Rhinology, 35, 22–27, 1997

Stereological estimation of blood vessel surface and volume densities in human normal and rhinitic nasal mucosa*

Don-Carlos Abrams[1], Stephen C. Toynton[2], Caroline Doré[3], Marie A. Emson[1], Peter Taylor[4], David R. Springall[1], Neil Pride[2], Jane Weston[1], Robert C. Schroter[5], Julia M. Polak[1]

[1] Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, London, United Kingdom
[2] Department of Respiratory Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, London, United Kingdom
[3] Department of Medical Statistics, Royal Postgraduate Medical School, Hammersmith Hospital, London, United Kingdom
[4] Department of Otolaryngology, Central Middlesex Hospital, Park Royal, London, United Kingdom
[5] Centre for Biological and Medical Systems, Imperial College, London, United Kingdom

SUMMARY

A technique is proposed for applying well-established stereological methods to study fixed nasal biopsy material to obtain an unbiased estimate of blood vessel surface and volume densities. Biopsies of the nasal mucosa from the anterior 10 mm of an inferior turbinate were obtained from 18 subjects (15 males, 3 females with a mean age of 28.5 years [range: 17-54 years]), ten of whom had perennial allergic rhinitis, and eight control subjects. The mucosal tissue volumes were estimated by water displacement. Zamboni's-fixed cryostat sections (10 μm thick), stained with haematoxylin and eosin, were examined histologically. Computerised images of randomly selected tissue sections were analysed with point-counting and intercept-counting techniques to determine large blood vessel volume and surface densities, respectively. There were no significant differences between the volumes of tissue analysed from the control and rhinitic subjects (p=0.35). The average volume density of the vessels was similar in the control group (6.17±1.41%) and the rhinitic group (7.8±5.59%; p=0.38), but with a greater variability in the rhinitic group. Surface density estimations were 3.14±0.74 mm^{-1} in the control group and 3.10±1.41 mm^{-1} in the rhinitic group. Therefore, on average, the volume and surface densities of the cavernous blood vessels in rhinitis were unaltered and there was no evidence of vascular remodelling.

Keywords: rhinitis, stereology, point counting, intercept counting, OpenStereo

INTRODUCTION

The nasal mucosa has an extensive system of cavernous vessels whose distension is controlled by throttle vessels (Widdicombe, 1994). Engorgement of these capacitance vessels reduces nasal patency and increases nasal airway resistance. In conditions such as the common cold or seasonal allergic rhinitis, release of histamine and other mediators leads to engorgement which is obvious on rhinoscopy and reverts to normal when the viral or allergic stimulus is no longer present. In subjects with chronic rhinitis, whether due to persistent allergen exposure (as with house dust mite) or without obvious allergen exposure, there is frequently chronic enlargement of inferior turbinates. Treatment with topical corticosteroid sprays may return appearances to normal, but many subjects either relapse quickly after stopping treatment or obtain no relief and require surgery to the inferior turbinate in an attempt to improve their symptoms.

The vasculature of the human nasal mucosa has been qualitatively studied (Kohlrausch, 1853; Dawes and Prichard, 1953; Cauna, 1982; Passàli et al., 1991), but it is not known whether the large cavernous capacitance vessels are altered in perennial

* Received for publication February 13, 1996; accepted August 20, 1996

rhinitis. In this study we apply stereological methods to obtain an estimate of large blood vessel density in nasal biopsies taken from subjects with chronic perennial rhinitis and, for comparison, biopsies from subjects undergoing nasal surgery for correction of anatomical abnormalities but without rhinitis.

MATERIAL AND METHODS

Subjects

Biopsies of the anterior end of an inferior turbinate were obtained, by the same surgeon (P.T.), under general anaesthesia in 18 subjects (15 males, 3 females) with a mean age of 28.5 years (range: 17-54 years). All were never smokers and had not had previous nasal surgery. Ten subjects had perennial allergic rhinitis (PAR) with variable nasal obstruction, rhinorrhoea, sneezing and nasal irritation present for more than 16 days per month and for more than 6 months per year for a minimum of three years. Diagnosis was confirmed by rhinoscopy and a position skin prick test to *Dermatophagoides pteronyssinus* and sometimes to other common aero-allergens. These subjects had turbinate amputation to improve the airway.

