# THE REID INDEX IN HEALTHY CATS

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## ABSTRACT

Asthma is a prevalent respiratory disease in humans and a similar condition has been reported in cats. It is difficult to diagnose feline asthma because the signs are often mistaken for other pulmonary or cardiac diseases, and the gross and microscopic lesions are subtle. The bronchial gland to bronchial wall ratio, also known as the Reid Index (RI), is commonly used to confirm asthma and chronic bronchitis in humans but this method has never been validated in cats. The objectives of this investigation are to establish the normal RI in healthy cats, and investigate the effect of bronchial sectioning and lung lobe on RI. The lungs of forty one clinically healthy cats obtained from P.E.I. Humane Society were examined at postmortem and fixed in formalin. Preselected sections of the right apical, right intermediate, right accessory, right diaphragmatic, left apical, and left diaphragmatic lobes were examined microscopically and the lungs were further classified as having no microscopic lesions or having minor lesions. Bronchi were cut either longitudinally or transversely and the RI was determined by linear measurement using J-image software (IJ) or by two-dimensional measurement using point count stereology (PCS).

Results showed that the RI in healthy cats is 61% ± 11.8 (using the mean of individual measurements in both groups of lungs with and without minor lesions, measured by linear measurement, and cut either longitudinally or transversely on bronchial view that have cartilage and cartilage was excluded) which is notably higher than the 40% reported for healthy human lung. There were minor but significant differences among lung lobes within the group of lungs with no microscopic lesions ($p$ = 0.034) and group of lungs with minor lesions ($p < 0.001$). There were no significant differences between the lungs with no microscopic lesions and lungs with minor lesions ($p < 0.946$). There was a significant difference between the right intermediate lobe in the group of lungs with no microscopic lesions and the right intermediate lobe in lungs with minor lesions ($p = 0.029$) with a higher RI in the group with minor lesions. The average of gland to wall ratio in all lobes except RIN lobe in the group of lungs with no microscopic lesions, cut longitudinally and measured by linear measurement was 50.44 ± 0.359.

The correlation between linear measurement and two-dimensional measurement was poor when cartilage was included in the measurements. Linear measurement and bronchial cross sections had more variation than two-dimensional measurement and longitudinal sections. Minimum of 12 measurements per cat (taken from different lobes) and a minimum of 15 measurements per lobe are needed to obtain a meaningful RI.

It was concluded from this investigation that the feline bronchial glands have a notably higher RI than human. Using the right intermediate lung lobe for RI calculation in cats should be avoided. Two-dimensional measurements should be preferred over linear measurements and longitudinal sections of the bronchial tree should be chosen over cross sections of bronchi. The least amount of variation in the RI is found when two-dimensional measurements are made in longitudinal sections. For a practical RI method, it is recommended to use the longitudinal sections measured by linear measurements in bronchial areas without cartilage. The measurements should be made in bronchial areas that do not contain cartilage or if present, cartilage should be excluded from the calculations.

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| | |
|---|---|
| APCs | Antigen presenting cells |
| AVC | Atlantic Veterinary College |
| BAL | Brochoalveolar lavage |
| CGRP | Calcitonin gene-related peptide |
| CNS | Central nervous system |
| 2-D | Two-dimensional |
| e-NANC | Excitatory non-adrenergic and non-cholinergic nerves |
| H&E | Hematoxylin and eosin |
| IJ | Image J analysis |
| i-NANC | Inhibitory non-adrenergic and non-cholinergic nerves |
| L-AP | Left apical lobe |
| L-D | Left dorsal lobe |
| μm | Micrometer |
| N | Number of cases |
| NANC | Non-adrenergic and non-cholinergic nerves |
| NKA | Neurokinin A |
| NKB | Neurokinin B |
| NO | nitric oxide gas |
| NOS | Nitric oxide synthase |
| PAS | Periodic acid-Schiff stain |
| PCS | Point count stereology |
| PEI | Prince Edward Island |

| | |
|---|---|
| PDGF | Platelets-derived growth factor |
| R-AP | Right apical lobe |
| R-AC | Right accessory lobe |
| R-D | Right dorsal lobe |
| R-IN | Right intermediate lobe |
| RI | Reid index |
| SD | Standard deviation |
| SP | Substance p |
| Th1 | Type 1 T helper cells |
| Th2 | Type 2 T helper cells |
| VIP | Vasoactive intestinal peptide |

# 1. GENERAL INTRODUCTION

## 1.1. The respiratory system

The respiratory system is morphologically and functionally divided into three compartments referred to as the conducting, transitional and exchange systems. The conducting compartment is the largest and most of it is located outside of the lung while the transitional and exchange compartments are in the lung. The transitional compartment is formed exclusively by the bronchioles which serve as connection between the bronchi in the terminal conducting system, and the alveoli or the exchange system in the lung (1). Each of these three compartments has anatomic and histological characteristics specifically involved in the process of respiration, and each one also expresses a characteristic response to injury. For instance, inflammatory response and wound healing in the conducting compartment are essentially the same whether these changes occur in the nasal cavity, trachea or bronchi (2).

### 1.1.1. The conducting system

The conducting system, as the name implies, is primarily involved in the conduction of air from the external environment into the lung where the exchange of oxygen and carbon dioxide takes place. Anatomically, the conducting system is composed of the nasal cavity, larynx, trachea and bronchi.

The nose has an internal and external portion. The external part projects from the face and has two openings called nostrils where the air enters the respiratory system (3). The size and shape of the nostrils, the surrounding skin, and the orientation notably varies among animals species. The skin covering the nostril is hairless and it is sharply demarcated in most domestic animals (1,4). This region remains moist from the flow of

secretion of the serous glands in nasal mucosa, as well as fluids from the nasolacrimal duct. Both secretions aid humidification of inspired air (1).

