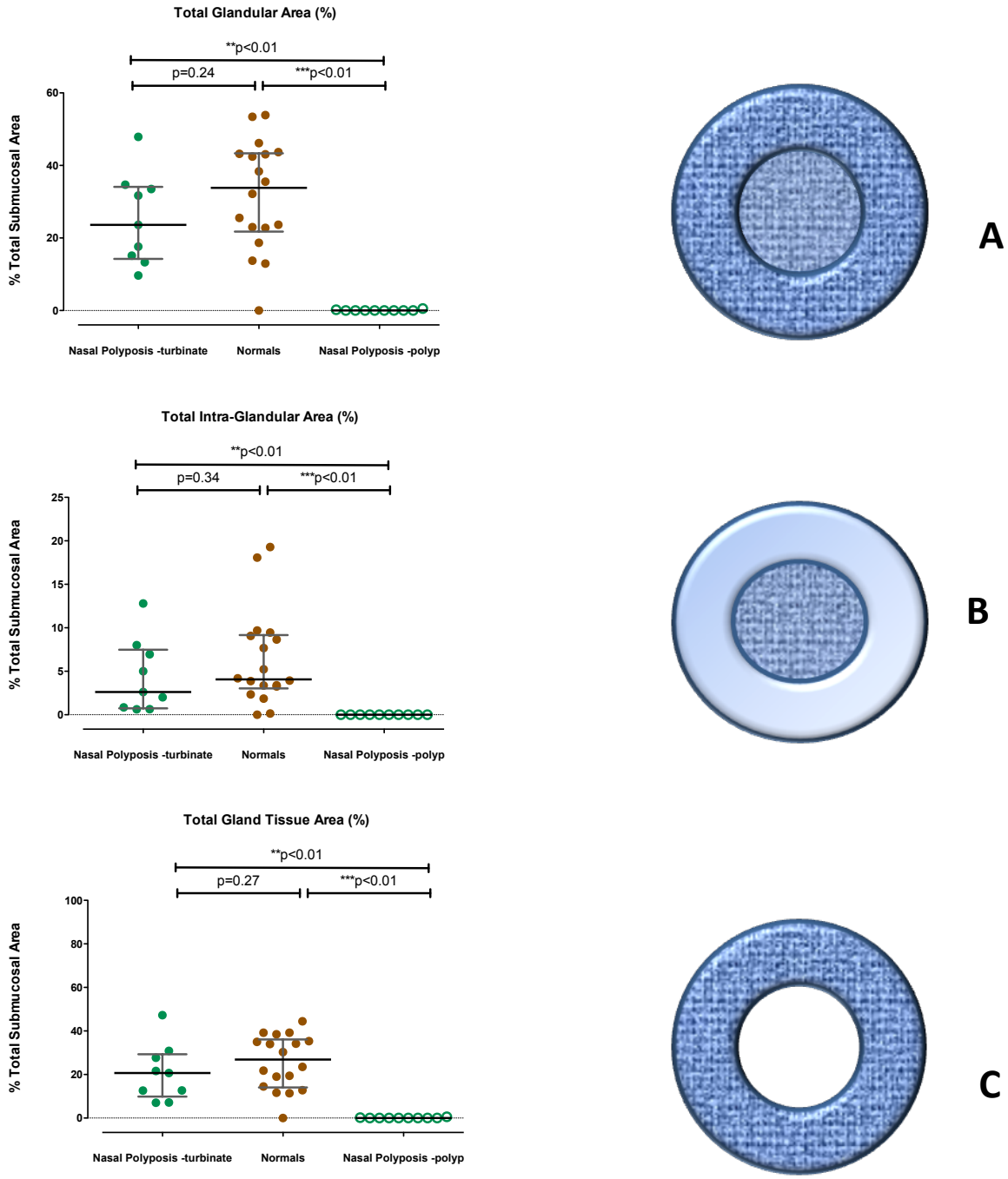
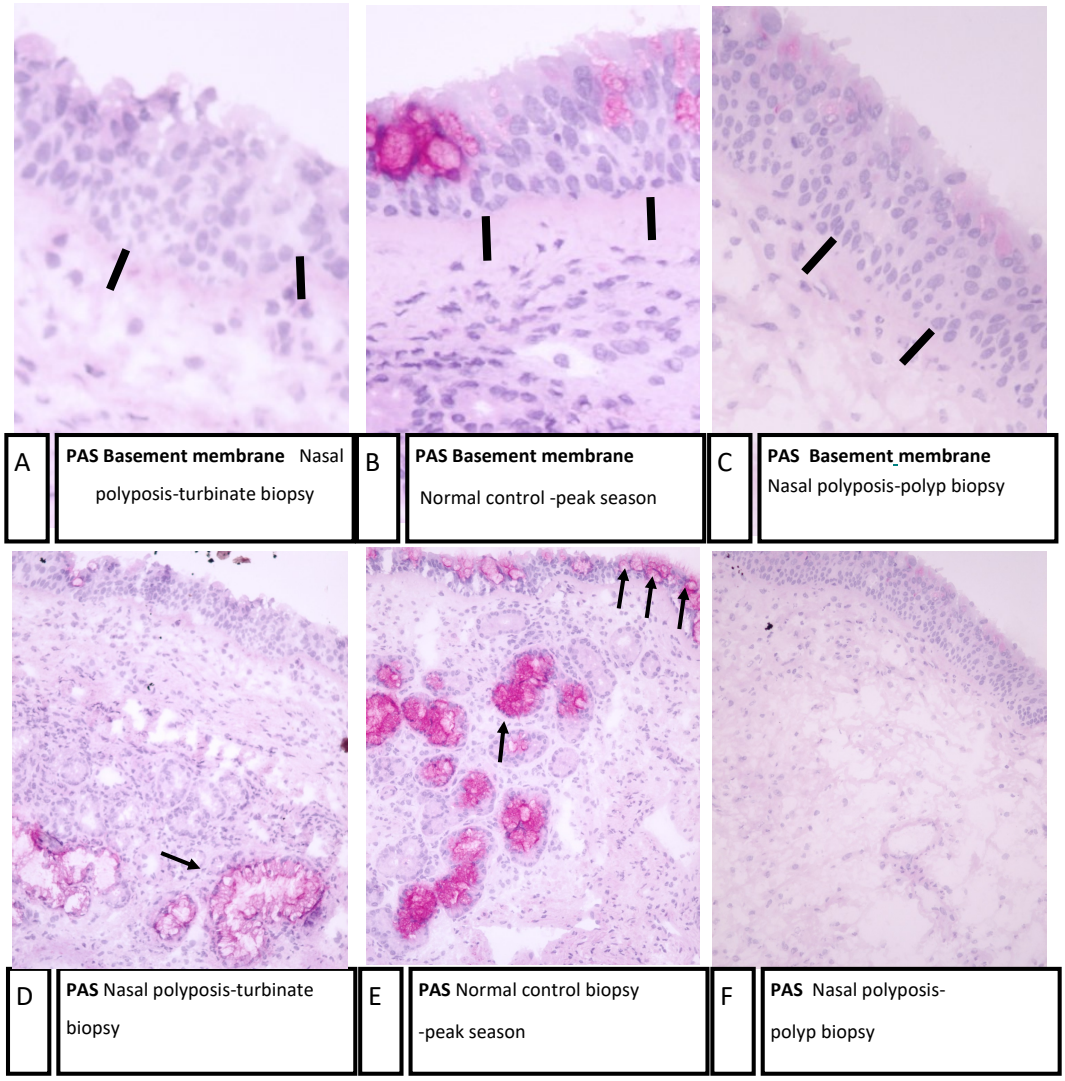


Figure 7.7: Submucosal Glandular Area: measured area is represented by textured blue background on schematic gland drawing, representing (A) gland tissue and secretory contents, (B) secretory content area only and, (C) glandular tissue only. Comparisons with normal samples were made using Mann-Whitney test. Paired nasal polyposis sample, n=9 were analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.



Photomicrograph 7.4: Periodic Acid Schiff staining (PAS) with haematoxylin counterstain. Basement membrane thickness was significantly reduced in nasal polyposis-polyp biopsies (C) compared to nasal polyposis-turbinates (A) and normal control biopsies (B). Black bar indicates the width of the membrane in normal control biopsies, the same sized bar extends into the submucosa of nasal polyposis- turbinate and polyp biopsies. Submucosal glands were absent in polyposis-polyps (F) whilst present to the same extent in turbinates from nasal polyposis subjects (D) and normal controls (E). Photographed using x20 lens.

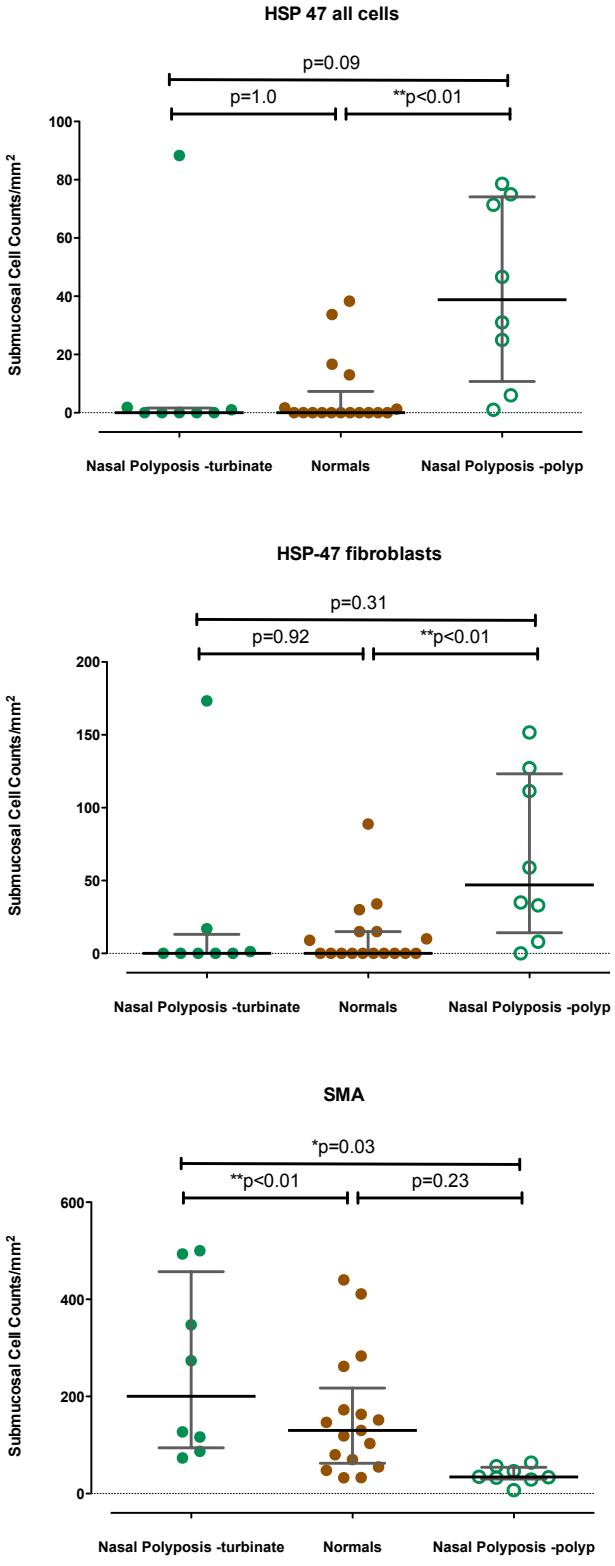
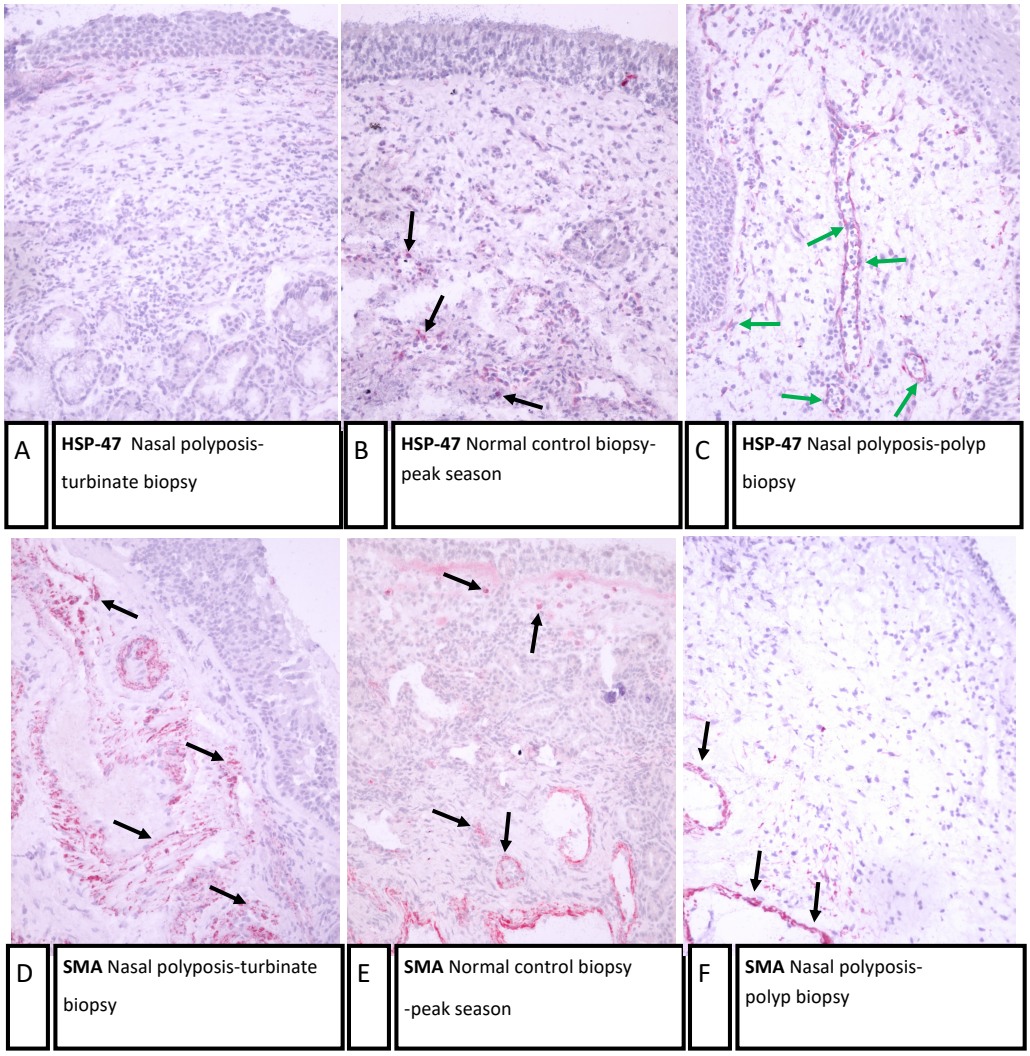


Figure 7.8: Smooth Muscle Actin and Heat Shock Protein-47 (measured in all cells and in a fibroblast cell subset): Paired nasal polyposis-turbinate n=8 and nasal polyposis-polyp samples n=8, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.