The remaining eight subjects had no history of rhinitis, negative skin prick tests to all aero-allergens and were undergoing other nasal surgical procedures such as septoplasty, submucosal resection of the nasal septum, and submucosal diathermy or trimming of the inferior turbinate bones. These subjects gave consent for an additional biopsy of the inferior turbinate.

The study was approved by Parkside Healthside Ethics Committee (EC2418). No subjects had received topical corticosteroid nasal sprays during the previous three months nor any other medication with known nasal effects (such as antihistamines and drugs with anticholinergic side effects) during the previous one month.

Surgical procedures

No topical vasoconstrictor was applied prior to biopsy. After medial fracture of an inferior turbinate, the anterior 10 mm was excised using turbinectomy scissors or in some subjects turbinate amputation was the definitive procedure. The nose was firmly packed for the following 24 h and there were no early or late haemorrhages, nor other complications.

Tissue processing

Surgically resected turbinates were fixed immediately in Zamboni's fixative (0.1 M phosphate buffer containing 2% (w/v) paraformaldehyde and 15% (w/v) saturated picric acid) for 4 h at room temperature and then stored in phosphate-buffered saline (PBS) containing 3% sucrose. The anterior 10 mm of the mucosa was dissected from the turbinate bone, washed thoroughly in PBS/sucrose and its volume measured by water displacement (Scherle, 1970). The mucosal tissue was then sliced into five blocks of equal thickness and isotropic uniformly random sections were cut (Mattfeldt, 1990). The mucosa was placed on a cork disc and surrounded by mounting medium (Tissue-Tek ®; Miles Inc., Elkhart, USA). The blocks were frozen by submersion in melting dichlorodifluoromethane (Arcton-12; ICI, Cheshire, UK) and then cooled to –196°C by immersion in liquid nitrogen and stored at –70°C.

With the aid of a random number generator (Press et al., 1988), random sections (10 μm) were cut using a Bright Cryostat. The sections were mounted on microscope slides coated with poly-L-lysine, left to dry for 1 h at room temperature and then stained with haematoxylin and eosin (Bancroft and Cook, 1984). Each biopsy was examined by the same pathologist (J.W.), who was blinded to the clinical diagnosis, for features of chronic rhinitis.

Image collection and analysis

From the 30 cryostat sections, ten were randomly selected. Two fields from each section were digitised using a computer programme called *OpenStereo* (Abrams et al., 1994). OpenStereo was also used for the subsequent analyses of the nasal mucosa images. The images were manually optimised for brightness and contrast and the cavernous vessels were identified and analysed by a single operator. Analysis of the images consisted of volume density (point counting) and surface density (intercept counting) assessments. The reference space was considered to consist of the whole tissue section excluding the respiratory epithelium (Figure 1). The extrapolation from area measurements to volume measurements is legitimate when correct stereological procedures have been used (Weibel, 1979; Gundersen, 1986, 1987; Gundersen et al., 1988, 1988a). The point density used for point counting was 225 points mm^{-2} (512 points per image; Figure 2) and the line density for intercept counting was 13 mm^{-1} (100 points per image; Figure 3). These densities were chosen because preliminary experiments indicated that they would produce results with relatively high reliabilities and low variances. Point counting (Chalkley, 1943; Cruz-Orive and Weibel, 1990) and intercept counting (Tomkeieff, 1945) are well described elsewhere.