Caudal to the nostril, the nasal vestibule has coarse hairs and the mucosa has abundant sebaceous and sweat glands (3). The two nasal cavities are separated by a well vascularized septum made up of a perpendicular plate of ethmoid bone above (dorsally), vomer bone, and vomeronasal bone and septal nasal cartilage beneath (ventrally) (3).

Internally, the nasal cavity contains the nasal conchae or turbinates which are bony ridges protruding laterally into the cavity and forming three air passage called superior, middle, and inferior meatuses (5,6) . All of the meatuses connect to the posterior nares allowing air to reach the upper respiratory tract through the pharynx (3). The nasal mucosa plays an important role in moistening and warming the inhaled air, and also involved in entrapping the water droplets during expiration (5).

The larynx, also called the voice box because it houses the vocal cords, is the next major structure of the conducting system connecting proximally (cranially) with the oral and nasal cavities and distally (caudally) with the trachea. The larynx is a cartilaginous tubular structure with a triangle shape, where the apex or thyroid cartilage is located in the anterior neck. When forced air passes through the larynx, the vocal folds vibrate creating sounds. When the food is swallowed, the larynx moves upward in opposition to epiglottis which prevents the entrance of food particles into the larynx (5). Overall, the morphological features of the larynx in domestic animals are similar to those in man, but there are a number of small differences among species (1).

The human trachea, commonly called the windpipe, connects proximally with the larynx and distally with the bronchi. It extends from the neck into the mediastinum along

the ventral aspect of the esophagus and enters into the thoracic cavity above the cardiac region at the level of the fourth to the sixth intercostal spaces. Distally, the trachea divides into two main bronchi (1) . In pigs and ruminants there is an additional tracheal bronchus proximal to the tracheal bifurcation that supplies the cranial lobe of the right lung (1).

The wall of trachea is formed by C-shaped cartilaginous structures known as tracheal rings. This cartilaginous structure protects the trachea from collapsing and provides flexibility so this organ can adjust during cervical movement. Both ends of the C-shaped tracheal rings are joined together by fibroelastic ligaments and bundles of trachealis smooth muscle (6).

The main bronchi enter the lung at the level of the sternal angle. The right bronchus is wider and shorter, enters the lung ahead of the left bronchus and carries about 55% of the air volume. Also, there are minor differences in bronchial anatomy between the right and left lungs. As examples, the right bronchus enters the lung behind the pulmonary artery and thus is called eparterial, while the left bronchus enters the lung below the pulmonary artery and is called hyparterial (7).

The main bronchi, also known as primary bronchi, divide forming five lobar bronchi collectively identified as secondary bronchi. The first three bronchial generations from the lobar bronchi are known as tertiary bronchi. These supply the rest of the lung lobes forming small lobar units called bronchopulmonary lung segments, or simple pulmonary segments (8). Each of these individual units is supplied by a specific bronchus and artery. In the human lung there are a total of 19 pulmonary segments for both lungs, 10 for the right and 9 in the left lung (7) and each of these segments is often

identified individually in radiology and pathology to localize topographically a particular lesion in the lung (3).

The left and right human lungs share many anatomical similarities but also have some differences based on bronchial branching. One main anatomical difference is that the left lung has only two lobes (upper and lower lobes) while the right lung has three distinct lobes (right upper, right middle and right lower) (7). Both upper lung lobes have an apical, anterior and posterior segments, while the lower lobes have an apical segment and anterior, lateral and posterior basal segments. The right lower lobe has one more segment named as medial basal or cardiac segment (7).

Another interesting feature in bronchial morphology is the asymmetry in bronchial branching. This asymmetry is referred to in lung anatomy as "pseudodichotomic" meaning that each individual bronchus has two branches of dissimilar diameter or length. The reason for this pseudo-dichotomy is not fully understood (7).

The lungs in domestic mammals are characteristically divided into lung lobes and share both morphological similarities and discrepancies with the human lung. In dogs for example, the lung lobes are noticeably divided by prominent and deep fissures and not surprisingly, the canine lung is more susceptible to lobe torsion than other species, while in cattle, the lobar fissures of the lungs are less prominent than in cats and dogs (1,4).

Like man, the left lung in all domestic mammals has two lobes, but because of the standing position of the animals is referred to instead of upper and lower as apical and diaphragmatic lobes. This type of lobation in animals is also based on bronchial branching, where the left lung has two main bronchi, the apical and diaphragmatic

bronchi. In contrast and based on bronchial branching, the right lung, depending on species, has four lobes known as the apical, intermediate, diaphragmatic, and accessory lobes. There are however, some important anatomical differences in the right lung among domestic species. In dogs and cats for example, the right lung has the apical, intermediate, diaphragmatic, and accessory lobes, whereas in the horse, the right lung has apical, accessory and diaphragmatic but lacks the intermediate lobe (1).

As reported for the human lung, lobar branching of bronchi and arteries in animals also divide the lung into smaller units or bronchopulmonary segments (9). However and in contrast to human medicine, the bronchopulmonary segments are rarely used in veterinary medicine to locate topographically neoplastic or inflammatory lesions in the lung (10). The rare use of broncho-pulmonary segments in veterinary pathology is likely because of the notable differences among domestic animals which make clinical application awkward.

**1.1.2.** The histology of bronchi

The tissues that constitute the bronchi and their lining mucosa have the same basic structure as those found in the nose, larynx, and trachea (2). Most of the conducting system is lined by pseudostratified respiratory epithelium containing secretory cells except for a few anatomical sites that also contain specialized non-ciliary epithelium such as at the mucocutaneous junction and the olfactory epithelium of the nasal cavity (8).

Respiratory epithelium is the most abundant type of airway epithelium covering over two thirds of the nasal cavity (8) and practically all segments of trachea and bronchi. Respiratory epithelium is primarily composed of pseudostratified columnar cells. Most

cells are ciliated, but there are also goblet cells, serous cells, brush cells, and basal cells (2).