Photomicrograph 7.5: HSP-47 staining (A-C) and α-SMA staining (D-F) using specific monoclonal antibodies with the ABC-AP technique. HSP-47 + and α-SMA+ cells visible as discrete red cells (black arrows or green arrows for HSP-47+ fibroblasts) with haematoxylin (blue) nuclear counterstain. Significantly more HSP-47+cells/fibroblasts were seen in nasal polyposis-polyps (C) than in turbinates from nasal polyposis subjects (A) or in normal control biopsies (B). α-SMA+ cells were more numerous in nasal polyposis turbinates (D) than in normal controls (E) or nasal polyposis-polyps (F). Photographed using x20 lens.

Turbinate tissue of nasal polyposis patients also exhibited thinning of the basement membrane without associated disturbance to the epithelial integrity, mucus staining intensity or percentage goblet cell area (figure 7.5 and 7.6). In contrast to NPp samples, turbinates possess submucosal glands with no demonstrable difference between NPt and normal controls. NPt samples showed enhanced lymphatics with all four markers of lymphangiogenesis statistically higher than non-atopic participants. Increases in vascular markers in the form of increased CD34+ cells ($p=0.08$) and statistically higher percentage vascular area ($p=0.04$) were seen in NPt samples, without change in VEGF signalling. MMP9 was less ($p=0.08$) with statistically more TIMP1 ($p=0.01$) and by extension, decreased ratios of MMP7/TIMP1 ($p=0.06$) and MMP9/ TIMP1 ($p=0.04$). Intensity of TIMP1 staining in submucosal glands was also increased ($p=0.03$). Expression of smooth muscle actin was (**$p<0.01$), but HSP -47 was not, significantly more in NPt samples versus normal nasal tissue. TGF-β, activin and pSMAD demonstrated no difference but ALK-4 was significantly more in nasal polyp turbinates than normal controls.

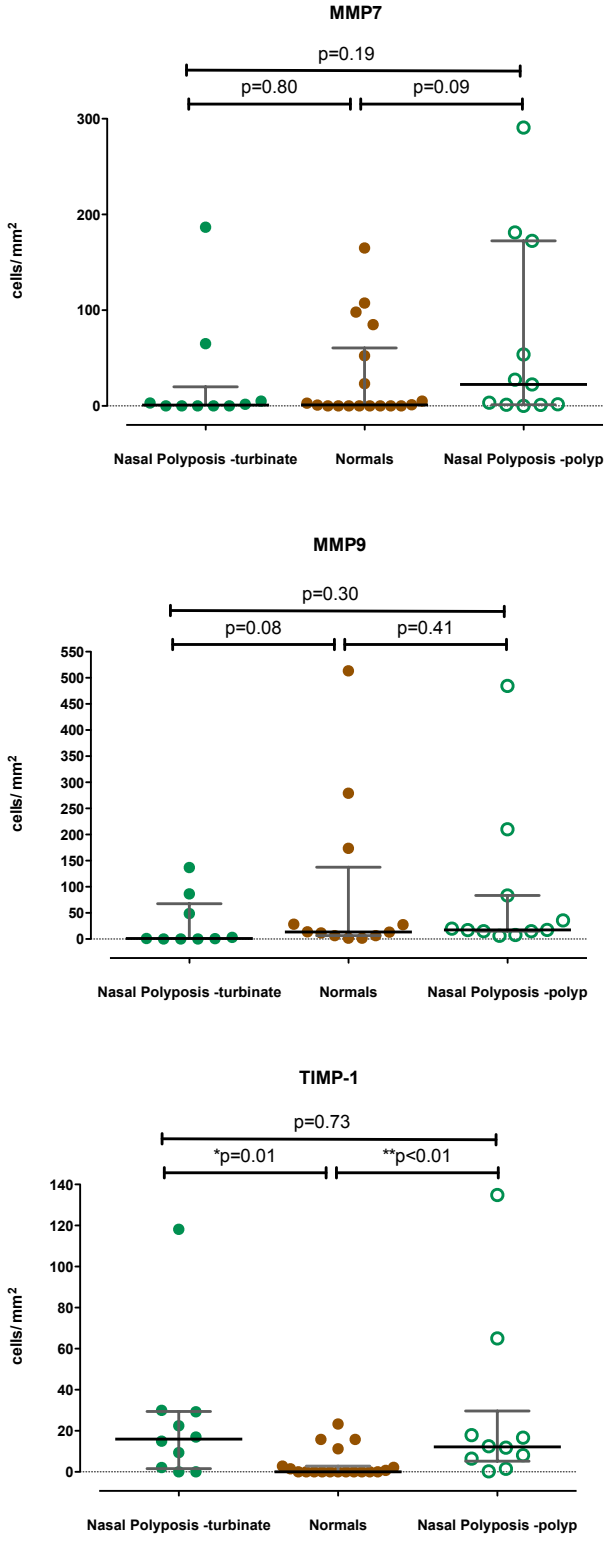


Figure 7.9: Matrix metalloproteases 7 and 9 with their inhibitor TIMP-1 /mm²: Paired nasal polyposis-turbinate n=9 and nasal polyposis-polyp samples n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.

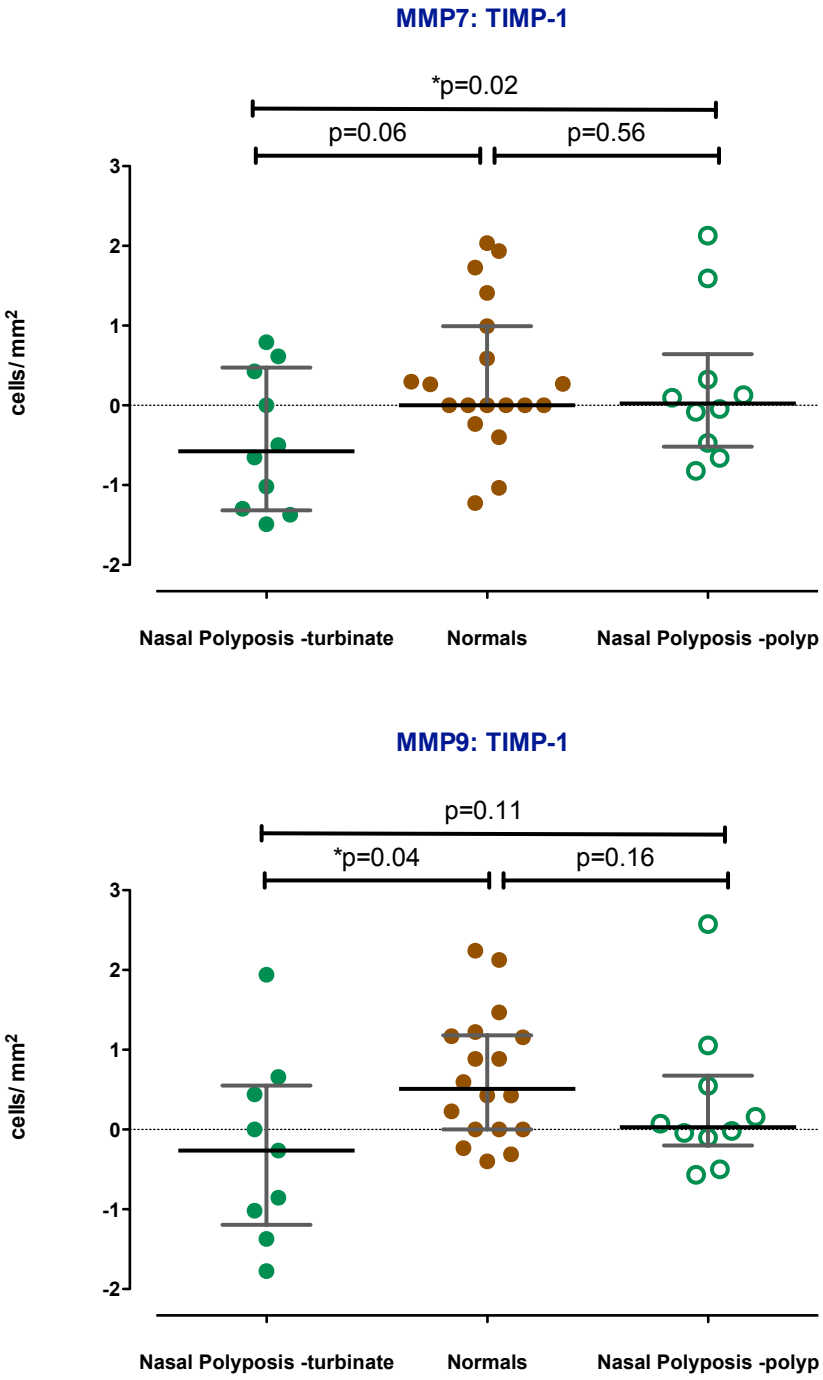


Figure 7.10: Matrix metalloproteases 7 and 9 ratio to TIMP-1/mm²: Paired nasal polyposis-turbinate n=9 and nasal polyposis-polyp samples n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.