Statistical analysis

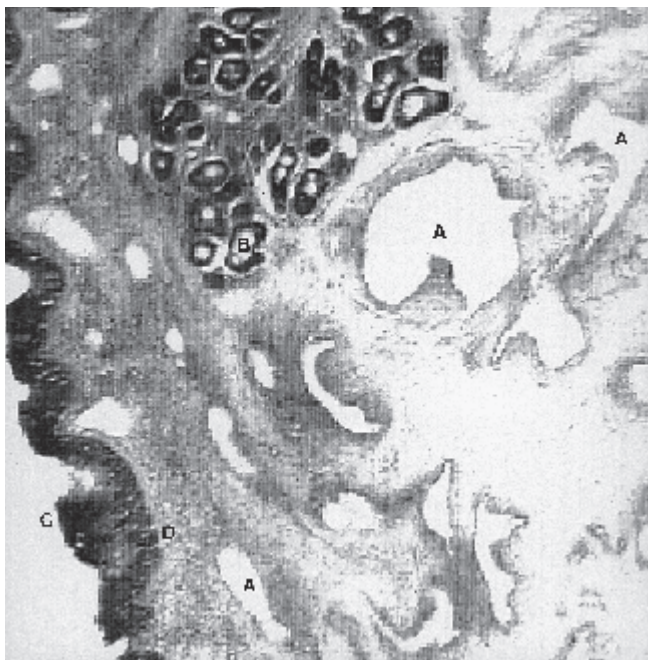

Figure 1. A digital image of a 10-μm section of nasal mucosa stained with haematoxylin and eosin. The cavernous blood vessel profiles, some of which are indicated by (A), vary in shape and size. The seromucous glands (B) are just beneath the respiratory epithelium (C) which covers the basement membrane (D).

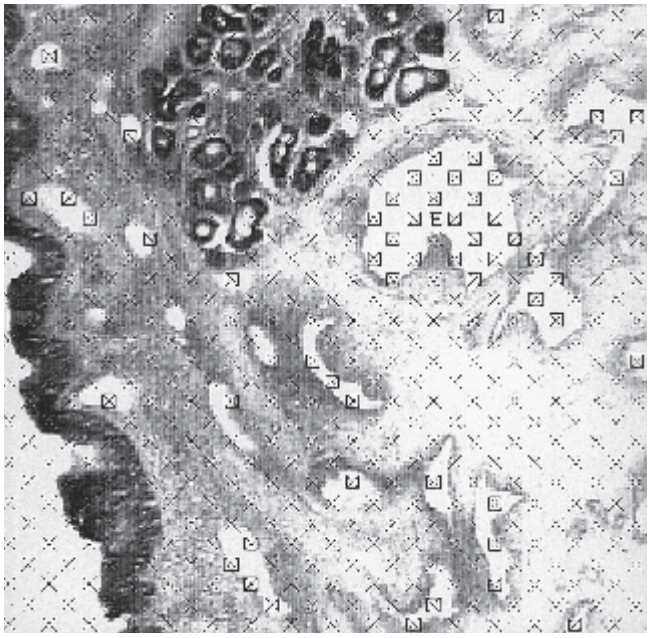

Figure 2. A digital image of the same nasal mucosa section is superimposed with a regular lattice of test points. Points that lay within the blood vessel profiles are selected and automatically enclosed within a square (E). The computer displays the points and squares in different colours for easy identification.

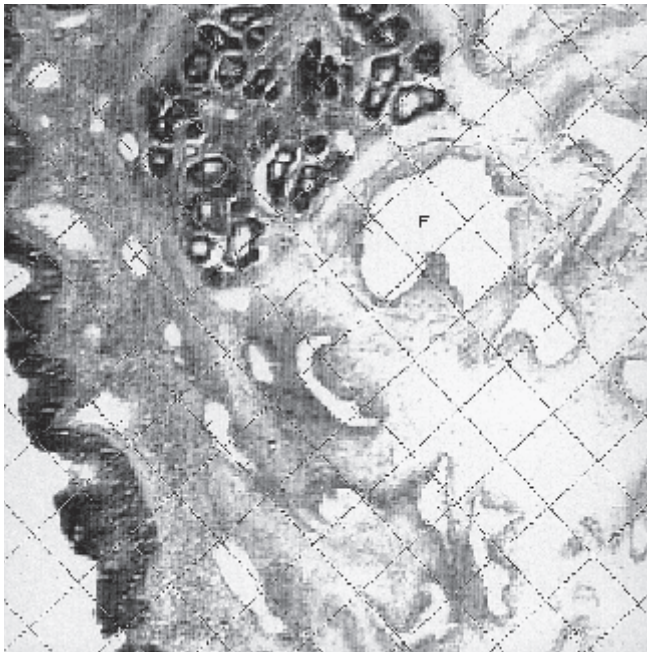

Figure 3. A digital image of the same nasal mucosa section is superimposed with a grid. Intersections between the grid and blood vessel profiles are selected and marked with a cross (F). The computer displays the grid and crosses in different colours for easy identification.