The ciliated cells in the conducting system are typically columnar in shape (8), 15-20μm in height (11) and have 200 to 300 motile cilia per cell (8). Each of these cilia is 6-8 μm in length and has a cross-sectional diameter of 0.3 μm. The cytoplasm of ciliated cells is firmly attached to the basement membrane and has complex interdigitations with the surrounding cells (11).

Non-ciliated secretory cells are numerically much less common than ciliated cells. Apical microvilli are typical structures seen in non-ciliated, ciliated and secretory cells. There is notable variation in the regional distribution and morphological appearance of secretory cells throughout the conducting system, differences that are best demonstrated using histochemistry. These histochemical differences relate to the biochemical composition of the secretory glycoproteins. For example, mucous epithelial cells contain sialated or sulfated acid glycoprotein (mucin) and appear electron-lucent in electron microscopy, while serous epithelial cells have less mucin and appear electron-dense. Secretory cells are typically tall and slender with variable number of mucous or serous granules depending on their secretory phase (8).

Goblet cells are the predominant secretory cell in the nasal, tracheal and bronchial mucosa in both humans and domestic mammals (8). Some studies suggest that goblet cells represent from 5-15% of the columnar cell population in the lung (12). Microscopically, these cells have prominent PAS and silver positive cytoplasmic granules which can be discharged into the lumen of the conducting system in just a few milliseconds (13). The granules of goblet cells are filled with high molecular weight

mucins produced in vestibules formed in the Golgi apparatus (12). These mucins are composed of various glycoprotein subunits formed by a peptide core connected by disulphide bonds forming the polymer (13). The carbohydrates make hundreds of oligosaccharide side-chains with the peptide core composing 90% of its weight (12).

Serous epithelial cells are another important type of secretory cells in the nasal and bronchial mucosa. These cells are histologically characterized by acidophilic cytoplasm with discrete granules or vacuoles and ultrastructurally characterized by electron-dense granules containing neutral glycoprotein. Serous cells are smaller in size than goblet cells and their secretions are more fluid with much less viscosity. In addition to secretory function, these cells are now known to play a role of stem cells in epithelial repair (14).

Another cell that is integral to the respiratory epithelium of the conducting compartment is the basal cell. These cells tend to be small and have a characteristic polyhedral shape. Basal cells are interspersed along the basal lamina and attach to other cells by desmosomes and to the basal lamina by hemidesmosomes. The cell turnover in the lung is very low. However, when injury to the lung epithelium occurs, the tissue has to be repaired as fast as possible (15). For many years the basal cell was thought to be the only "mother cell" or progenitor cell involved in repair, however, recent investigations have shown that replacement cells do originate from the other cell types (8) such as ciliated cells and non-ciliated, secretory cells (14,15).

A rather peculiar cell type present in the respiratory epithelium of the conductive compartment is the so-called brush cell. This unique type of cell is also referred to in the literature as caveolated, multivesicular or fibrillovesicular cells. However, because these

cells are not easy to identify on routine microscopic examination they are rarely described either in the healthy or diseased lung. In the respiratory system brush cells are present in the conducting, transitional and exchange systems but their numbers appear to be smaller in the bronchi as compared to the nasal cavity, trachea, bronchioles and lung. Ultrastructurally brush cells are characterized by a tuft of long and thick microvilli (~120-140/cell), hence their name of brush cells (16). Although their function remains unclear, some histologists suggest that brush cells have sensory receptors associated with endings of trigeminal nerve (8).

In addition to the secretory cells normally present on the surface of respiratory epithelium, the mucosal membranes of all segments of the conducting system, from nasal cavity to bronchi, have abundant glands embedded in the submucosa except for the region of the larynx where the true vocal cords are located (2).

Even though mucus is produced by both tracheobronchial glands and goblet cells, the volume of mucus secreted by the seromucous glands is about 100 times larger than from goblet cells (17). The number and the size of the glands differ notably among domestic animals. Glands are abundant in the trachea of most species like man, cow, sheep, goat, cat, and dog. However, glands are scarce in rat and are found only in the first portion of the trachea until the third cartilaginous ring. In mouse, glands are found only in the border between larynx and trachea, and there are no glands in rabbit (18).

Although the bronchial and tracheal cartilages are both composed of the same type of hyaline cartilage, the shape and size of the cartilage varies. In the trachea, cartilage is well curved forming almost complete cartilaginous rings, while in the bronchi, cartilage forms incomplete segments anatomically known as plates. These

plates are located in the submucosal connective tissue. As the bronchi bifurcate they typically become smaller to the point that cartilaginous plates are almost non-existent in distal bronchi near the bronchial-bronchiolar junction. Near the terminal bronchi these plates are finally replaced by small fragments of cartilage called plaques (19).

The bronchial wall also contains smooth muscle cells which play a major role in the regulatory mechanisms of airflow by means of bronchoconstriction and bronchodilation (10,20). Like cartilage, smooth muscle cells within bronchi vary in shape and size depending on the airway caliper. Normally, the smooth muscle in the trachea is present as large bundles of fibers which eventually transform distally into slender fibers within the bronchial laminae muscularis mucosae (8). It has been estimated that smooth muscle in the healthy human lung constitutes only 4.6±2.2% of the bronchial wall volume (21). In major bronchi the smooth muscle fibers are in the inter-cartilaginous space or on the luminal side of the cartilage. In smaller airways such as bronchioles, the smooth muscle fibers always form a complete cross-sectional ring (8).

#### **1.1.2.1.** Bronchial glands

There is notable variation in the distribution of the glands amongst domestic animals. For instances, felines have glands not only in nasal cavity, larynx, trachea, primary and secondary bronchi, but also in tertiary bronchi and as distal as the proximal bronchioles (22). In goats, the glands are embedded within adipose tissue, while there is less adipose tissue around the glands in sheep (18). Glands often fill the gaps between the cartilaginous plates in bronchi and as the plates decrease in size, the glands also decrease in size despite an increase in available connective tissue volume. Typically, glands are larger and abundant in primary and secondary bronchi, and smaller and more

scarce in the tertiary bronchi (22) suggesting a strong correlation between the airway diameter and the gland volume (18).