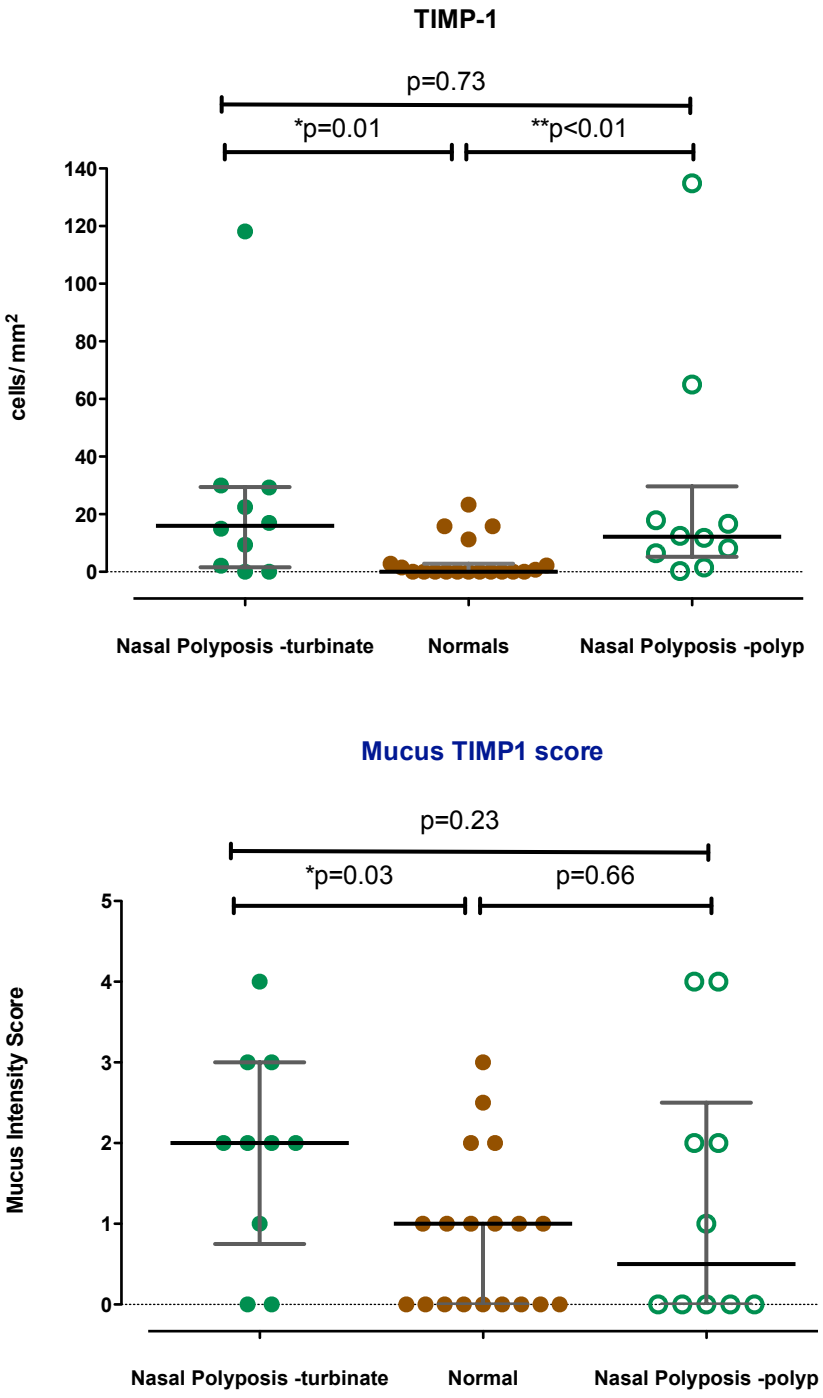
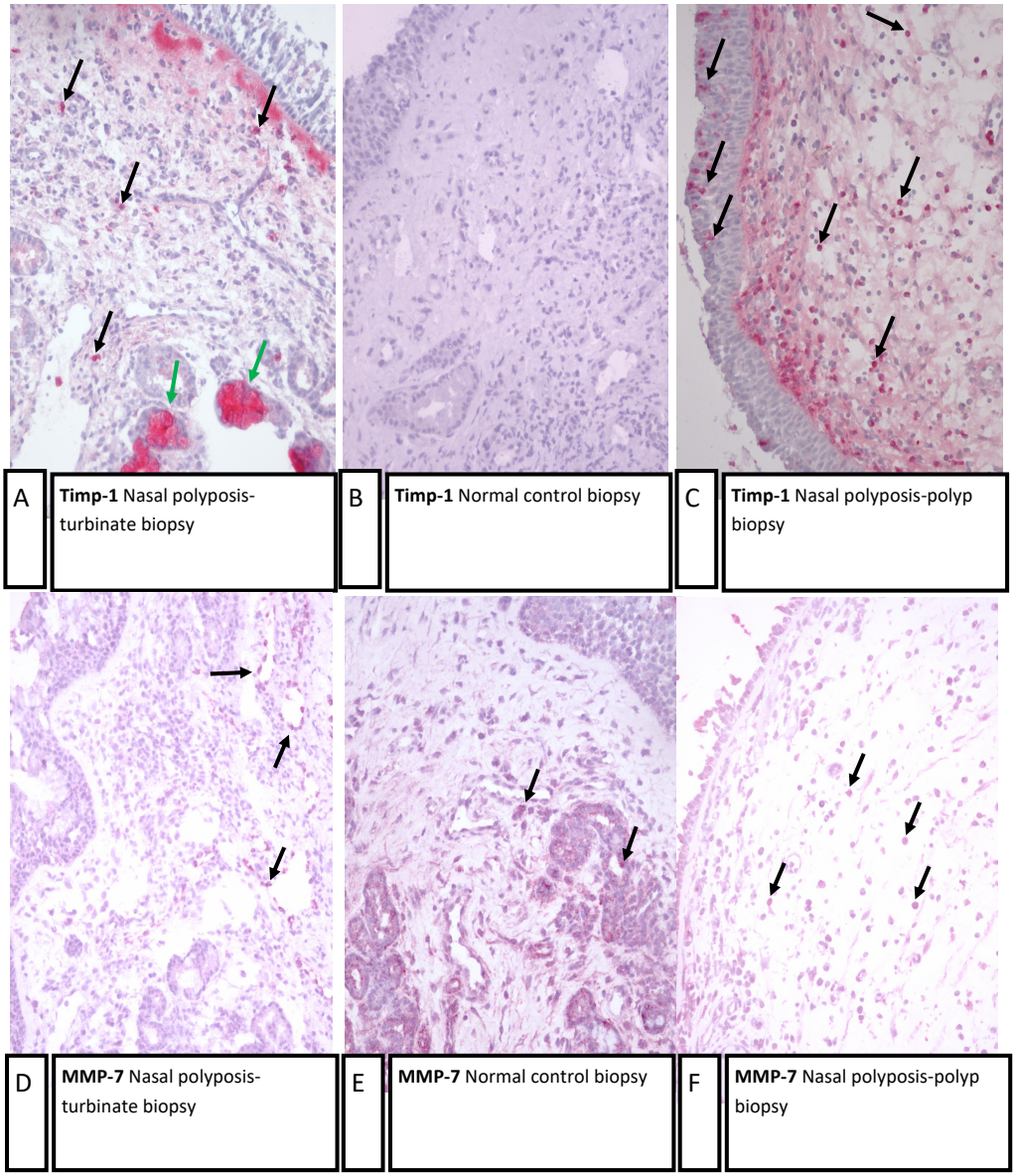
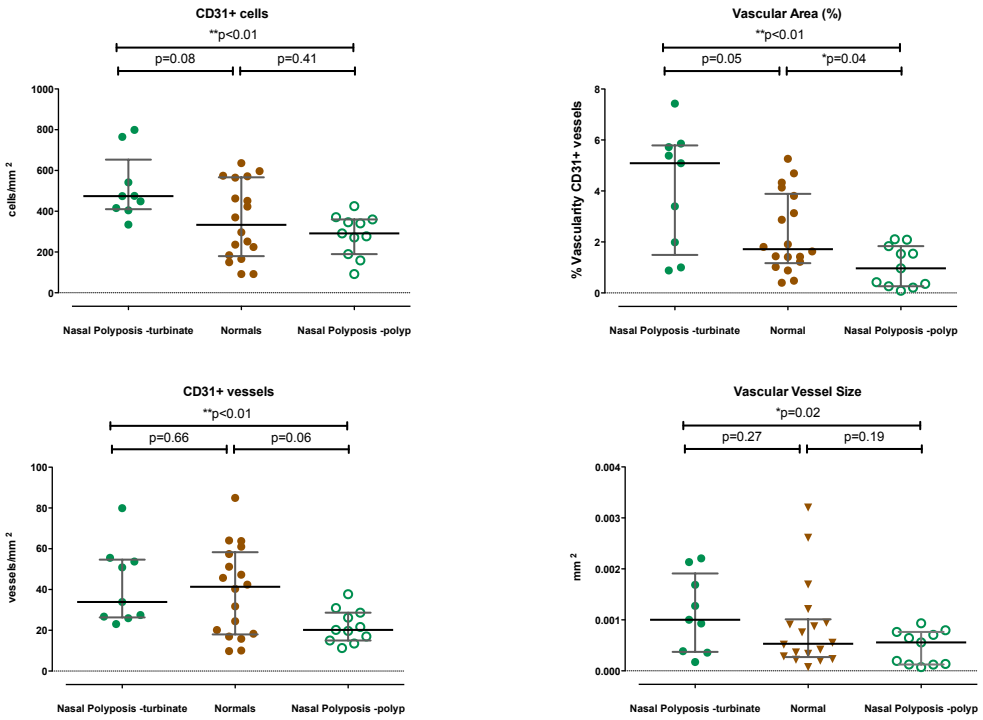


Figure 7.11: TIMP-1 cells/mm² and intra-glandular mucus TIMP-1 staining intensity score: Paired nasal polyposis-turbinate n=10 and nasal polyposis-polyp samples n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.



Photomicrograph 7.6: Timp-1 staining using monoclonal antibody (A-C) and MMP-7 staining using monoclonal antibody (D-F) with the ABC-AP technique. Timp-1+ and MMP-7+ visible as discrete red cells (black arrows) with haematoxylin (blue) nuclear counterstain. Timp-1+ mucus indicated with green arrows. Significantly more Timp-1+cells in nasal polyposis turbinates (A) and polyps (C) than in normal control biopsies (B). MMP-7 was found in similar levels nasal polyposis turbinates (D) and polyps (F) as well as in submucosa of normal controls (E). Photographed using x20 lens.

A



B

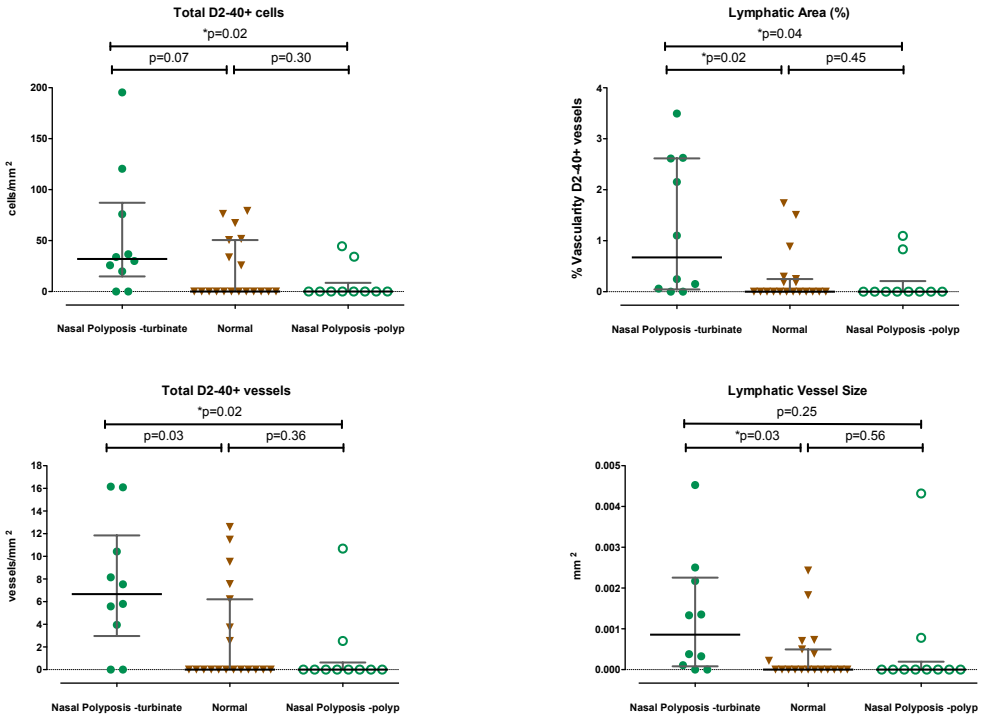


Figure 7.12: Angiogenesis (A) and Lymphangiogenesis (B) markers: Counted as total number of cells and vessels/mm², vessel area (% total submucosal area) and vessel size(mm²). Paired nasal polyposis-turbinate n=10 and nasal polyposis-polyp samples n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.

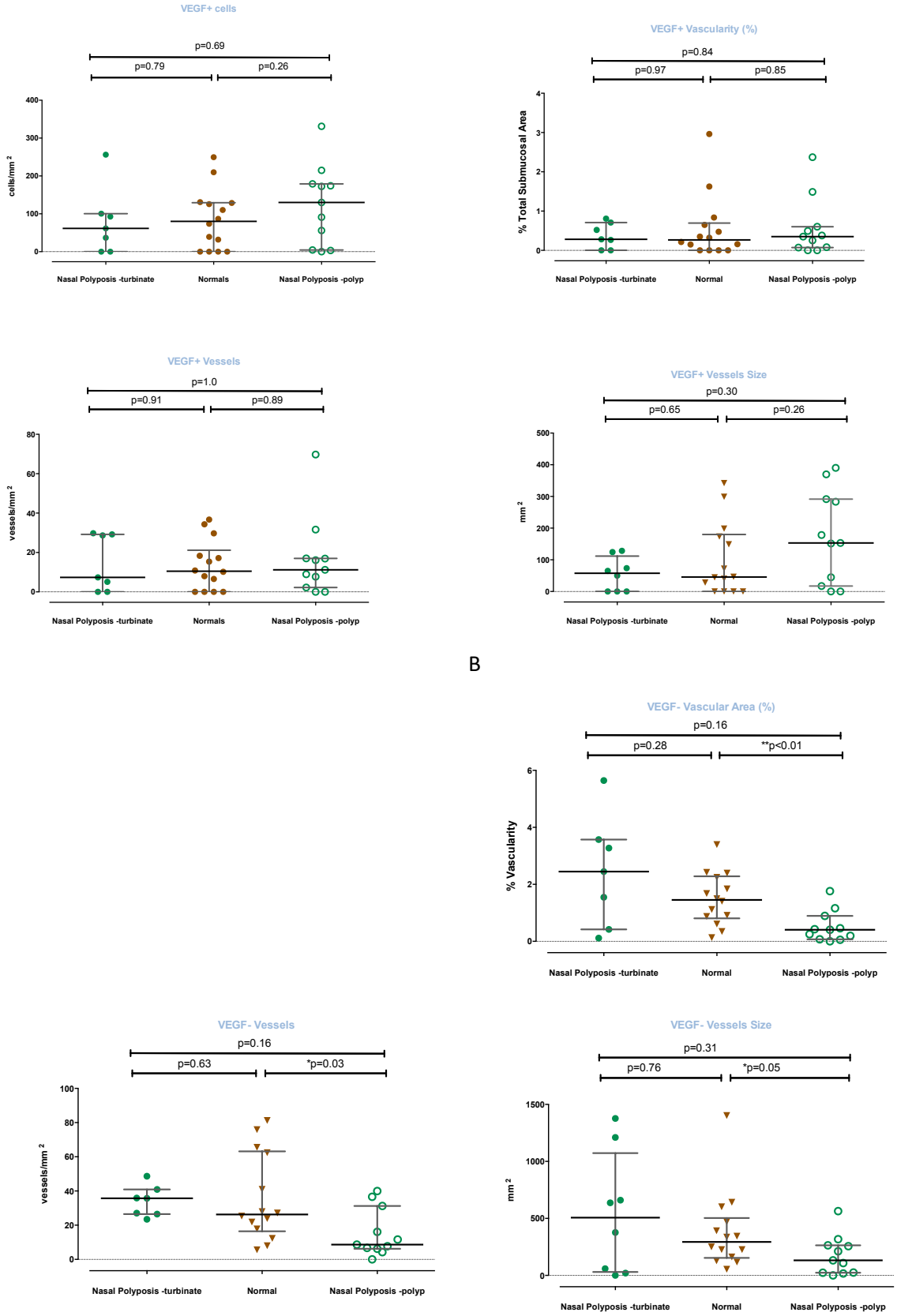


Figure 7.13: VEGF+ (A) and VEGF- (B) vessels: Paired nasal polyposis-turbinate n=10 and nasal polyposis-polyp samples n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=19) were made using Mann-Whitney test. Significance was accepted as p<0.05.