All data were tested for normality using the Shapiro-Francia W' test (Royston, 1983). The volume of rhinitic tissue analysed was compared with the volume of normal tissue analysed using a two-sample *t* test. The mean value for each subject was calculated. The blood-vessel volume density data for the two groups were analysed using Welch's *t* test and the *F* test to compare the group means and the variances of the subject means, respectively. This was repeated for the intercept-counting data. Genstat (Statistics Department, Rothamsted Experimental Station, Hertfordshire, UK) was used to perform the residual-maximum-likelihood procedure (REML; Robinson, 1987) to calculate the estimated components of variance for the point-counting and intercept-counting data. The software package called "N" was used to perform sample size calculations (IDV; Munich, Germany).

RESULTS

Histology

Eight of 10 subjects with perennial allergic rhinitis had significant mucosal inflammation and five had an eosinophilic infiltrate. Three of eight control subjects had significant mucosal inflammation and two had an eosinophilic infiltrate, hence it was not possible to predict confidently which biopsies originated from subjects with clinical symptoms of rhinitis on those histological features.

Tissue volumes

The average volume of the anterior 10 mm of tissue from control subjects was 0.219±0.082 cm^3 and 0.310±0.179 cm^3 in the rhinitic subjects. The variances of the two groups were not significantly different ($F_{(9,7)}$=1.05, p=0.49), following reciprocal transformation. The residuals were normally distributed, with a W' value of 0.950 (p=0.37) as shown by the Shapiro-Francia W' test (Royston, 1983). A two-sample *t* test on the transformed data showed that the mean volumes were not significantly different (p=0.35).

Point counting

The estimated mean vascular volume densities were 6.17±1.41% in the control and 7.82±5.59% in the rhinitic subjects. The values for each field for each subject are plotted in Figures 4 and 5. The distribution of the residuals was found to be positively skewed and remained skewed after logarithmic transformation. Hence, subsequent statistical analyses were performed on the non-transformed data.

The variances of the subject means was larger in the rhinitic group than in the control group, ($F_{(9,7)}$=15.12, p=0.0008). Because the variances were unequal, Welch's *t* test was used to

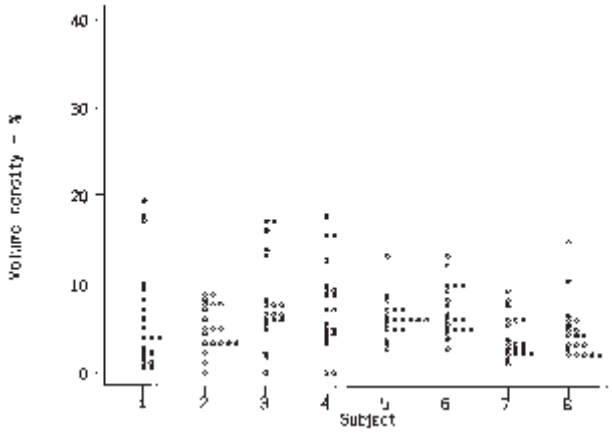

Figure 4. A scatter plot showing the vascular volume density for all the fields measured in the eight control subjects. Each measurement is represented by a circle.

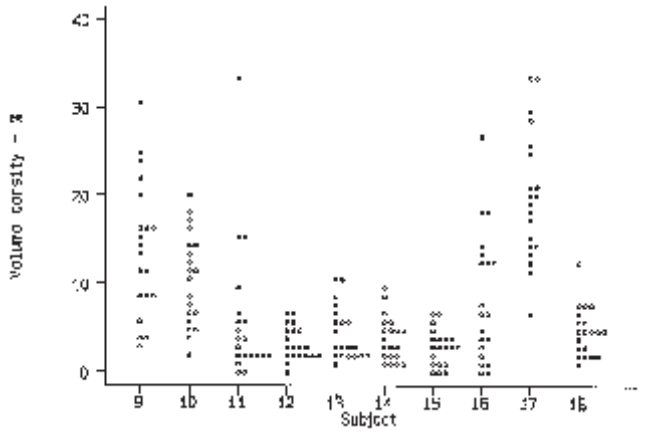

Figure 5. A scatter plot showing the vascular volume density for all the fields measured in the ten rhinitic subjects. Each measurement is represented by a circle.