The gland's secretions empty into the secretory tubules that eventually drain into collecting ducts and finally reach the bronchial lumen. The secretory tubules are lined by two main types of functional cells: the serous and mucous cells. Beneath these cells there are myoepithelial cells (17).

The glandular secretions are chemically complex. Serous cells, as their name implies, secrete watery material and anti bacterial substances such as lysozymes, lactoferrin, and secretory IgA (23,24) with less protein or mucus (22,25) while the mucous cells secretions are rich in gel-forming mucins (25). The number and types of the cells in the glands, and the nature of their secretions, vary not only among animal species but also on the level of the bronchial tree where the glands are present (19). For instances, in all regions of the feline trachea, submucosal glands are composed of 61-94% secretory cells; serous cells compose the majority of these cells (26). Serous cells have well developed endoplasmic reticula, more mitochondria, and smaller and fewer granules than mucous cells (27). Serous cells compose 50% of the respiratory epithelial glands in man. Mucous cells are less numerous than serous cells in normal bronchial glands (27,28). This seemingly trivial point will become relevant in discussions of mucus cell proliferation during chronic respiratory stress.

Serous cells are also more numerous towards the distal portion of the glandular tubule, while the mucus cells are in the proximal part of the tubule towards the lumen (29). This position gives serous cells an important function of secreting a watery fluid

that washes out and hydrates the mucin gel component secreted in the proximal tubules by the mucus cells, which is drained by the collecting ducts out of the gland (25).

Serous cells have a characteristic triangular shape with the nucleus generally located in the basal region of the cells. The apical part of these cells has large numerous electron dense eosinophilic secretory granules (17), 100-180 nm in diameter on the electron microscope (30), while the base of the cell has a high amount of endoplasmic reticulum that imparts a basophilic staining property on the light microscope (17).

Mucous cells are characterized by basal nuclei (17). Their apex is filled with large mucin granules (7) that compress the cell's organellar contents to the basal part (30). Their apical surface is covered by short microvilli (17). Respiratory epithelial glands may bifurcate twice or more, yet they always end with serous cells (30).

Myoepithelial cells resemble smooth muscle in that their cytoplasm has myofilaments composed of actin and myosin (31). Myoepithelial cells are located between the glandular epithelial cells and the adjacent basement membrane; they are connected to the other secretory cells by desmosomes (17). Myoepithelial cells are only seen in the acini and proximal collecting ducts and are absent in the distal duct that opens to the bronchus lumen. Mechanism of myoepithelial stimulation varies between animal species (31). When activated, myoepithelial cells contract helping to drain the secretion into the lumen (17).

The epithelium of the collecting duct has columnar cells that have microvilli on their apical surface (25,29). The nucleus is ovoid and located in the centre with prominent nucleolus. These cells have densely packed mitochondria and a well developed Golgi apparatus. They are found adjacent to the glandular mucus cells. Myoepithelial cells are

present beneath the epithelium of the collecting duct. The duct lumen is 110 μm wide which is larger than that in the secretory tubules; an average value for serous tubules is 5 μm and for mucus tubules is 25 μm. Bronchial collecting duct cells may play a part in fluid and ionic regulation (32).

Close to the bronchial lumen, the duct is ended by ciliated pseudostratified columnar cells (17) that are similar to the ciliated epithelium of the mucosa, hence the term ciliated duct (25,29). In the rat, the pseudostratified columnar cells are the only type of cells found in the collecting duct (33). The collecting duct rarely has a distended cavity in the submucosal space (25,29).

The submucosal glands are controlled by parasympathetic (cholinergic), sympathetic (adrenergic), and non-adrenergic non-cholinergic nerves (NANC). Parasympathetic neural pathways are the dominant motor controls of submucosal glands in airways of all domestic animals including man (34).

The main neurotransmitter of parasympathetic cholinergic nerves is acetylcholine (Ach) (35). The nerves stimulate submucosal gland secretion via muscarinic receptors found on both mucous cells (mucin) and serous cells (fluid, electrolytes) (34,36). .

The sympathetic nervous system is less predominant than the parasympathetic system. The main neurotransmitter of sympathetic nerves is noradrenaline (35). It stimulates bronchial glands via α- and β-adrenergic receptors. These receptors selectively regulate airway submucosal glands. For example, α-adrenergic agonists act on serous cells causing fluid secretion, while β-adrenergic agonists act on mucous glands causing mucus secretion. There is variation in adrenergic stimulation among animal species in

that α1-adrenergic agonist phenylephrine has a small effect on the sheep, pig, and man. However, the cat, ferret, and dog respond strongly to phenylephrine (37).

NANC neural effect is mediated by neurotransmitters released from the classic parasympathetic nerves. NANC neurotransmitters are small molecular weight peptides. The inhibitory NANC (i- NANC) system releases Vasoactive intestinal peptide (VIP), VIP related peptides, and nitric oxide gas (NO). VIP peptides are colocalized with Ach (34) in parasympathetic nerve terminals (35). The motor control is mediated by VIP receptors. VIP Inhibits mucus secretion, suggesting that this peptide regulates acetylcholine release (34). NO is a gaseous neurotransmitter formed by conversion of L-arginine and oxygen to L-circulline via nitric oxide synthase (NOS). NO is released by simple diffusion from the NOS containing nerves around the submucosal glands (35). It is thought that NO facilitates fluid secretion indirectly by increase of blood flow to the glands via vasodilation (25).

The excitatory NANC (e- NANC) system releases neuropeptides such as substance p (SP), neurokinin A and B (NKA and NKB), and calcitonin gene-related peptide (CGRP). These neuropeptides are released from nonmyelinated sensory c-fibers (34,35). Some of the c-fibers release neuropeptides from the peripheral terminals without involvement of CNS (38), forming a separate neural system called local axon reflex (34). The local axon reflex can stimulate the gland secretion in both mucous (34) and serous cells (25) in isolated trachea and bronchi, forming an intrinsic pathway (39). SP-containing nerves can be found around the submucosal glands. SP-inducing nerve terminals are sparse compared with VIP–containing nerves (35).