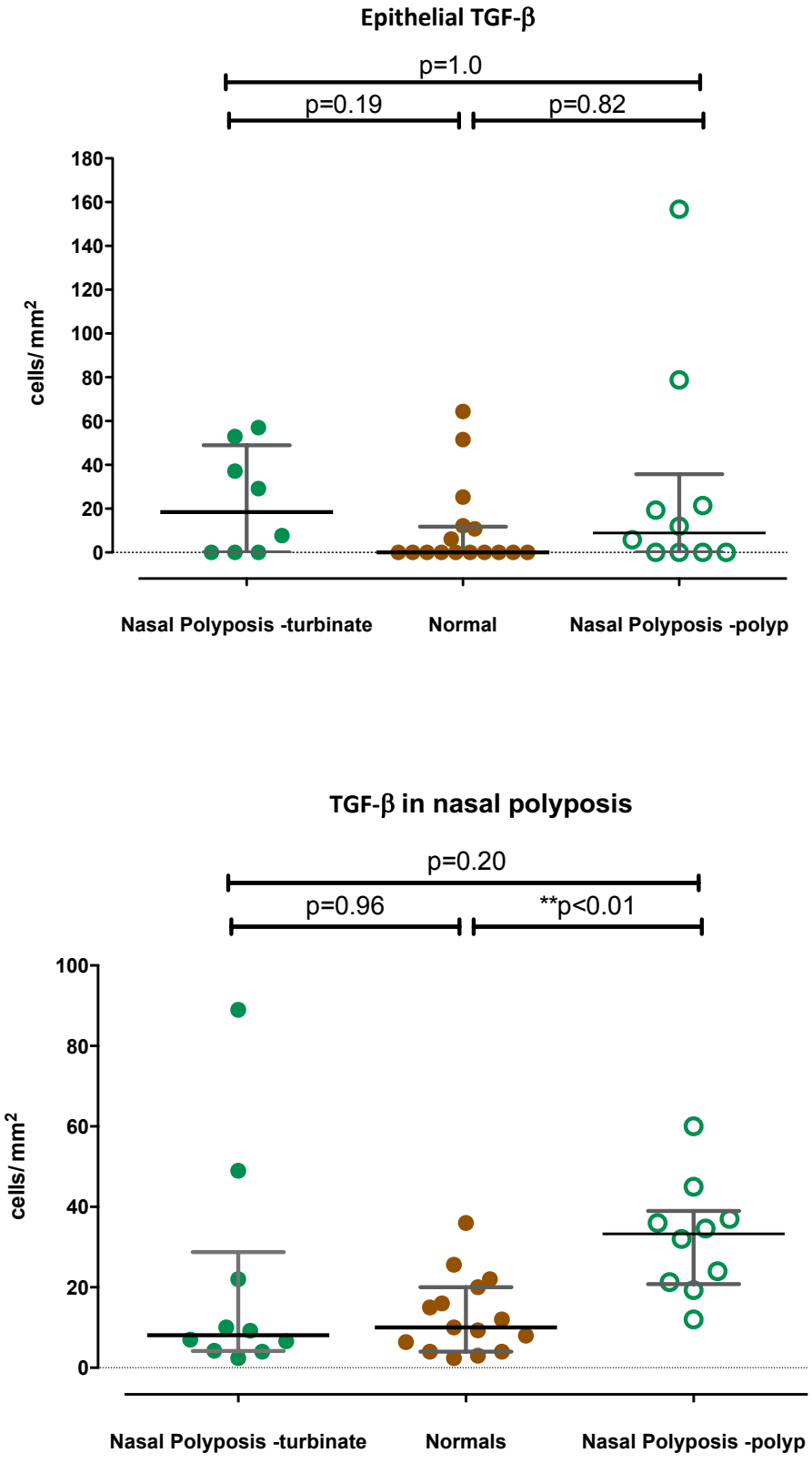


Figure 7.14: TGF-β: epithelial and submucosal cell counts/mm². Paired nasal polyposis-turbinate n=10 and nasal polyposis-polyp samples n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=16) were made using Mann-Whitney test. Significance was accepted as p<0.05.

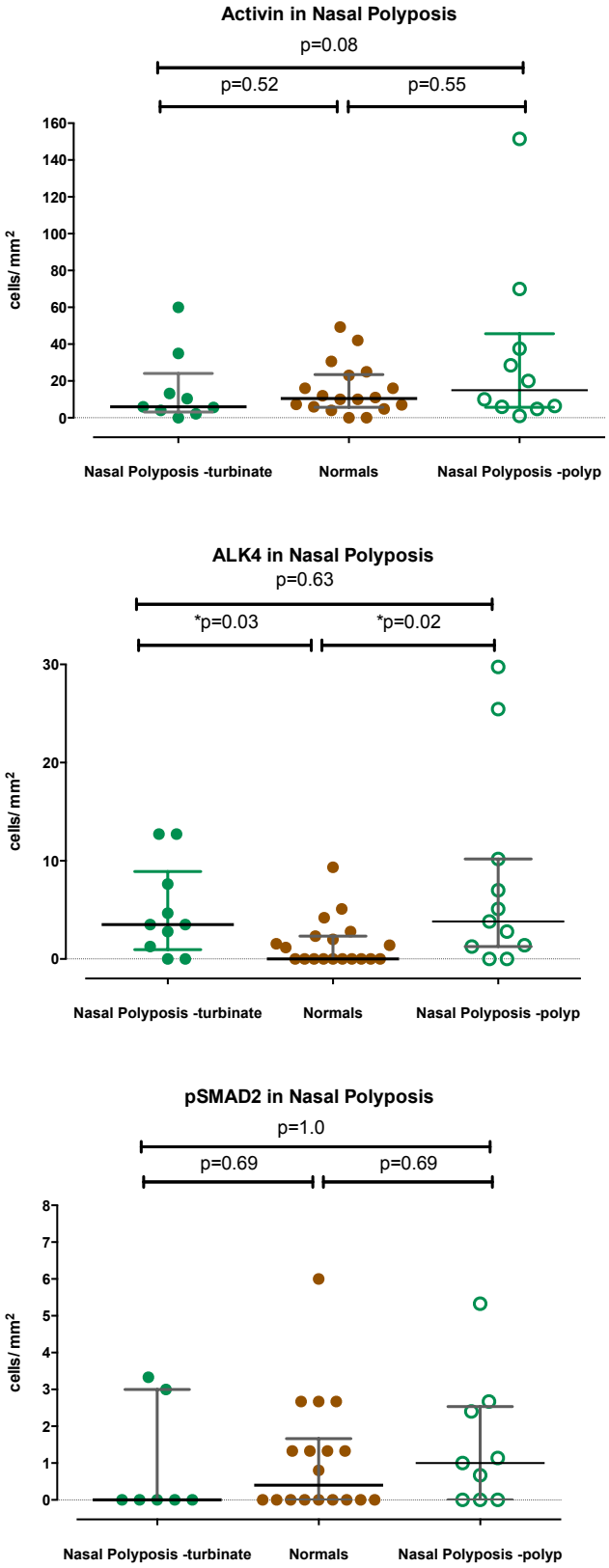


Figure 7.15: Submucosal activin, ALK-4 and pSMAD-2 cell counts/mm². Paired nasal polyposis-turbinate n=10 and nasal polyposis-polyp samples n=10, were compared using the Wilcoxon signed rank test. Comparisons between the individual nasal polyposis groups and normal samples (n=16) were made using Mann-Whitney test. Significance was accepted as p<0.05.

Discussion

Inflammation

Nasal polyposis presents a unique interface with epithelium abundant in inflammatory infiltrate. With enhanced expression in polyps and turbinates, the submucosa is rich in eosinophilic and neutrophilic infiltrate with accompanying increase in CD4 cell numbers in polyps only. With enhanced basophil and macrophage expression, and possible augmented antigen presentation, polyps in particular provide a rich source of Th2 cytokines and allergic inflammation. Th2 cells transfer and mediate allergic inflammation between the nose and lung. Lower airway challenge in mice produces eosinophilia and ICAM-1 expression as a result of Th2 cell lung priming (278). Such persistence of Th2 specific cellular infiltrate may explain the marked link between upper and lower airways disease in nasal polyposis. Treatment of either significantly improves clinical symptom of both, an effect likely to be mediated by alteration of Th2 mediated responses. Nasal polyposis group under investigation contained highest number of asthmatics at 38% of any study group, but this did not relate to higher eosinophil counts in either inferior turbinate or polyp samples in this cohort. It has previously been shown that CRS patients have higher eosinophil counts than normal controls but no difference exists between allergic and non-allergic CRS sufferers (753).

Mast cells are present in excess in the epithelia of polyps only, in contrast to findings in turbinate samples of same individuals or allergic rhinitis. This may demonstrate ongoing migration/ efflux in the absence of antigen induced degranulation, underscoring redundancy of such process in nasal polyposis. Nasal polyposis patients have increased collective mast and basophil cell counts both in their polyp and adjacent turbinate tissue bearing no correlation with allergic status (754). In combination with eosinophils, macrophages and neutrophils, mast cells provide a rich source of remodelling mediators such as TGF-β. Alterations of epithelial architecture dependent changes in polyp topography with budding and new polyp formation will rely on localised TGF-β responses. The latter will be determined by presence of inflammatory infiltrate in both epithelium and submucosa as, eosinophils are the predominant source of TGF-β with majority of eosinophils contributing to its synthesis in nasal polyps (755).

The dichotomy of eosinophil and neutrophilic polyps is contradictory, with evidence showing no significance to clinical outcomes (231). With such abundance of both eosinophils and neutrophils in both polyp and turbinate samples, it can be postulated that both cells types will play a significant role in pathogenesis with combined effects playing greater role than each individual cell type *per se*. Both NPp and NPt samples are remarkable for the persistence of inflammatory infiltrate which is likely to

drive a Th2 cytokine response in this disease. Other cytokines such as IL-8 may also play a part in neutrophil chemotaxis (IL-8 was significantly more abundant in nasal polyps than normal controls with **p<0.01) although there was no difference post allergen challenge in the RAC protocol. The influence of IL-8 over neutrophil cell numbers may have variable significance in nasal polyps as opposed to allergic rhinitis (756).

Remodelling

Epithelium

No evidence exists of epithelial damage as a result of persistent allergic inflammation or active polypoidal disease. With no alteration in TGF-β, there was a lack of direct EMTU activation despite a robust Th2 hallmarked tissue response. There was a suggestion of enhanced expulsion of goblet cell contents in NPt samples with reduction in mucus staining intensity although this was below statistical significance. This indicated ongoing expulsion of mucus contents, more marked in turbinates than polyps themselves.

Although TGF-β did not increase in the epithelium, myofibroblast transformation may be ongoing independently with increased HSP-47 in polyps and increased SMA in turbinates, hence indirect evidence of EMTU activation. This is likely to not be solely reliant on TGF-β signalling with variable epithelial responses playing their part. A genome wide meta-analysis study suggested correlation between allergic rhinitis and TSLP variants (254) whilst expression of TSLP was increased in nasal polyps with chronic rhinosinusitis (255). In the absence of epithelial TGF-β alteration in both polyp and turbinate tissue of nasal polyp patients, mechanical insults are either insufficient by themselves, or are of inadequate magnitude to elicit TGF-β mediated epithelium driven activation and remodelling even in the presence of eosinophilia and Th2 cytokine environment. Epithelial tight junctions may be deficient in response to circulating cytokines and allow for dysregulated control by breach of integrity (757).

Basement Membrane

Basement membranes demonstrate heterogeneity and disease specificity (678). Both polyps and turbinates of nasal polyposis participants demonstrated significant epithelial thinning; a finding significantly more marked in polyps. As evidenced in lung budding and alveolar development, thinning of basement membrane is localised to areas of greatest cell turnover allowing for changes in expansile growth forces and budding (758). Localised alteration in TGF-β signalling allows for isolated thinning of the basement membrane with enhanced tissue synthesis on either side, initially forming a cleft with

subsequent buds of tissue on either side. In pulmonary morphogenesis isometric tensile forces across the basement membrane are a prerequisite. Such tensile forces during active nasal polyp disease may similarly enhance cell growth and extracellular matrix remodelling and concomitant neo-polyp formation (758).

Epithelial damage and increases in basement membrane thickness correlate with epithelial eosinophilic infiltrate in polyp samples (759). A thinned basement membrane will possess decreased tensile strength, be stiffer and exist in an altered TGF-β rich milieu (681). This is in the context of active changes in tissue topography with enhanced MMP expression, coalescing to aid budding and expansile tissue growth. With tubular structures such as the lower airways, increased resistance to intraluminal pressure is supported by folding of the mucosa (760). Whilst this is advantageous in lower airways disease, it serves no purpose in the nose. By folding of the epithelium space within an airway, and resistance to further epithelial expansion, is maximised. Such lack of epithelial folding in the nose may contribute to absence of resistance to expansile forces during polyp formation which are left to proliferate unabated.

The mechanisms behind thinning of the basement membrane may be replicated in nasal polyps where growth and branching of grape-like polyps undergo periods of rapid growth. Thinning of basement membrane exists in both turbinate and polyp samples of nasal polyposis but is statistically greater in the latter where histological changes tip over into frank polyp formation.

Collagen and fibroblast transformation

Unrelenting tissue stress caused by persistent oedema drives alterations in collagen synthesis as evidenced by increased HSP-47 in NP-p (when compared to normal) and enhanced SMA in NP-t (when compared to polyp samples). Myofibroblast transformation evidenced in turbinate samples by increased SMA was not seen in polyps. Although there were fibroblasts in both polyps and turbinates of patients with nasal polyposis, they are likely to be phenotypically different as polyp fibroblasts demonstrate high propensity for collagen synthesis in the absence of transformation to a myofibroblast phenotype (761). This appears to be the responsibility of turbinate fibroblasts which, in turn, do not produce collagen to significantly enhanced degree. Differences in fibroblast phenotype and presence of physical stretch stimuli, and prevailing cytokine/ TGF-β milieu are likely to alter degree of collagen synthesis in each tissue locale, even within the same disease state (762).

TGF–β and ALK-4 expression were statistically enhanced in polyps when compared to normals, with activin levels greater in polyps than turbinates. Such upregulation of TGF–β and activin signalling will

influence collagen production, with variable responses in deposition (451). Optimised ECM conditions will enhance collagen synthesis (717). Mechanical stretch forces induced by marked tissue oedema seen in polyps will encourage collagen synthesis, demonstrating increased HSP-47 in such tissues. Maintenance of these changes coupled to altered phenotype in turbinate fibroblasts may see optimized response to TGF-β and activin, with notable myofibroblast transformation in turbinates. In this scenario, airway oedema activates fibroblast to enhance collagen synthesis (763) (764) in polyps, with concomitant increase in expression of TGF-β and activin, providing an engine to drive myofibroblast transformation in turbinates where tissue architecture responses to tissue oedema manifest in altered fibroblast response. With polyp tissue lacking such transformational fibroblast ability and without 'ruching' of the epithelium, there are no mechanically restrictive forces to limit tissue expansion. Differences in extracellular matrix characteristics between turbinates and polyps in nasal polyposis imply altered thresholds for biomechanical forces needed to release matrix bound TGF-β (763). As such, polyps are responsible for ECM collagen synthesis, and turbinates demonstrate iteration into a myofibroblast phenotype, with possible attempts at contraction and thereby physical limitation of polyp mediated tissue expansion.