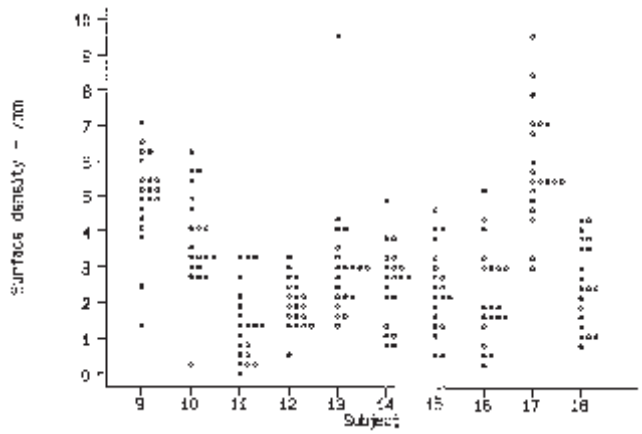

Figure 7. A scatter plot showing the luminal surface density (in mm^{-1}) for all the fields measured in the ten rhinitic subjects. Each measurement is represented by a circle.

compare the group means There was no significant difference in the mean volume density between the two groups (p=0.38).
The REML procedure showed that the variance between sections was not significantly different in the control and rhinitic group (Table 1) and that the components of variance between subjects and fields were much larger in the rhinitic group.

Table 1. The point-counting variance components of vascular volume density with associated standard errors (in parentheses) for each group is shown.

component of variance	control	rhinitis
between subjects	1.10 (1.08)	28.6 (14.2)
between sections	2.94 (1.77)	3.34 (2.95)
between fields	12.0 (1.90)	25.0 (3.54)

Intercept counting

The mean estimated surface density for the rhinitic group was 3.14±0.74 mm^{-1} and 3.10±1.41 mm^{-1} in the control group. The values for each field for each subject are plotted in Figures 6 and 7. The distribution of the residuals was found to be normal.

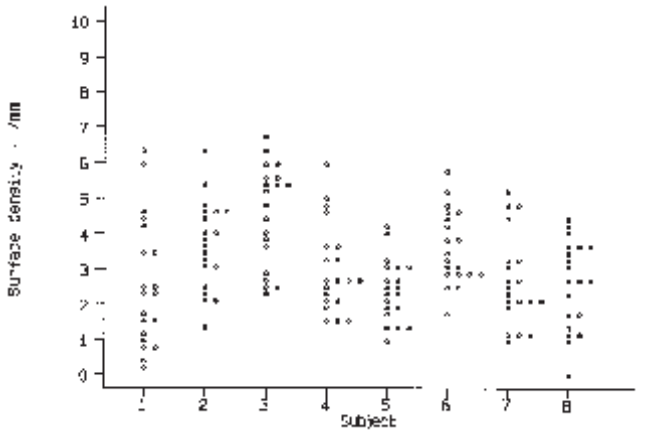

Figure 6. A scatter plot showing the luminal surface density (in mm^{-1}) for all the fields measured in the eight control subjects. Each measurement is represented by a circle.

The variances of the subject means was larger in the rhinitic group compared with the control group ($F_{(9,7)}$=3.64, p=0.05). Because the variances were not equal, Welch's *t* test was used to compare the group means. There was no significant difference in the mean surface density between the two groups (p=0.94).
The REML analysis showed that the variance component between fields was similar in the control and rhinitic group (Table 2) and that the components of variance between subjects and sections were much larger in the rhinitic group.

Table 2. The intercept-counting variance components of vascular surface density with associated standard errors (in parentheses) for each group is shown.

component of variance	control	rhinitis
between subjects	0.47 (0.30)	1.88 (0.94)
between sections	0.04 (0.19)	0.47 (0.18)
between fields	1.62 (0.26)	1.22 (0.17)

Sample size calculations

Sample size calculations were performed using the data from the volume density experiment. The results showed that for a difference in the means of 1.65% (as detected by this experiment) to be significant at the 5% level with a power of 90%, 294 subjects (147 subjects per group) would be required. Further investigations revealed that with the number of subjects analysed in this experiment, a difference in the means of 7% could be detected at the 5% significance level with a power of 90%. However, since the data are not normally distributed the sample sizes required should be increased by 10% (Lehmann and D'Abrera, 1975).

DISCUSSION

Numerous previous studies and reviews have examined the organisation of the nasal mucosa and its vasculature. These have been directed at describing the anatomical architecture of the microvasculature (Dawes and Prichard, 1953; Änggård, 1974; Cauna, 1982; Passàli et al., 1992) and its physiological control (Widdicombe, 1990, 1994). To the best of our knowledge no

previous attempts have been made to quantify the density of vessels within normal or diseased human nasal mucosa. Vascular density measurements have been obtained for other organs, particularly in neoplastic diseases of the breast, where attempts have been made to correlate vascular density with invasion and outcome (Protopapa et al., 1993; Van Hoef et al., 1993), prostate (Brawer et al., 1992), cervix (Kohno et al., 1993) and oesophagus (Porschen et al., 1994).