**1.2.** Bronchial diseases

The most common bronchial diseases that affect the bronchial glands are chronic bronchitis and asthma. However, the clinical and pathological definitions often overlap significantly between the two conditions (40).

### **1.2.1.** Chronic bronchitis

Chronic bronchitis is a common disease that affects about 10-25% of the adult population. It has a higher prevalence in persons 40 years or older as well as in men compared to women. Chronic bronchitis is well documented in workers with jobs where there is exposure to dust and in individuals living in highly polluted cities. The frequency of chronic bronchitis in industrial workers is generally double that of the normal population (41).

Chronic bronchitis generally develops following chronic irritation of the airways after prolonged inhalation of exogenous particles such as those present in cigarette smoke (90% of chronic bronchitis patients are smokers) in man, grain dust, silica particles (42), sulfur dioxide or other air pollutants (43).

In domestic animals, chronic bronchitis has been reported in cats and dogs. In dogs, chronic bronchitis is most common among middle to older-aged dogs (44,45) and is more common in small breeds (43,45). Chronic bronchitis is clinically characterized by repeated episodes of cough that last at least three months in two consecutive years (42, 43) and is typically accompanied by excessive mucus secretions (41,42).

Productive cough is the major symptom of chronic bronchitis as a result of the irritation as well as the accumulation of mucous secretions in the airways. Airway obstruction may be accompanied by dyspnea. More severely affected patients may also experience hypercapnia, hypoxaemia, and cyanosis (41,43,46).

Prolonged bronchial irritation leads to a series of changes in the mucosa which culminates in chronic inflammation. Clinical investigations using bronchoalveolar lavage (BAL) have provided some insights regarding the cellular changes that take place in airways during chronic bronchitis. According to these studies the cellular composition of BAL fluid changes dramatically and affected individuals have significantly higher numbers of mononuclear cells, neutrophils (41,43) and sometimes eosinophils (41).

Mucus hypersecretion in chronic bronchitis results from hypertrophy of submucosal glands and hyperplasia of goblet cells (42). Enlargement of submucosal glands is not only because there is an increase in the number of the cells, but also because of an increase in the cell size. Some studies have shown a slight increase of the proportion in mucous cells at the expense of serous cells, although this change was not statistically significant (41).When the number of serous cells is reduced, the respiratory tract is more prone to bacterial colonization, and not surprisingly, patients with chronic bronchitis are more susceptible to secondary bacterial infections (23,24).

There is still controversy regarding the significance of bronchial smooth muscle in chronic bronchitis. Some studies reveal increased volume density of bronchial smooth muscle in patients with chronic bronchitis; however, this change was not general, but restricted to the bronchus of the left lower lobe (41). Increase in the smooth muscle mass is due largely to hyperplasia of the muscle fibers (24,41).

Prolonged bronchial injury is accompanied by epithelial cell proliferation, hyperemia, and mucosal edema. In severe cases polyps may form and protrude into the bronchial lumen (43). These changes are not only in larger bronchi but also in smaller bronchi and bronchioles. Prolonged exposure to irritants in airways smaller than 2-3mm

in diameter alters the epithelial mucosa, eventually culminating in goblet cell metaplasia. The term metaplasia is preferred over hyperplasia because healthy bronchioles lack goblet cells. In chronic bronchiolar injury, Clara cells normally present in the mucosa disappear and are replaced by goblet cells. This metaplastic change causes excessive production of mucus in a region of the airways with a very limited capacity to clear secretions by the mucociliary apparatus (42). As a result, mucus accumulates in the bronchiolar lumen forming large plugs that eventually cause airflow obstruction and severe respiratory signs (42,43).

Bronchiolar mucosa undergoing persistent injury develops squamous metaplasia, dysplasia, lymphocytic infiltration and fibrosis of the bronchiolar walls (42) and irreversible destruction with dilation of bronchi and bronchioles, a condition known as bronchiectasis (24). There can be a collapse in the main bronchi and segmental bronchi during expiration (44) because of weakness of the bronchial wall due to chronic infection (43). Finally, emphysema is the dominant lesion after moderate to severe airway obstruction. This later change develops when the damage reaches the gas exchange parenchyma distal to terminal bronchioles and it is characterized by permanent enlargement of the airspaces with destruction of alveolar walls (42). Interestingly, emphysema is rarely seen in dogs (43).

### **1.2.2.** Bronchial asthma

Asthma is a chronic inflammation with widespread contraction of bronchi that affects about 5 % of the human population. It has a higher prevalence in young people (3). The prevalence of asthma has gradually increased in the last thirty years (41), especially in western countries (42), even though some other studies show little change in

prevalence for some other areas of the world (41,47-50). Risk factors associated with asthma include atopy, genetic predisposition, and severe respiratory infections during the early days of life (41). In general, there are some factors that can trigger but do not initiate the disease such as bacterial and viral infection (41). The largest population of asthma-related fatalities occurs in older patients in spite the fact that asthma is much more prevalent in young people (7).

In domestic animals, cats are the most predominant species affected with asthma. Cats at risk of developing feline asthma are generally young to middle age (51) ranging from two to eight years. Females appear to be more susceptible than males (52,53). Some studies show that the Siamese breed is overrepresented (51,52). Qualitative factors in the feline pulmonary system make cats more susceptible to asthma: airway smooth muscles are more abundant; the ratio of smooth muscle to the bronchial wall thickness is larger in cats than in any other species; and ciliated epithelial cells extend further down the airways (55).