Matrix Metalloproteases and their inhibitors

The regulatory effect of increased TIMP-1 is preserved in nasal polyposis, with raised TIMP-1 present in both polyps and turbinates. Such attempts at repair are ongoing although incomplete as evidenced by increased vasculature and lymphatics in CRSwNP turbinates (NPt) samples. Prolonged *de novo* synthesis of TIMP-1 by epithelial and submucosal cells is a likely requirement if an increase in staining intensity is to be discernible (765). TIMP-1 has two glycosylated sidearms which will stain with PAS and changes in staining intensity are readily visible (766). With persistence of submucosal inflammatory cells and perseverance of glandular hypertrophy, presence of epithelial goblet cell TIMP-1 reflects, and is likely to influence, submucosal regulatory processes. Imbalance of TIMP/MMP7 is evidenced in CRS (767). TIMP increased in both CRS (maxillary sinus biopsies) and nasal polyposis (ethmoidal sinus derived polyp tissue) (527). TIMP-1 enhances fibroblast cell growth (509) and may encourage phenotypic transformation. TIMP-1 anti-angiogenic and anti-apoptotic with cell cycle arrest properties discourages alteration of established tissue structure (506).

Epithelial MMP expression is enhanced in nasal polyposis patients; an effect that is more pronounced in polyp tissue itself as opposed to nasal mucosa from CRSwNP subjects (768). This study supports these finding as MMP7 was significantly increased in NPp samples without concomitant alteration in MMP9. MMP9 inhibition enhances wound repair (769), whereas upregulation of MMP7 encourages epithelial cell migration after injury (770). MMP7 augments E-cadherin ectodomain shedding (671)

and disrupts syndecan expression (771), inducing conformational change in integrins, thereby facilitating wound repair. When activated, MMPs can cleave extracellular matrix and basement membrane and are one of the fundamental activators of TGF-β (709). TGF-β in turn upregulates gene expression of a number of pro-fibrotic genes such as SMA-α (408). In this study, increased MMP7 in polyp tissue may have influenced fibroblast behaviour and had a bearing on collagen synthesis. The effect was localised as HSP-47 and by extension, collagen synthesis, was only upregulated in polyps and not seen in adjacent turbinate tissue, where different ECM remodelling environment exists.

MMP9 was unchanged in polyps but was decreased in turbinates, reflecting differences in activation of regulatory processes. Reduced activation of MMP9 (particularly when coupled to increased TIMP1) allows for reparative processes. When seen in the light of increased myofibroblast phenotype, turbinates in nasal polyps make considerable and partially effective attempts at regulating alterations in tissue architecture begotten by persistent tissue oedema and polyp derived TGF-β driven remodelling.

MMP7 did not alter in turbinates but with decreased MMP7/ TIMP-1 ratio and increased TIMP-1, remodelling is regulated. Increased MMP7 in the polyps is likely to drive, at least in part, angiogenic changes seen in turbinates. Concomitant increase in TIMP in both polyp and turbinate tissue works to partially stabilise, and downregulate, new vessel growth (772). Myofibroblast transformation, as evidenced by increased smooth muscle actin, is likely to act as an abundant source of TIMP-1.

A significantly higher level of IL-8 was seen in nasal polyposis participants when compared to both normal mucosa and allergic rhinitics (**$p<0.01$). IL-8 is angiogenic (773) (774) and an activator of MMP mediated dysregulation (775). Such colocalisation and coactivation of angiogenesis, with coexistent ECM remodelling will differ between polyp and turbinate tissues, but in both instances, matrix metalloproteases and their inhibitor TIMP-1 were altered.

Vascular and Lymphatic Changes

Contrasting signals of remodelling, including vascular and lymphatic changes, are manifest in polyps and turbinates of nasal polyposis patients resulting in altered topography of these two tissue types in this unique upper airway disease. Polyps, devoid of lymphatics, glands and possessing very few blood vessels, nonetheless maintain VEGF signalling. Indeed, polyps may be more reliant on effects of VEGF than turbinates. NP-t and NP-p samples had similar levels of VEGF+ vessels but VEGF negative vessels were significantly reduced in polyp versus turbinate samples. Within the context of mechanical alterations, a plethora of pro and anti-angiogenic factors will be playing their part (776). Polyps more

so than turbinate samples, will be prone to such alteration of tissue architecture with their extracellular matrix made up mainly of pseudocysts. In this shifting topography, VEGF may guard against disturbance of blood vessels as a stabiliser of blood vessel components (777). In the absence of such protection, changes in tissue turgor, polyp growth and alteration of collagen fibrillar content, may all coalesce to pull apart and destroy fragile vasculature not endowed with the pro-angiogenic influence of VEGF. As such, reliance on VEGF to maintain vascular integrity may be of greater significance in polyps than turbinates of nasal polyposis.

Sources of VEGF are variable, with fibroblasts and epithelial cells relying on TGF-β, whilst eosinophils respond to IL-5 and GM-CSF (222). In nasal polyposis where in conjunction with a primarily eosinophilic drive, rich in secreted IL-5 and coupled to polyp derived TGF-β secretion, the VEGF+ signal was maintained despite decreased vascularity of polyp tissue. There was no difference in VEGF+ vascular tissue at both cellular and vessel level. As mentioned above, there are few VEGF negative vessels in polyps, accounting for the difference in overall vascularity of this tissue compared to allergic and non-atopic individuals. This would further imply reliance on a sustained, possibly regulatory aspect, of VEGF in nasal polyps where reliance on signalling is needed to prevent near total loss of vasculogenic drive.

In a chick model, neutralisation of eosinophil and mast cell derived VEGF reduced angiogenesis by 55% and 30% respectively indicating need for persistent eosinophilia ideally coupled with generalised inflammatory cell response to maintain neovascularisation (695). As nasal polyps themselves are lacking in VEGF negative vessels, such signals will be delivered to, and have effect on, VEGF positive vessels in both polyps and turbinates of nasal polyposis patients enhancing new vessel formation. In this study, this enhanced vessel formation was evidenced in turbinates (NP-t) by increased blood vessels in absolute terms, as measured by percentage vascular area ($p=0.05$) versus normal controls. In contrast, in polyps (NP-p samples) there was significantly less CD31+ve vascular area ($*p=0.04$) and decreased percentage of total vascular area ($p=0.06$) but the blood vessels that were present in nasal polyps were VEGF+ve.

There may be a discrepancy between increased levels of transcription (not measured in this study) and quantifiable VEGF protein levels. There is evidence of higher expression of VEGF receptor at mRNA level in nasal polyps, but this is not coupled to higher VEGF-A levels detectable at cellular level via immunohistochemistry (778). Nonetheless, increased vascularity both of blood, as well as lymphatic vessels, is a characteristic of turbinates of nasal polyposis patients. Conversely, near complete absence of lymphatics, glands and marked paucity of blood vessels typify polyps of the same patients (Figure

7.12). There exists different but inter-related topography, cytokine and cellular profiles between polyps and turbinates in nasal polyposis. TIMPS, which have MMP independent anti-angiogenic properties (506) demonstrate a persistent rise in turbinates, reflecting a considered attempt at abrogating neo-vascular changes. Albumin rich exudation into polyp pseudocyst, with clinical evidence of rhinorrhoea in nasal polyps may be combated with enhanced lymphatic drainage seen in turbinates.

Although there is paucity of evidence of lymphangiogenesis in asthma, it is possible to speculate on a bidirectional relationship between tissue turgor, protein rich extracellular fluid exudation with rise in interstitial pressure and the combined effect of these on tissue fibrosis, and remodelling (681). Lymphatic dysfunction is demonstrated in conjunction with fibrosis in severe clinical situations where disturbed lymphatics cause clinical symptoms of lymphoedema (779). Within this context, Th2 derived cytokines were found to be crucial in disease development; abrogation of IL-13 and IL-4 prevented development of fibrosis and impeded the deterioration of lymphatic dysfunction. In further support of the inverse relationship between tissue fibrosis and lymphatic drainage, treatment with collagen decreased fibrosis and prevented development of lymphatic dysfunction in a mouse model (780), demonstrating that sound attempts at reinstating normal tissue/ extracellular matrix architecture will combat the development and the sequelae of exudation.

Within the context of nasal polyposis, polyps themselves, devoid of lymphatics and lacking in substantial vascular component, will be unable to effectively clear tissue exudate enhanced by localised MMP driven extracellular matrix degradation. With insufficient TIMP levels to counteract this change, and with VEGF levels comparable to normal tissues, there exists a persistent attempt at maintaining vasculature in these tissues. Increases in HSP-47, signalling enhanced collagen secretion serves to rescind fibrotic damage resulting from prolonged rise in tissue turgor. Conversely, turbinates of nasal polyp patients, possess enhanced vessels in order to aid drainage and maintain overall vascular supply. It is possible, that this increase in vessel formation may aggravate the increased extravasation, establishing an incomplete and faulty repair mechanism. The Th2 rich environment with concomitant rise in eosinophils is likely to further contribute to fibrosis, thereby impeding drainage of pseudocystic polyp tissue.

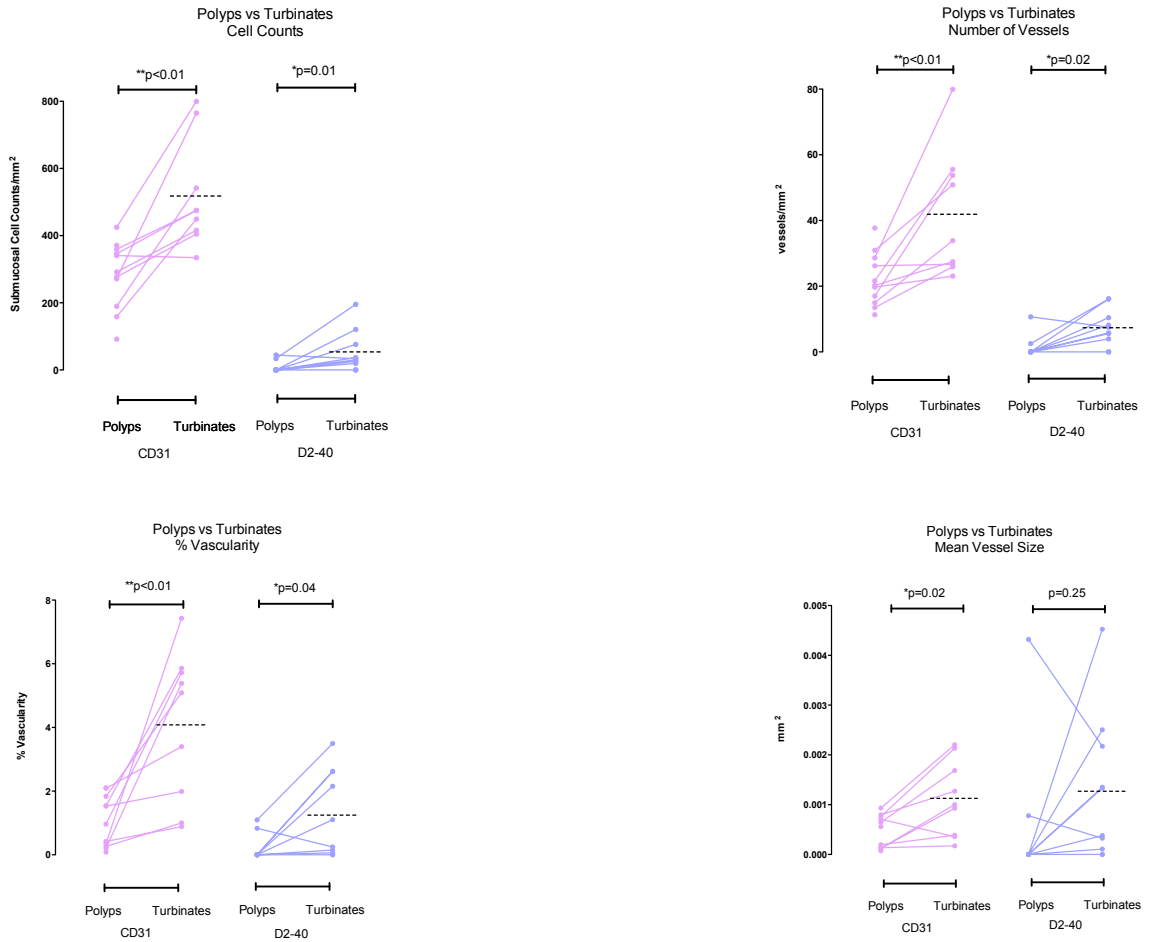


Figure 7.16: Comparison of lymphatic and blood vessels polyps versus turbinates of nasal polyposis. Paired samples were analysed using the Wilcoxon signed rank test. Significance was accepted as p<0.05.

TGF-β and family

TGF-β, activin, and ALK-4 signalling was markedly altered in nasal polyposis when compared to normal controls. ALK-4 was significantly more abundant in both polyps and turbinates, with polyps also demonstrating enhanced TGF-β. In addition, activin signalling was significantly higher in polyps versus turbinates in this unique upper airway disease. Following allergen challenge in asthma, CD3+ and SMA-α positive myofibroblasts strongly colocalise ALK4 (781). In the context of SMA-α differentials between turbinate and polyp tissue, it can be postulated that fibroblasts having undergone transformative change to a myofibroblast phenotype, now actively engage in activin regulatory signalling. In turbinate samples of nasal polyposis patients, increased SMA-α was coupled to an increase in ALK-4, replicating in polyposis, findings seen in asthma. With mast cell numbers constant, neutrophils are more likely to contribute to the activin rise seen in polyp tissue. Neutrophil numbers were increased in the turbinates of nasal polyposis patients, further influencing receptor levels, with increased ALK-4 in both polyp and turbinate tissue of nasal polyposis patients.

Increased TGF-β, activin and pSMAD2/3 are present in nasal polyp samples with the two signals showing significant correlation (737). In a murine explant model of lung morphogenesis, SMADs are necessary for lung branching (782). Different concentrations of TGF-β have distinct effects on nasal polyp fibroblast function, with lower concentrations enhancing proliferation and higher levels encouraging SMA expression in an *in-vivo* model (783). It may be postulated that different TGF-β levels will influence alterations of fibroblast phenotype in nasal polyposis in differing ways. Inflammatory cell derived TGF-β in the context of a Th2 cytokine rich milieu, affects collagen synthesis in polyps with enhancement of myofibroblast transformation in turbinates. Furthermore, enhanced activin signalling in polyps versus turbinates (p=0.08) will further drive pro-fibrotic fibroblast activity, possibly via IL-25 dependent mechanisms (784).