Effects of various angiogenesis-inducing agents have also been studied (Sorbo et al., 1994; Danesi et al., 1993). To date, most methods have been performed manually although some studies have utilized image-morphometric methods for vascular density measurement (Brawer et al., 1992; Visscher et al., 1993). Our technique greatly reduces the time taken to quantify vascular density. The production of photographs is not necessary, but the digitised histological slides allow recall for re-assessment, use in other studies and for peer review.

The luminal surface density of the large cavernous blood vessels was estimated in order to determine whether there was any change in the luminal surface area of these blood vessels present in PAR. Such a change could represent an increase (or decrease) in the number of blood vessels or an increase (or decrease) in the cross-sectional area of the blood vessels or both. In conjunction with the vascular volume density estimates, the surface density data were used to determine whether there was any change in the number of blood vessels present in the diseased tissue. This was necessary because it was important to determine whether a change in the number or size of blood vessels had occurred. In this study, the vascular volume density estimations did not detect a significant change in the volume density of the blood vessels; similarly, no significant change in the surface density of the blood vessels had occurred. If, however, there was no change in the vascular volume density but a change in the surface density, then obviously this change would not be detected by vascular volume density estimates. An example of how this situation could arise would be if the number of blood vessels increased but the size of these vessels (namely the cross-sectional area) decreased to such an extent that the overall volume of the blood vessels was unchanged.

The large cavernous blood vessels were relatively easy to differentiate from the subepithelial vessels and are thought to be largely responsible for acute changes in inferior turbinate volume. The subepithelial capillary network was not visible at the working magnification of ×9. The large vessel density in these biopsies averaged 6-7% of the total tissue volume. Because it was not possible to maintain intravascular pressure, these estimates may underestimate vascular density in particular sites of the nasal mucosa. Similarly, the surface density estimates, which indicate the amount of luminal surface area present, may also be underestimated. To obtain an estimate closer to *in vivo* conditions, freezing of the turbinate prior to biopsy or perfusion of a feeding vessel at physiological pressures would be necessary. Either technique would be difficult to perform.

There was no significant differences in mean vascular density between biopsies from control and PAR subjects, but vascularity was much more variable in PAR than control subjects and two PAR subjects (Nos. 9 and 17) had larger values than any of the controls. These subjects had no specific clinical features distinguishing them from other subjects of the PAR group.

The volume measurements performed on the anterior 10 mm of the inferior turbinates indicate that there is no significant difference in the amount of tissue analyzed between the two groups. Therefore, hypertrophy of the nasal mucosa is unlikely to be prominent within the anterior portion of the turbinate. However, only the anterior tip of the turbinate has been analyzed, so only changes in the cross-sectional area of the mucosa would be detected while any increase in the length of the turbinate or the nasal mucosa would not be identified. The histological analysis has shown some overlap between the two groups. In summary, the results overall do not support the existence of an increased vascular density in PAR; conceivably, however, the *in vivo* state of distension of the cavernous vessels could be greater in PAR due to changes in neural or other control.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Health and Safety Executive for the support of Stephen Toynton.