Generally, asthma is defined as chronic inflammation with reversible narrowing of the airway caused by inhalation of an allergen. Asthma is clinically characterized by wheezing, dyspnea, and cough (42,56) especially at night and early morning. Asthma can be triggered (but not caused) by exposure to cold weather (42) or exercise (42,57). *Mycoplasma* microorganism has been associated with the disease in cat and man (24,58). One study found that 4 out of 9 cats with asthma had *Mycoplasma* isolated from the lungs, suggesting that this organism may exacerbate bronchial edema and contraction of airways (51,52). Bronchial asthma is rarely fatal but some individuals can die suddenly

during an episode of status asthmaticus (42) or due to complications with secondary infection (59).

The pathogenesis of bronchial asthma has been studied for many years and there are still many enigmas to be resolved. A genetic predisposition to type I hypersensitivity may be the underlying mechanism which leads to airway inflammation and remodeling (42,59). The relationship between asthma and genetics has not been studied or documented in feline asthma other than through breed predisposition previously mentioned (51-54).

There are many putative effector cells involved in the pathogenesis of asthma including; eosinophils, mast cells, macrophages, T lymphocytes, neutrophils, and epithelial cells. The immediate phase of asthma starts when allergens like pollen are inhaled. Allergenic substances are first trapped in the mucosa and then carried to the lymph nodes by antigen presenting cells (APCs), stimulating a Th2 response and releasing cytokines. These cytokines stimulate B lymphocytes to produce IgE which binds to specific receptors on mast cells that are normally present in the bronchial submucosa. Once stimulated by the binding of antigen to IgE, the activated mast cells release pro-inflammatory cytokines that cause influx of neutrophils, macrophages, lymphocytes, basophils and eosinophils into the airways mucosa (42).

#### **1.2.2.1.** Cellular changes

Mast cells are one of the most important effector cells in asthma and responsible for many of the inflammatory changes and clinical signs seen in asthmatic patients. Once activated, mast cells release potent vasoactive mediators (42) such as histamine (41) that produces bronchoconstriction on bronchial smooth muscles, dilates bronchial blood

vessels, opens the intercellular tight junctions of the endothelial cells increasing vascular permeability (edema), and has a small effect on epithelial cells causing an increase of mucous secretion. In addition, initial inflammation facilitates deeper penetration of antigen to submucosal mast cells which stimulate vagal receptors causing further bronchoconstriction, one of the cardinal changes of this disease. A recent study supports the argument that infiltration of mast cell in the bronchial smooth muscle relates to airway hyperresponsiveness. This association seems to be independent of the disease severity, treatment, and airway wall structural remodeling (60).

When eosinophils are activated, they produce cationic proteins which lead to epithelial damage and increased responsiveness in the smooth muscle (41). This inflammatory reaction causes oxidative stress and further contributes to airway injury (57). Even though eosinophils infiltrate and degranulate in the airways during asthma, these cells are not specific markers for asthma (41).

#### **1.2.2.2.** Bronchial glands

Bronchial glands are involved in the pathogenesis of asthma. Change in bronchial gland size is associated with asthma; moreover, patients that die of status asthmaticus after a short period of asthma symptoms have larger bronchial gland measurements (41). A recent study found that the ratio of the myoepithelial cell area to the total bronchial gland area is increased significantly in fatal asthma compared to the non-asthma control. Electron microscopy of fatal asthma cases showed hypertrophy of myoepithelial cells with increased intracellular myofilament actin (31).

#### 1.2.2.3. Bronchial lesions in asthma

Gross lesions in asthma are rarely seen in autopsies except in patients with severe asthma whose lungs are overdistended and have small areas of atelectasis and thick mucus plugs in major airways (42,51). Histologically, the lungs of patients with asthma show typical changes of bronchial remodeling including thick basement membrane, increase in size of submucosal glands and bronchial smooth muscles (42). Eosinophils and mast cells are often seen in the bronchial mucosa (42,59).

Sloughing of the bronchial epithelium is frequently seen in bronchial biopsies taken from asthmatic patients. Some studies indicate that the degree of desquamation is more severe in patients with persistent asthma than in those with intermittent asthma. Loss of epithelium in the bronchial mucosa exposes nerve endings, leading to mediator release by subepithelial cells (61). The sputum of asthmatics contains clusters of detached epithelial cells known as Creola bodies. In case of complete desquamation of the epithelium, the basal cell layer regenerates resulting in squamous metaplasia (41).

One of the most characteristic microscopic changes in the bronchial asthma is the thickening of the basement membrane. However, this change is not specific for asthma since it also can be seen in other bronchial diseases such as chronic bronchitis and bronchiectasis (41). The bronchi of asthmatic patients have also increased numbers of fibroblasts in the lamina reticularis which appears to correlate with the thickness of the subepithelial layer (41).

Accumulation of mucus plugging the airways is another morphologic change frequently seen in asthmatic patients, particularly those who die in status asthmaticus. Autopsy reports in these fatal cases indicate that the airway lumen is plugged with thick

tenacious mucous and this mucous secretion extends to the segmental airways but rarely reaches the respiratory bronchioles (41). The mucous plug contains large numbers of crystals composed of eosinophilic membrane proteins known as Charcot-Leyden proteins, and also forms coiled mucus casts that contain shed epithelium known as Curshmann's spirals (42). The sputum of asthmatic patients has a distinctive increase in viscoelasticity which is due to an increase of glycoprotein concentration and serum protein (41).

In severe cases of asthma, there is excessive production of mucus due to hyperplasia of bronchial glands and goblet cells causing airway obstruction and trapping of air in the lung. Mucus obstruction of some airways also has a valve effect, creating an imbalance between air entering and exiting the lung, which eventually leads to atelectasis (51). As the disease progresses inflammatory and obstructive changes result in airway fibrosis and parenchymal destruction leading to emphysema and loss of pulmonary elasticity (62). In addition, the bronchial walls are weakened by chronic inflammation resulting in irreversible bronchial dilation, a condition known as bronchiectasis (51).