Alterations in localised and diffuse TGF-β will affect both the epithelium as well as the submucosa with the former playing an important role in new bud and possibly polyp formation, and the latter linking into collagen synthesis and attempts at resolution of inflammation and fibrosis. Within the epithelium, localised TGF-β signalling stunts epithelial growth, thereby forming a cleft with more pronounced growth on either side. Well established in lung budding and development, this specific role in polyp formation has not been shown. This study did not examine localised variations in epithelial TGF-β but it is possible to hypothesise that factors similar to those in lower airways disease may be playing their part. In the submucosa, TGF-β will rise in an attempt to balance collagen synthesis and thereby enhance fluid drainage from MMP endowed pseudocysts, all the while aiming but possibly failing to

completely abrogate fibrosis. Activin is likely to play an active part in this effect on polyp resident fibroblasts.

Within turbinates, increases in responsiveness to activin levels as seen by enhanced ALK-4, will facilitate fibroblast responses at this site. With increased SMA, it may be postulated that contractile forces, which may alter mechanical forces within the extracellular matrix and thereby enhance polyp TGF-β release restrict tissue growth and polyp size. This study demonstrates the ongoing and active signalling of TGF-β, activin and enhanced receptor level ALK-4 in both polyps and turbinates, with distinctive 'signatures' at each of these sites, further underscoring the unique nature of this upper airway disease.

Summary of chapter

Inflammatory cell infiltrate is persistent but varies in profile between polyp and turbinate samples of nasal polyposis patients with more pronounced infiltrate in polyps than turbinates. Polyps are rich in CD3 and CD4 positive cells. There was no difference in eosinophilic infiltrate in either polyp or turbinate samples of nasal polyposis participants. Both polyps and turbinates have significantly thinned basement membranes when compared to normal controls with such thinning more prominent in polyp tissue.

Within polyps, there exists evidence of increased collagen synthesis dovetailing into augmented MMP7 levels and a rise in TIMP-1. Increased collagen synthesis together with enhanced fibrosis is suggestive of attempts to stiffen polyp tissue. Tensile forces exert local influence during morphogenesis and embryonic development (758). Such focused alterations in biomechanical stress may similarly influence neo-polyp formation in this nasal disease (735). Attempts at abrogating such change were seen in the form of increased TIMP-1 which will counterbalance MMP mediated alteration in the extracellular matrix as well as possessing independent anti-angiogenic properties. In combination with TGF-β, TIMP-1 will work to limit the remodelling of polyp tissue topography, caused by and resulting in, marked increase in tissue/ pseudocyst exudate.

Polyps and turbinates differ markedly in their vascular, lymphatic and glandular structures. Polyps are near devoid of any lymphatics and glands and possess very little vascular content. The vessels that are present are VEGF-a positive, indicating possible reliance on such signalling to maintain vasculogenic drive. Increased vascular and lymphatic markers in turbinates (when compared to normal controls) could support a reduction in tissue turgor and limit fibrosis. Upregulated TGF-β signalling would also affect myofibroblast transformation in turbinate tissue. With increased SMA in turbinates versus

polyps, and increases in turbinate ALK-4 expression, it can be postulated that fibroblasts having undergone transformative changes to a myofibroblast phenotype, actively engage in activin regulatory signalling.

The summation of these changes will be crosstalk between fibroblasts, epithelial and inflammatory cells causing MMP and TGF-β expression to effect collagen synthesis. Polyps may serve as the 'engines' that drive long-term vascular and lymphatic remodelling changes in turbinates with concomitant TGF-β expression. Albeit insufficiently, turbinate tissue in turn will attempt to limit destruction of tissue architecture within polyps (785). With distinctive cellular and histopathological signatures in polyps and turbinates, remodelling is a persistent and longstanding change in this unique upper airway disease providing a positive control for the study of remodelling in the nose.

Chapter 8 : Safety & Tolerability of RAC protocol

Introduction

In this chapter the safety, patient acceptability and clinical sequelae of four consecutive turbinate biopsies with nasal lavage is presented.

The nose as a highly accessible organ can be biopsied with relative ease. Early surgical treatments would not always recognise the importance of maintaining functional anatomy and physiological integrity of the nasal mucosa. With the advent of endoscopic sinus surgery greatly aiding detailed visualisation of the post-operative healing nose, the effects of mucosal loss are better appreciated. In the context of sampling nasal mucosa for research, Fokkens has demonstrated a safe method of nasal tissue biopsy with minimal epithelial disruption using a tissue forceps bearing her name (786). These are similar to angled cup forceps used in middle ear surgery, with sharp biting action, resulting in high integrity biopsy specimens. The non-intrusiveness of this method is such, that it is capable of preserving the fluid layer overlying nasal epithelium (787). Gerritsma forceps which are also an angled biting cupped instrument, will produce similar results (788). Since then, the Fokkens forceps (or alternatives in the form of endoscopic biopsy forceps) have been widely used in research. Pain and epistaxis are the most troublesome possible clinical consequence of a single nasal biopsy. To date, no study has looked at the feasibility of 4 consecutive nasal biopsies, possible side-effects and non-troublesome clinical symptoms.

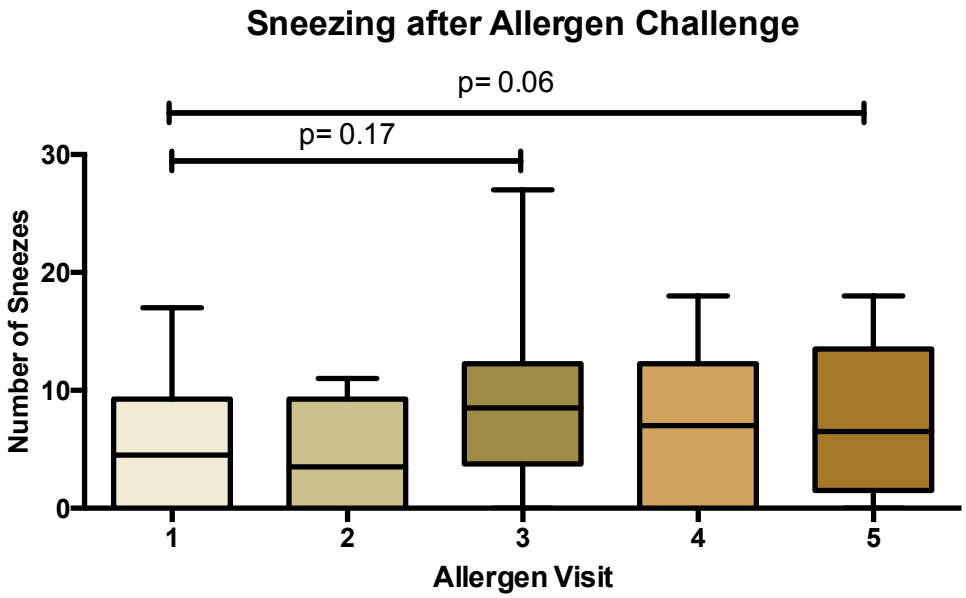
For this RAC protocol to be adopted as a sound model of allergic rhinitis, it must be easy to carry out whilst being acceptable to participants.

Results

Data is presented on ten participants. Each underwent 4 biopsy visits with each visit consisting of one nasal lavage and 3 biopsies on the ipsilateral side.

Clinical response to multiple nasal allergen challenge

Allergen challenge elicited a strong immediate hypersensitivity reaction clinically demonstrated by rhinorrhoea and sneezing (Figure 8.1). The number of sneezes and the weight of secretions in the first 30 minutes of allergen inhalation did not show any statistically significant change with repeated allergen administration over the course of five challenge visits, although both seemed to demonstrate increased clinical priming at third visit ($p=0.17$ for sneezing and 0.06 for rhinorrhoea), findings reflected by the subjective Total Nasal Symptoms Scoring and Visual Analogue Scales.



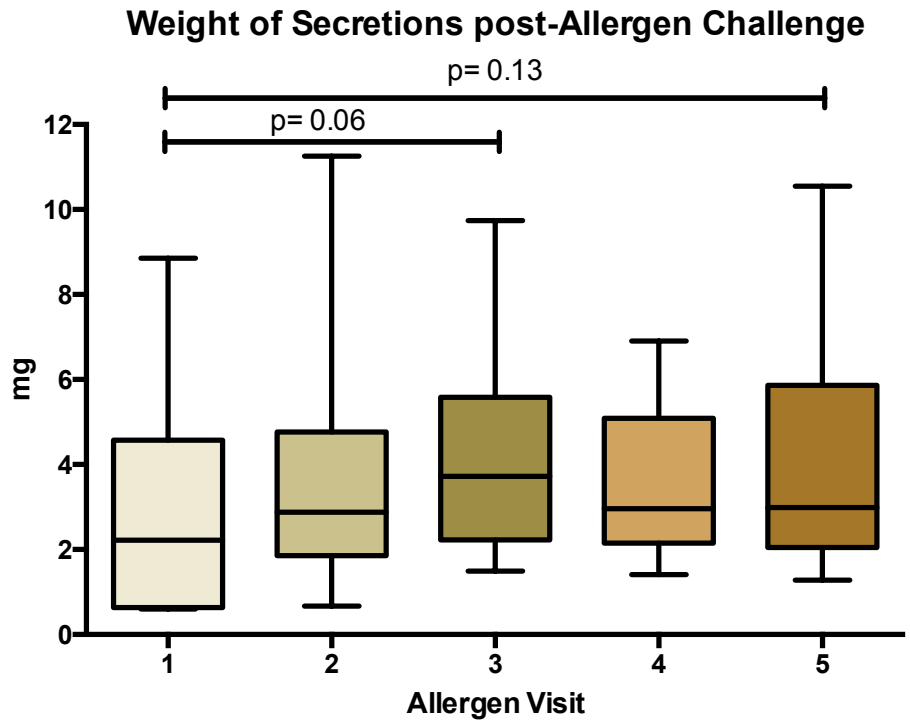


Figure 8.1: Symptoms of sneezing and anterior rhinorrhoea post allergen challenge at 5 allergen challenge visits. Multivariate analysis was via Kruskal Wallis test, with comparison between 1st and 3rd challenge visits via Mann-Whitney test.

Objective versus subjective measurements of clinical response and complications

Visual analogue scale reported pain scores were low at every visit (highest median=2 on the last visit, lowest median=1 on visit 2 and 3, with maximum score of 5). Pain at time of biopsy did not increase with each visit (Figure 8.2). In the twenty-four hours after each visit, the pain experienced more than doubled when compared to pain at the time of biopsy itself but showed no statistically significant deterioration as the study progressed (highest median=4 on the 4th/ last visit, lowest median=2.5 on visit 3, with maximum score of 9). Variability of pain scores was greater in the 24hrs post biopsy when compared to pain scores during the biopsy itself. The first 24 hours were the worst in terms of pain experienced (median=1 day for all visits except visit 3 where median=12 hours). 'Worst day' related to pain experienced due to the biopsy. Many head and neck procedures will result in pain scores that are worse several days after surgery as opposed to immediately after (789).

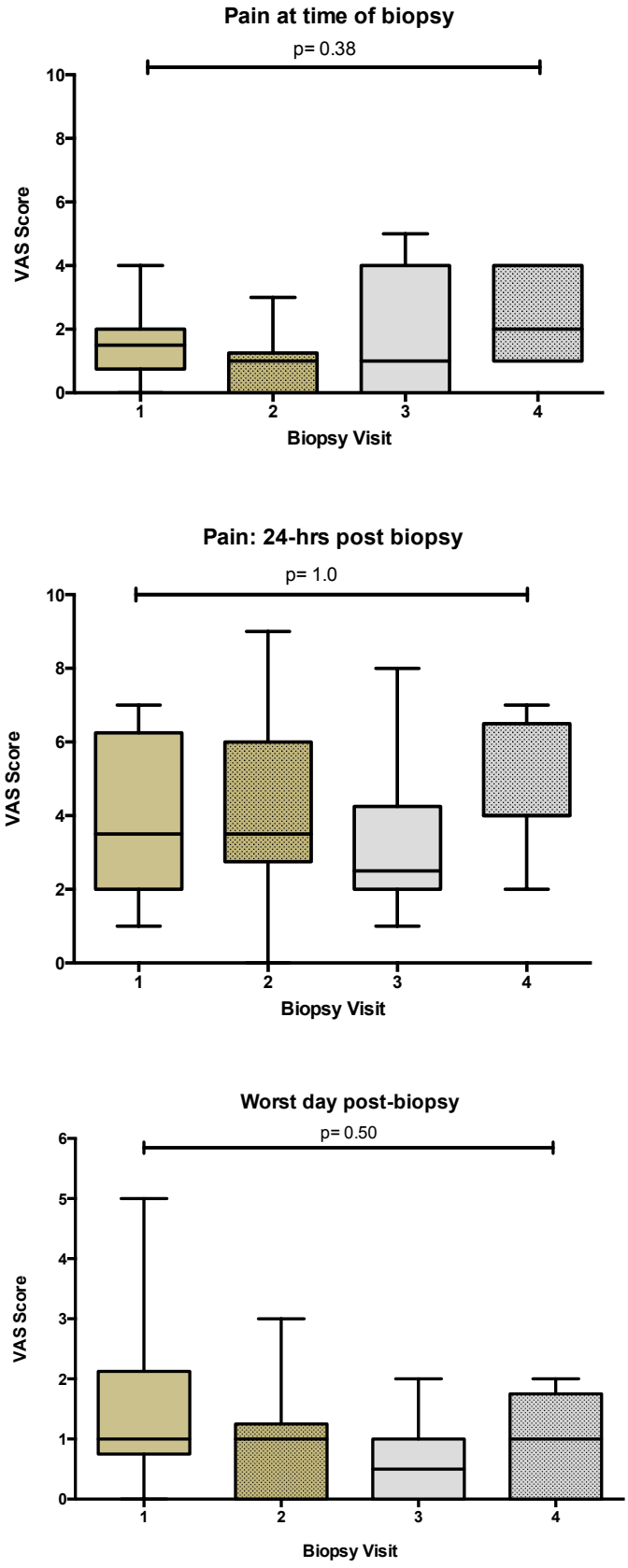


Figure 8.2: Assessment of pain using Visual Analogue Scale (VAS) score of 1-10: during biopsy, 24 hours after and week following biopsy. Differences were analysed using Mann Whitney test between 1st and 4th biopsy visit

Pain and post-procedure recovery

Participant reported anterior rhinorrhoea and obstruction showed no increase with study progression (figure 8.3). Anterior rhinorrhoea was marked with highest score (median=6) at the last visit. Although not statistically significant, nasal obstruction was also highest at the last visit (median score=7). Time taken to fully recover from the biopsy did not increase with study progression (highest median=6 days after the first visit). The longest reported time to full recovery was 13 days following the last visit. Three biopsy episodes resulted in a post procedure bleed. All settled on phone advice using conservative measures in keeping with standard clinical epistaxis advice. There were no other complications or adverse events associated with 4 consecutive biopsies.