REFERENCES

1. Abrams DC, Facer P, Bishop AE, Polak JM (1994) A computer-assisted stereological quantification program. Microsc Res Tech 29: 240-247.
2. Änggård A (1974) Capillary and shunt blood flow in the nasal mucosa of the cat. Acta Otolaryng (Stockh) 78: 418-422.
3. Bancroft JD, Cook HC (1984) Manual of Histological Techniques. Churchill Livingstone, Edinburgh, pp. 18-21.
4. Brawer MK, Bigler SA, Derring RE (1992) Quantitative morphometric analysis of the microcirculation in prostate carcinoma. J Cell Biochem 16H (Suppl): 62-64.
5. Cauna N (1982) Blood and nerve supply of the nasal lining. In: DF Proctor, I Andersen (Eds.) The Nose: Upper Airway Physiology and the Environment. Elsevier Medical Press, Amsterdam, pp. 45-69.
6. Chalkley HW (1943) Method for the quantitative morphologic analysis of tissues. J Natl Cancer Inst 4: 47-53.
7. Cruz-Orive LM, Weibel ER (1990) Recent stereological methods for cell biology. Am J Physiol 258: L148-L156.
8. Danesi R, Del Bianchi S, Soldani P, Campagni A, La Rocca RV, Myers CE, Paparelli A, Del Tacca M (1993) Suramin inhibits ßFGF-induced endothelial cell proliferation and angiogenesis in the chick chorioallantoic membrane. Brit J Cancer 68: 932-938.
9. Dawes JDK, Prichard MML (1953) Studies of the vascular arrangements of the nose. J Anat 87: 311-322.
10. Gundersen HJG (1986) Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J Microsc 143: 3-45.
11. Gundersen HJG (1987) The efficiency of systematic sampling in stereology and its prediction. J Microsc 147: 229-263.
12. Gundersen HJG, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A, West MJ (1988a) The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96: 857-881.
13. Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Møller, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A, West MJ (1988b) Some new, simple efficient stereological methods and their use in pathological research and diagnosis. APMIS 96: 379-394.
14. Kohlraush O (1853) Ueber das Schwellgewebe an den Muscheln der Nasenschleimhaut. Arch Anat Physiol : 149-150.
15. Kohno Y, Iwanari O, Kitao M (1993) Prognostic importance of histologic vascular density in cervical cancer treated with hypertensive intraaterial chemotherapy. Cancer 72: 2394-2400.

16. Lehmann EL, D'Abrera HJM (1975) Nonparametrics: Statistical Methods Based on Ranks. McGraw-Hill International Book company, USA.
17. Mattfeldt T, Gerhard M, Gharenbaghi H, Moller P (1990) Estimation of surface area and length with the orientator. J Microsc 159: 301-317.
18. Passàli D, Buccella MG, Vetuschi A, Bellussi L (1992) Nasal vascularization: Experiences using the microcorrosion technique in human foetuses. Rhinology 30: 81-88.
19. Porschen R, Classen S, Piontek M and Borchard F (1994) Vascularization of carcinomas of the oesophagus and its correlation with tumour proliferation. Cancer Res 54: 587-591.
20. Press WH, Flannery BP, Teukolsky SAZ, Vetterling WT (1988) Numerical Recipes in C: The Art of Scientific Computing. Cambridge University Press, New York, pp. 210-211.
21. Protapapa E, Delides GS, Revesz L (1993) Vascular density and the response of breast carcinomas to mastectomy and adjuvant chemotherapy. Eur J Cancer 23A: 1391-1393.
22. Robinson DL (1987) Estimation and use of variance components. Statistician 36: 3-14.
23. Royston JP (1983) A simple method for evaluating the Shapiro-Francia W' test of non-normality. Statistician 32: 297-300.
24. Scherle W (1970) A simple method for volumetry of organs in quantitative stereology. Mikroskopie 26: 57-60.
25. Sorbo J, Jakobsson A, Norrby K (1994) Mast-cell histamine is angiogenic through receptors for histamine-1 and histamine-2. Int J Exp Physiol 75: 43-50.
26. Tomkeieff SI (1945) Linear intercept, areas and volumes. Nature 155: 24.
27. Van Hoef ME, Knox WF, Dhesi SS, Howell A, Schor AM (1993) Assessment of tumour vascularity as a prognostic factor in lymph node negative invasive breast cancer. Eur J Cancer 29A: 1141-1145.
28. Visscher DW, Smilantz S, Drozdowicz S, Wykes SM (1993) Prognostic significance of image morphometric microvessel enumeration in breast carcinoma. Analyt Quantit Cyt Hist 15: 88-92.
29. Weibel ER (1979) Stereological Methods, Volume 2. Academic Press, London.
30. Widdicombe JG (1990) Comparison between the vascular beds of upper and lower airways. Eur Respir J 3: 564s-571s.
31. Widdicombe JG (1994) Control of airway vascular beds. In: M Kaliner, PJ Barnes, GHH Kunkel, JN Baraniuk (Eds.) Neuropeptides in Respiratory Medicine. Marcel Dekker, New York, pp. 373-397.

Don-Carlos Abrams
Department of Histochemistry
Royal Postgraduate Medical School
Hammersmith Hospital
DuCane Road
London W12 0NN
United Kingdom

ANNOUNCEMENT