### **1.2.3.** Glandular changes in bronchial injury

#### **1.2.3.1.** The bronchial secretion and the mucociliary transport

Mucociliary transport is the physical movement of mucus in the respiratory system by which the deposited particles and gases are removed. The process of mucociliary clearance is the most important defense mechanism of the conducting system and involves the nasal cavity, trachea and bronchi. Mucociliary transport is facilitated by the mucociliary escalator (59) composed of ciliated cells and a mucous bilayer lining of the airway surface (63).

The mucus is a complex non-homogenous mixture (64) of water, glycoprotein, immunoglobulins, lipids, and electrolytes. This mixture is formed by goblet (mucus) cells, serous cells, and submucosal glands. The upper layer forms the gel and the lower layer, which is in direct contact with the cilia (59), forms the sol (59,64). Both layers have glycoproteins with different features. The sol layer acts as a liquid and is characterized by low viscosity and elasticity as it has less oligomerized glycoproteins. The gel layer is highly viscous and elastic (65).

The gel layer is transported partly by the coordinated beating of the cilia (65). Each ciliated cell in the conducting system has around 250 cilia (59).The cilia beat continuously forming a series of waves with each wave formed at approximately 1000 strokes per minute (59). The strong rapid movement of cilia transports the gel layer from the airways (64,65) toward the oropharynx where it is swallowed (65).

#### **1.2.3.2.** Glandular hypertrophy

Chronic bronchitis and asthma are hypersecretory diseases characterized by excessive mucus secretion from the submucosal glands and to a lesser extent from goblet cell hyperplasia or metaplasia. Reactive glands increase in size in trachea and bronchi, and this glandular enlargement can be estimated by calculating the relationship between the size of the glands relative to the size of the bronchial wall. This gland to wall relationship is known as the Reid index. The Reid index in a normal human bronchus is less than 0.4 (42), while patients with chronic bronchitis have indexes as high as 0.7 (66).

Mucus hypersecretion is a feature of chronic bronchitis and asthma. Mucus hypersecretion in the trachea and bronchi results mostly from the hypertrophy of

submucosal glands (42). Enlargement of submucosal glands is due to an increase in the number and size of the cells.

**1.3.** Morphometry

The linear measurement and 2-dimensional measurement have been used in human pathology to quantify the morphologic changes in bronchial diseases. These two quantitative methods permit meaningful statistical comparison between normal and diseased groups. Before dealing with application of linear measurement and 2-D measurement in bronchial pathology, it is necessary first to review their origin and relevance in biomedical science. The linear measurement was first proposed by Reid in 1960 to objectively evaluate the severity of bronchial hyperplasia by means of measuring the bronchial gland to bronchial wall thickness (66).

Stereology is a branch of science that uses a two-dimensional planar section to predict three-dimensional components in an object (67). Stereology was first established by the geologist Delesse in 1848 to approximate the contents of a particular mineral in a rock. For instance, it was used in mining to determine the content of gold or quartz in rock. Starting in 1916 geologists used stereology (Delesse principle) to study rock composition (68).

Galgolev in 1933 (69) and Thomson in 1930 (70) were the first to emphasize that volume density can be estimated by a random point counting. Chalkley in 1943 (71) applied this geological concept to biology and in particularly to histology. Many researchers have published applications of stereological methods to cell biology and medicine, most notably Weibel and his group (67).

Stereological methods generate numerical values as continuous variables that could be accurately analyzed by statistical methods. These are powerful tools that yield unbiased quantification and can be used for any organelle, cell, tissue or organ in the body (67).

**1.3.1.** The Reid index by linear measurement

Reid index (RI) is the ratio of bronchial glands to the bronchial wall. RI can be calculated by a linear measurement with a one dimensional level that assesses the bronchial gland thickness. This method had been used in human pathology to compare the severity of bronchial hyperplasia between patients with bronchitis and healthy individuals. Even though the Reid index is widely used to study bronchial diseases, there are still concerns about the variability of results in this method. Objective decisions about the measurement should be taken for consistency when assessing the ratio to minimize inter-and intra-observer variation (41).

There are many sources of error when estimating the RI. There are normal variations in the size of the glands not only between healthy individuals but differences in various regions of the same lung. Furthermore, glands in the same bronchus may vary in size. Another source of variation detected in the RI is the method in which the measurements are made. For instance, some bronchial glands are located between the basal lamina and the internal aspect of the cartilage while others may be behind the cartilage (41), the split between the perichondrium and the cartilage can lead to an error since the exact point for the measurement is not easy to recognize (72). Some authors suggest that the glands that lie outside cartilage should not be measured (41) and the internal border of cartilage should be taken as a starting point in all measurements (72).

The bronchi should be excluded from the calculation when there is separation of subepithelial tissue from cartilage. Some researchers recommended limiting the measurement in bronchi that range in size from 2.5-7.5 mm because in bronchi below 2.5mm it is not easy to find measurable glands, and the differentiation between the obstructive and non-obstructive airways is difficult (73). The bronchi larger than 7.5 mm show a higher variability between normal and abnormal lungs (73). In the site of measurement, bronchial wall should be measured on the exact line used to measure bronchial glands, and the cartilage should be parallel to the epithelium (41).

In addition to assessing the RI on human bronchial glands, RI has been evaluated on feline tracheal glands. Jeffery in 1978 determined the RI in tracheal glands of four pathogen-free cats and found an index of 0.65 (74) which is higher than the value of RI in human (.33) (66). Unfortunately, the bronchi were not evaluated in this study. A search of several databases did not show any papers in which the linear measurement had been applied to the feline bronchi to date. Reid had found that linear measurement of mucus glands in normal bronchi ranged from 0.14-0.36 with average of 0.26 and that the ratio is larger in young children than in older children and adults. In chronic bronchitis, the linear measurement ranged from 0.41-0.79 with average of 0.59 with no overlap between healthy people and people with bronchitis (41). Other studies had found that linear measurement overlapped in patients with bronchitis and patients without bronchitis (75) and the increase in the linear measurement in chronic bronchitis is small (66,73). There is quite a difference in the range of the ratio in other studies as reported by Scott (76) who found that the mean of linear measurement in patients with bronchitis was 0.49, while it was 0.40 with others without bronchitis (41). These results were similar to those

achieved by Thurlbeck and his colleagues (0.52 in people with bronchitis and 0.40 in people without bronchitis) (77).