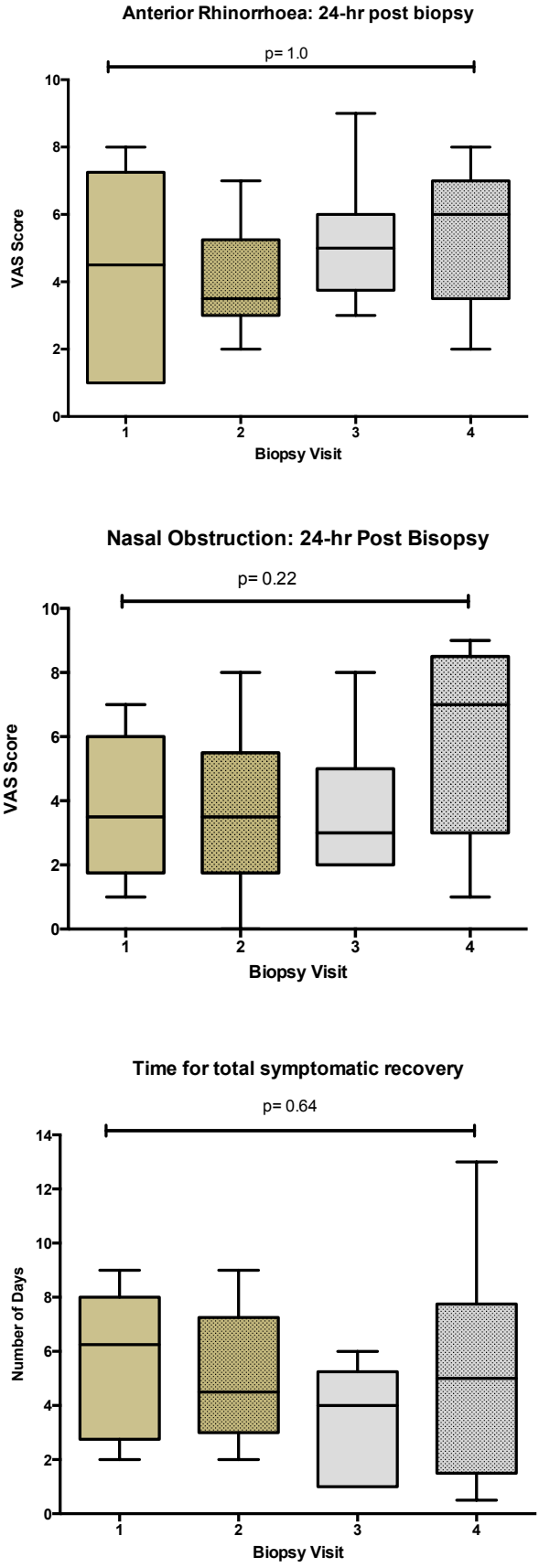


Figure 8.3: Symptoms post-biopsy: anterior rhinorrhoea, nasal obstruction and time to full recovery. Differences were analysed using Kruskal Wallis test. Differences were analysed using Mann Whitney test between 1st and 4th biopsy visit.

Discussion

This study demonstrates that four consecutive nasal biopsies, each preceded by a local allergen challenge, over a two-week period are safe, sustainable and well tolerated. Such a repetitive allergen challenge protocol was not associated with increased morbidity over time and resulted in only three instances of self-resolving epistaxis in the few hours post biopsy. This RAC protocol was validated by the increased inflammatory cell recruitment at 24 hours after each allergen (versus diluent) challenge. Such histopathological evidence of allergen response dovetails into significant enhancement of clinical responses as measured by Total Nasal Symptom Scoring and Visual Analogue Scale. The allergen threshold dose was selected by the criteria of dose producing five or more sneezes and 2mg or more of nasal secretions and this positive clinical response was sufficient to elicit inflammatory tissue changes. This will provide more reliable criteria than studies using a combination of clinical symptoms (590) (790). This RAC study design validates a longitudinal allergen challenge model which can be used to study inflammatory changes with concomitant alteration in some signalling molecules of remodelling such as the TGF-β family. In addition, validation of staining methods provides tested procedures for future studies examining remodelling markers as demonstrated in nasal polyposis turbinate samples.

The use of TNSS and VAS scoring in this study demonstrate the ability of these, and the Quality of Life questionnaire to be used as an appropriate tool for accurately recording severity and duration of disease as per the ARIA classification (791). In this study we used both to assess clinical responsiveness to repetitive challenge, with both TNSS and VAS demonstrating sound reproducibility with objective measurements of weight of secretions and number of sneezes. Weight of secretions showed a priming effect between first and third visits, with number of sneezes also increasing at these time points. The data in this study demonstrated both subjective and objective measurements of priming. As no biopsies were taken after the third challenge, no comment can be made regarding cellular inflammatory response. Nonetheless, enhanced allergic cellular recruitment links in with subjectively worsening symptoms as measured by TNSS and VAS.

As quality-of-life questionnaires such as the Juniper mini RCQLQ assess past symptoms they cannot be used to assess clinical response. Assessing the relevance quality of life questionnaires during the seasonal portion of this study did demonstrate correlation with TNSS and VAS and validated the use of these symptomatic assessment tools in rhinitis research both during natural and induced allergen challenge studies.

There are no human studies in the literature utilising longitudinal repetitive allergen challenge models with repeated nasal biopsies. Previous studies using more than one nasal biopsy examine single allergen challenge models (176). With the ability to not just quantify but to localise cell types and to present the overall topography of tissue pathology, full thickness tissue biopsies are the gold standard in the study of nasal allergy. Although cell numbers between brushings and biopsy do correlate (792), biopsy is superior to nasal smear in identifying inflammatory infiltrate (793). Method and site of nasal tissue/ secretion sampling influences cytokine and cellular profile yield. Superficial sampling reveals a highly neutrophilic picture whilst deeper tissue sampling such as curettage or cytobrushing which extend to the submucosa encompass eosinophilic and mast cell fractions (794) (795). This study of longitudinal repetitive allergen biopsies following repetitive nasal allergen challenge to mimic allergic rhinitis, was well tolerated with two participants withdrawing after the first biopsy visit. Epistaxis is a common occurrence after all nasal trauma, iatrogenic or otherwise, and with three participants experiencing self-limiting episodes, this model of induced allergic rhinitis produces minimal morbidity. The biopsy experience itself caused little pain with quick recovery after the initial 24 hours post visit.

Summary of chapter

This chapter summarises hitherto unreported repetitive nasal biopsies linking in with repetitive nasal allergen challenge. Four nasal biopsies with concomitant nasal secretion collection, following on 24 hours from nasal allergen challenge, was well tolerated and lead to high quality tissue and fluid samples in persistent severe allergic rhinitics. By obtaining more than one biopsy at each biopsy point, we ensured differently preserved tissue samples were in our armamentarium for future tissue stains. This is the first study of its kind demonstrating multiple biopsies at each visit, performing multiple allergen challenges and repetitive nasal tissue and fluid samples in the most severe clinical group of allergic rhinitics. Such a model authentically reproduces clinical findings of natural pollen exposure.

Chapter 9 : General Discussion

One of the main aims of this study was the development of a human model of induced allergic rhinitis via repetitive allergen challenge, to establish and confirm the presence and the degree of remodelling in allergic upper airway disease. Developing a well-tolerated method of repeated sampling following allergen challenge, was vital for accurate assessment of histopathological changes. No murine model could faithfully replicate a heterogenous group of nasal diseases such as allergic rhinitis and chronic rhinosinusitis with nasal polyps, not least as no established animal model for CRS exists. Development of a human model that is able to reproduce the disease and provide a conduit for therapeutic intervention is vital for future development of treatment modalities. Establishing the presence and degree of nasal remodelling using both natural and induced allergic rhinitis and comparison with chronic rhinosinusitis with nasal polyposis, allows for a deeper understanding of these two different disease states. Whilst sharing some key Th2 inflammatory type signatures, they vary vastly in their remodelling profile.

This study demonstrates the feasibility of longitudinal nasal challenge studies and the tolerability of multiple biopsies over a two-week duration. Although more demanding than the single allergen challenge model, it may provide a more faithful reproduction of the pollen season. Investigative in nature, it presents a template for use in studying therapeutic interventions. It potentially allows for future detailed study of cellular responses to prolonged allergen exposure, in particular for examination of dendritic and T cell profiles. Meticulous patient profiling and selection, careful preparation pre-biopsy, close monitoring with dedicated aftercare allows for prolonged allergen challenge with multiple longitudinal sampling and for multiple samples at each time. Collection of nasal secretions facilitates quick, accurate and economically viable cytokine profiling. A repeat study might look at cellular and cytokine response half- way through this model, i.e. at day 5 when priming of the early phase was at its most marked. Such a study would allow for closer examination of the priming process and in particular would be useful to look at the involvement of inflammatory, dendritic and T regulatory cells with examination of the TGF-β family at this intermediate time point. The study also demonstrates the importance of site sampling in nasal polyposis and presents the fullest picture to date of remodelling in nasal disease.

This study answers the overarching question of whether remodelling exists in the upper airways and if so, under what conditions and to what extent. Whilst remodelling does indeed manifest in the nose, it will do so to differently and to variable extents as shown by data in CRSwNP and allergic rhinitis. It has previously been shown that CD4+, but not CD45+ or CD8+, increase with rise in neutrophils and eosinophils after single allergen challenge, but the loss of epithelial integrity, and epithelial thinning

are absent in allergic rhinitics following allergen challenge (156). Remodelling hallmarks as seen in asthma, with basement membrane thickening, goblet cell hyperplasia, epithelial discontinuity, subepithelial fibrosis and new endothelial vessel formation, are not a feature of severe persistent allergic rhinitis as demonstrated comprehensively for the first time by both induced and natural models of exposure. In a unique study of polyp and turbinate biopsies from the same CRSwNP individuals remodelling was clearly manifested differentially with polyps possibly driving remodelling in the turbinates. In both allergic rhinitis and CRSwNP, TGF-β and activin levels and their signalling was dysregulated with alterations at both receptor and intracellular level.

Immunohistochemistry is a semi-quantitative method of analysis and is an inherent weakness of this study. In addition, no functional data is available to support the alterations in remodelling markers/ TGF-β signalling seen in nasal polyps and allergic rhinitis. Notwithstanding the above, a comprehensive picture of remodelling within the upper airways was demonstrated. Sampling of natural allergen exposure after the very peak of the pollen season allowed for investigation of both inflammation and remodelling as it occurred. By using established severe persistent allergic rhinitics, as defined by ARIA, the study utilised highly mono-sensitised individuals to challenge out of season. There was no difference in epithelial and submucosal mast cell numbers between natural and induced models and such cells were primed for effective degranulation at modest allergen loads. In both instances, either during the season or after 10 days of repetitive allergen challenge, mast cells would be coated in IgE and would be highly responsive to crosslinking induced degranulation. This was evidenced by excellent clinical symptom severity scores during both protocols. During natural seasonal exposure, pollen counts are variable and despite some peaks, do provide occasional respite from high allergen load. Above threshold dosing ensures repetitive allergen induced rhinitis adequately and longitudinally stimulating the allergic response over a number of weeks with evidence of priming. In both cases robust Th2 cellular infiltrate was seen with more marked responses in the induced model. Both groups saw reliable Th2 cytokine secretion. TGF-β and activin signalling was dysregulated in both natural and induced models with representations of variable responses likely to be determined by temporal influences as well as the absolute and total allergen load over time. Natural seasonal exposure saw a coupled decrease of submucosal TGF-β with similar changes in SMAD2 without concomitant alteration in activin or ALK-4. Induced repetitive challenge produced significant increases in TGF-β, activin, ALK4 and SMAD2. It can be postulated that these temporal alterations were in response to the magnitude and duration of allergen exposure, with prolonged and supra-thresholds manifesting as persistent elevations in TGF-β and activin signalling. The regulatory role of activin will be manifest in similar levels in allergic rhinitics and non-atopic individuals. Activin was seen to rise quickly after inflammatory insult and needs ongoing stimulation for sustained elevation. TGF-β increases slowly and then wanes to

baseline levels with possible overcorrection of regulatory pathways once allergen load is negligible. With variable type I receptor signalling, alterations in TGF-β and activin will manifest a pleotropic immunohistopathological picture of ALK and pSMAD response. Elevations of both were seen with repetitive exposure with waning of SMAD2 in line with a drop in TGF-β in natural seasonal exposure. This study demonstrates the dysregulation of TGF-β, activin ligand ALK-4, and signalling pathway components in nasal allergy by outlining alterations in response to disease activation via either natural or induced allergen challenge.

This thesis offers unique and novel insights into the remodelling changes seen in nasal polyposis, it notes how these differ between polyp and turbinate samples from the same individual and how these findings are related collectively to the nasal mucosa of allergic rhinitic and normal non-atopic control individuals. Epithelial integrity is maintained throughout the nose in CRSwNP despite the fact that the basement membrane is markedly thinned. In keeping with findings seen during lung morphogenesis and bud formation, thinning of the basement membrane putatively allows for invagination and new polyp formation. Airway wall thickening as is seen in asthma, offers protection from bronchial hyperreactivity and may prevent sudden and potentially fatal responses in asthma. Ruching of the airway epithelium with thickening of the airway wall may also be protective in lower airways disease but is not relevant in the nose. Clinically, childhood wheezing following HRV infection is a strong predictor of asthma at 6 years (796) demonstrating relevance of airway function to airway pathology later down the line. Remodelling in asthma will lead to progressive loss of function (797). Using high resolution CT and ultrasound bronchial probes, bronchial hyperresponsiveness (BHR) was found to be inversely related to airway thickness (798) (799). This may lead to unopposed muscular airway constriction and lead to brittle asthma and death. Such protective basement membrane thickening has the deleterious effect of chronic irreversible airway obstruction (800). These dynamic alterations are absent in the nose where polyp growth is unrestricted by airway diameter and where thickening of the basement membrane confers no improvement in clinical symptoms. Indeed, the basement membrane of polyps was significantly thinner than that of turbinate samples in CRSwNP and in both cases thinner than that found in normal non atopic control turbinates. Pseudocysts in polyps exist at the expense of glands, lymphatics and almost complete absence of blood vessels. VEGF may play an integral part in the maintenance of overall vessel integrity, as there are virtually no VEGF negative blood vessels in polyps.

There is evidence of persistent collagen production in polyps with the presence of myofibroblasts in both turbinates and polyps. It is not clear whether these transform from resident fibroblasts or are the product of influx from circulating CD34+ cells. Colocalising CD34 with SMA-α may answer this

question. Such segregation of upregulation of collagen and myofibroblast induction hints at interconnected but individual changes in polyps and turbinates. Blood and lymphatic vessels are increased in turbinates. Enhanced MMP signalling in polyps with concomitant increase in TIMP1 (and decrease in MMP:TIMP ratio) in turbinates facilitates the continuous alteration of polyp architecture with tissue growth and expansion. Increases in extracellular matrix turnover via MMPs, thinning of basement membrane, and increased collagen production may stiffen the expanding polyp. These mechanisms may exert biomechanical forces, which could cause release of extracellular TGF-β. TGF-β might be released from its latency binding peptides at lower ECM stiffness than is required for *de-novo* α-SMA synthesis, i.e. in response to tensile forces TGF-β precedes myofibroblast differentiation (376). This would support the idea that polyp derived TGF-β is driving alterations in the fibroblast phenotype in turbinates. Allergen and biomechanical stress will not be the only factors affecting TGF-β signalling. It has previously been shown that TGF-β and activin with increased evidence of SMAD2/3 mediated signalling, are present in nasal polyp tissue at baseline and further enhanced by noxious stimuli such as cigarette smoke in chronic rhinosinusitis (737). Release of active TGF-β with a concomitant rise in activin could abrogate some of the above changes associated with polyp formation. It is possible to postulate that polyp produced TGF-β and activin will affect remodelling changes manifest in turbinate tissues of CRSwNP. This would make polyps the engine that drives remodelling in turbinates of nasal polyposis. This may explain the fundamental differences between chronic rhinosinusitis with (CRNwNP) or without polyps (CRNsNP) both in terms of clinical picture as well as cytokine and cellular profile. (801).

Although histologically allergic rhinitis and CRSwNP share many similarities this thesis underscores some important differences. With increased epithelial and submucosal TGF-β following two weeks of induced versus natural allergen exposure, enhanced activin, ALK4 and SMAD2 expression, a tendency towards EMTU activation was present in the upper airways. Signalling was short lived, quickly returning to baseline with even relative respite in allergen load. In asthma, the remodelled airway is a chronic finding with no abrogation of topographical changes, despite differences in clinical phenotype. Crucial differences between the nose and lung exist. The lung is continuously involved in changing expansile and contractile dynamic forces, whereas the nose undergoes very limited alterations in the form of intra-luminal vascular vessel volume. In lower airways disease, biomechanical strain in the form of bronchoconstriction, is able to effect remodelling changes in isolation without the need for eosinophil recruitment (627). Thickened airway walls may serve a protective mechanism against smooth muscle forces (802). Different anatomic airway sites respond in variable degrees to both active and passive contractile forces with smaller lower airways seemingly being most sensitive (803). There is complete absence of circumferential smooth muscle layer in the

nose and lack the smooth muscle resident mast cells which provide a vital aspect in drive to remodelling. With these two main differences, it may be postulated that EMTU activation in the nose will be less robust when present, and less persistent overall, than in the lower airways. This thesis supports such a hypothesis as evidence of epithelial TGF-β driven signalling does exist but with decrease in allergen load over time, it wanes relatively quickly.

An increase in epithelial TGF-β is an allergen specific response and not witnessed in nasal polyps, either in polyp or in turbinate samples. As epithelial TGF-β signalling drives epithelial mesenchymal trophic unit activation, TGF-β mediated changes will be initiated and localised to the submucosa in polyps. Effects of TGF-β signalling, balancing pro-inflammatory, fibrotic and suppressive tendencies are determined by the nature of the insults (e.g. allergic or mechanical), the site of secretion, the target cell affected and the duration of damage. In allergic rhinitis, activation of EMTU will take place at sites of mucosal injury where the epithelium may bear the greatest injury. On these occasions, alteration of epithelial architecture and signalling to the submucosa are essential communicating pathways to effect protective change. In contrast, nasal polyps are intrinsically predisposed to alteration in submucosal topography, with concomitant mechanical shearing forces. Release and activation of TGF-β in these instances is more likely to result in epithelial-mesenchymal transition, as opposed to EMTU activation, and further alteration of tissue architecture which still manifests as remodelling. These two different disease states result in fundamentally different manifestations of TGF-β activation and signalling. Similarly, the multifaceted nature of tissue responses to activin A is likely influenced by tissue type, number of allergen challenges, and the type of provocation where OVA or HDM/ grass stimulation affect varies in its TLR initiated epithelial response (804). Effects of activin on alterations of T helper cell tissue profile is likely to be dose dependent as well as on prevailing cytokine conditions; for example relatively lower concentration of TGF-β, in the presence of activin, drives Th9 cell differentiation in IL-4 co-stimulated explant murine model of CD3+CD4+ Tcells (805) which in turn may stimulate airway mast cell recruitment *in vivo*.

Combinatorial interaction of TGF- β with ALK2 receptor is implicated in epithelial to mesenchymal differentiation (806). With activation of both TGF-β and activin pathways, switching of TGF-β to ALK-1 signalling may prevent depletion of common SMAD2/3 and allow for simultaneous and possibly synchronous effects. Following allergen challenge CD3+ and SMA-α positive myofibroblasts strongly colocalise ALK4 (781). In the context of differences in SMA-α positive cell numbers between turbinate and polyp tissue, it can be postulated that fibroblasts having undergone transformative change to a myofibroblast phenotype, now actively engage in activin regulatory signalling. Due to variability in receptor/ ligand interaction, expression of SMADs may not change in the face of enhanced TGF-β

receptor activity (481). Whilst TGF-β may work synergistically with Th2 cell derived cytokines to enhance feedback eosinophil recruitment (723), any subsequent collagen deposition (TGF-β augmented, or otherwise) may not differentiate between severity of airway disease. In other words, degree of collagen deposition does not seem to correlate with degree of airway hyperreactivity (677) in the lower airways. As such, collagen deposition may contribute little to upper airway clinical severity. It can be postulated that instead of contributing to deteriorating symptoms in the nose, enhanced collagen deposition, particularly considering upregulated MMP function, is needed to limit clinical severity by attempting to abrogate unchecked polypoid expansion.

The study protocol presented here provided an optimum balance between duration of allergen exposure and dose, driving allergic inflammation, and bringing forth altered TGF-β signalling in upper airways (807). Priming in the context of induced models of allergic rhinitis, is likely to need prolonged provocation. One week of exposure may not be sufficient (609), with mouse models needing 3 weeks. In human models of asthma, three challenges 48 hours apart were insufficient to elicit evidence of priming (808). Our model provides an optimal study duration with Th2 signature inflammation, activation of remodelling signalling pathways whilst keeping faithful to histopathological and topographical changes seen in allergic upper airway disease.

Different modalities of nasal tissue sampling have been utilised in the past. Nasal lavage cytokine levels coupled with nasal symptom scoring provides an accurate benchmark of Th2 driven inflammation (809). The protocols presented in this thesis support the validity of nasal cytokine measurement as a rapid, reliable and reproducible mechanism of establishing secretory cytokine profile using the MesoScale Device.(810-813) More recently there have been advances in nasosorption techniques such that CE marked, sterile and individually packaged medical grade kits are commercially available by Hunt Developments (Midhurst, UK). These include a handle/lid, SAM and tube that are manufactured in clean rooms with no allergen and no LPS and have been quality controlled for absorption and elution. Hansel *et al* amongst others has demonstrated its use in grass pollen allergic rhinitis and asthma. Future uses of this SAM may extend to measuring complement, coagulation and fibrosis.

Comparison of eosinophil and mast cell populations by cytobrush (absolute cell numbers) and nasal secretions (ECP and tryptase) in allergic rhinitis, and chronic rhinosinusitis with or without polyps, demonstrates increases in secretion markers with ECP higher in nasal polyps (versus both allergic rhinitis and chronic rhinosinusitis without polyps) and tryptase higher in allergic rhinitis (vs CRS and controls) but with neither marker correlating with a rise in their respective inflammatory cell counts

(182). Furthermore, eosinophil counts vary depending on nasal site with inferior turbinates containing less than middle turbinates but more than the septum (182). Inflammatory granulocyte secreted proteins may show a rise in secretions without evidence of corresponding rise in cellular profile. This may be due to site differences during nasal sampling (with some showing less variation between disease states) or due to degranulation during collection and staining (814). The full thickness nasal biopsy method utilised in these studies is the gold standard for understanding whole tissue changes, the relationship of cells to other structures, and is ideally placed to help us understand the interplay between allergic inflammation and remodelling.

Possible future work related to this thesis would include the study of other TGF-β family members in allergic rhinitis and CRSwNP. Evaluating how other signalling pathways are altered will be crucial in fully understanding the dysregulated TGF-β family signalling in nasal remodelling, particularly as it pertains to polypoid disease. Special focus of investigation should include BMP-7, which plays an anti-fibrotic role in asthma (742) (815). Co-localisation with eosinophils which are the main cellular source of BMP-7 in asthma will help elucidate its role in the nose. Upregulation of ALK-4 in allergic rhinitis and in nasal polyposis and indeed in prolonged induced allergen exposure dovetailed into changes seen in activin levels. TGF-β is more prevalent in polyps and post repetitive allergen challenge reflecting alterations seen in SMAD2 levels. Collectively this suggests that in nasal disease, ALK4 but not pSMAD2 responses are activin driven, partially replicating findings in asthma (735). It hints at preferential signalling of TGF-β through ALK-5 which would utilise intracellular SMAD2. Establishing relative ALK1 and ALK5 levels will further elucidate the alterations in TGF-β and activin signalling in the upper airway.

Further study could focus on the interplay between dendritic and T cells. Dendritic cells play a crucial role in the sensitisation and maintenance of the allergic phenotype, determining sensitisation or tolerance after repetitive allergen challenge in mice and men (816). In allergic rhinitics, dendritic cells favourably enhance Th2/Th17 differentiation but have abrogated Th mediated IL-10 secretion or intrinsic IFN-α synthesis both locally but also in the systemic circulation (817). TSLP stimulated myeloid dendritic cells are phenotypically distinct in their cytokine profile to those stimulated without a TSLP rich environment. A TSLP rich milieu enhances eotaxin-2, IL-8 and TARC secretion versus the TNF-α, IL-6 and IL-1b secretory cytokine profile of non-TSLP stimulated dendritic cells (818). Genome wide meta-analysis studies suggest correlation between allergic rhinitis and TSLP variants (254) whilst expression of TSLP is increased in nasal polyps with chronic rhinosinusitis (255). High IL-17 in murine model of asthma is associated with neutrophilic, eosinophil poor and steroid resistant asthma (660). Examining the interplay between nasal epithelium, dendritic and T cells and priming in the context of

TSLP, IL-33 and IL-25 mediated responses will better elucidate the role of the nasal epithelium in orchestration of Th2 driven allergic and remodelling responses via epithelial and T cell cross talk.

A regulator of G1 cyclins, $p21^{waf}$ prompts cell cycle arrest and is enhanced by TGF-β, and is found in asthmatic airway epithelia and may form an aspect of EMTU activation seen in lower airways disease (819). With no rise in epithelial TGF-β, intact and robust epithelial turnover is present in allergic rhinitis, as following initial loss with expulsion of goblet cell contents, there is complete restitution of epithelial integrity. It would be helpful to further define cell cycle changes in polypoid nasal mucosa and allergic rhinitis given there are no alterations in epithelial integrity. Clinical symptoms of persistent severe allergic rhinitics can on occasion continue out of the season with little to no allergen exposure. Further examination of neurologic causes of nasal hyperreactivity and its relationship to allergic rhinitis in particular will improve our understanding of both conditions (820) (821) . The areas outlined above will further improve our understanding of the allergic upper airway (822) and provide future investigative directions.

In summary, this thesis demonstrates that allergic inflammation does not *ipso facto* drive remodelling in the upper airway. Such allergic inflammation needs to be coupled to mechanical distention with contraction of tissue architecture and be persistent in its duration to affect histopathological change. Despite sustained symptoms, periods of quiescence are likely to provide sufficient relief to abrogate remodelling in allergic rhinitis. There exists a subjective alteration of perception of clinical severity as can be seen with chronic disease with possible persistence of neuronal pathways and neurogenic inflammation. As seen in asthma, inflammation and remodelling are uncoupled (823) in the upper airways (824). This study demonstrates the link between an allergic inflammatory infiltrate, altered tissue vasculature with oedema and extravasation, changes in extracellular components, partly mediated by MMP dysregulation and the altered TGF-β signalling pathways and how these coalesce to affect remodelling in nasal polyposis and altered TGF-β family expression in allergic rhinitis without alterations in tissue topography. This thesis comprehensively outlines the changes seen in both nasal pathologies (allergic and polypoid) and begins to paint a picture of these individual pathologies. It further provides a sound, reliable and reproducible protocol for the study of therapeutic interventions in allergic rhinitis. Collectively this thesis provides a unique and novel insight into the remodelling changes characterising upper airway disease.

Chapter 10 : Bibliography

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