**1.3.2.** Reid index by two-dimensional measurement

One of the methods that are used to estimate the bronchial gland thickness is the two-dimensional measurement using point count stereology. Point count stereology is a multipurpose technique. It has two dimensional measures in which a lattice can be applied on the bronchi to assess mucous gland proportion (41).

There are some advantages in using this technique on bronchi, for example, glands that are located behind the cartilage can be counted; artifacts such as separation between subepithelial mucosa and cartilage can be omitted from the bronchial wall; and the involvement of two dimensional measures makes the method more meaningful for assessing the ratio of bronchial glands to the bronchial wall. Random points fall on all glands, not just selected glands, so a total proportion of the glands is measured with no bias. 2-D measurement can provide information about other components in the bronchial wall such as the proportion of the cartilage or muscles, in addition to glands (41). A mucous gland proportional method is able to discriminate between patients without bronchitis and patients with bronchitis. 2-D measurement can be used on biopsy specimens; however additional tissues should be taken as multiple random samplings are required (78).

Some disadvantages of 2-D measurement are: it is difficult to determine the boundary of the bronchial wall; any change in the points that fall on the wall will change the proportion (41); and it is a time consuming technique (78).

Reid index using 2-D measurement on lungs of people without bronchitis ranges from 7.6-16.7% with an average of 12.7%, while that people with bronchitis has an average of 27.8%. There are some variations among authors such as Mullen and his colleagues that had found the proportion of the glands using 2-D measurement in the central airways is 4-5% in lungs without bronchitis (79) while Scott and his colleagues found that 2-D measurement is 33% in lungs with bronchitis (76). Some studies found that there is good correlation between linear measurement and 2-D measurement such as Scott ($r = 0.67$) (76), and Takizawa and Thurlbeck ($r = 0.86$) (78), while Jamal and his colleagues did not find a correlation between the two methods when there is a severe obstruction in the bronchial airways (80).

A stronger correlation with more accurate results was found in 2-D measurement of patients with chronic bronchitis as compared to the linear measurement. Also there was a stronger correlation between 2-D measurement and amount of sputum produced in patients with chronic bronchitis as compared to the linear measurement (41).

### **1.3.3.** Morphometry in cats

Linear measurement and 2-D measurement have been used in human pathology (76). Those measurements help human pathologist to systemically assess the glandular changes in bronchial injury. On the other hand, there have been no systematic evaluations of glandular changes in bronchial injury of cats to date.

## **1.4.** Objectives

The objectives of this investigation were: 1- establish the normal bronchial gland to wall ratio (Reid index) in cats; 2- evaluate and compare two different methods of bronchial measurements, linear and 2-D measurements; 3- evaluate and compare the

effect of bronchial sectioning, cross and longitudinal sections; 4- evaluate and compare the RI when cartilage is included or excluded from the calculations; 5- investigate if there are differences in the RI between pulmonary lobes in cats; and 6- determine the minimal number of bronchial measurement required for proper determination of the RI.

**1.5.** Hypothesis

The Reid index can be used to objectively evaluate the feline bronchial glands.

## 2. Materials and Methods

### 2.1. Cats

Fifty eight clinically healthy cats obtained from the PEI Humane Society were humanely euthanized by intramuscular sedation with Ketamine/Xylazine, followed by intravenous administration of barbiturates. All cats had been clinically evaluated and observed by the PEI Humane Society veterinarian. Following euthanasia, the cats were transported to the diagnostic laboratory of the AVC, weighed and put in the postmortem cooler until postmortem examination. Forty one cats were used in the study , while seventeen cats were excluded because their lungs tissues were either inflamed or did not meet the criteria for bronchial morphometry.

#### 2.1.1. Necropsy

A routine postmortem examination with emphasis on the thoracic cavity was done in each cat within 24 hours of arrival at the AVC. Cats were placed in left lateral recumbence and digital photographs were taken with an identification number. After dissecting the skin through the midline, the thoracic cavity was opened by cutting with scissors along the costo-chondral and costo-vertebral junctions and the diaphragm. Once the thorax had been opened, the lungs were visually examined, palpated and photographed *in situ* with their identification number.

#### 2.1.2. Heart

After removing the pericardium, the heart was resected at the base, separated from the remaining thoracic pluck and blood from the cardiac chambers was removed by flushing the heart with tap water. The heart was weighed using a digital scale and then

placed *en toto* in 10% buffered formalin. The heart to body weight ratios were calculated in all cats.

**2.1.3.** Lungs and bronchial swabs

The lungs used for the cross sectional study (n = 14), along with the trachea and esophagus (thoracic pluck) were removed from thorax and placed on a plastic dissecting board. The trachea was cut longitudinally with scissors along the midline starting approximately 5 cm cranial to the bronchial bifurcation. After the tracheal lumen had been exposed, a sterile swab was carefully introduced into the airway and passed caudally slightly beyond the bronchial bifurcation. The mucosa of a main extra-pulmonary bronchus was swabbed (one swab per cat), and the swab was sent immediately for routine bacteriological culture to the bacteriology laboratory, Diagnostic Services, Atlantic Veterinary College.

**2.1.4.** Lung lobes fixation and sectioning for histopathology

The lungs were fixed *en toto* by simple immersion in 10% buffered formalin to mimic the routine postmortem technique typically done in diagnostic laboratories. Once in the formalin, the lungs were placed on a shaker and left in the fixative for at least 24 hours.

Two studies were conducted to determine the method of choice that would provide the best bronchial sections for microscopic examination. Twenty seven pairs of lungs were trimmed longitudinally by sagital sections along the bronchial axis, and fourteen pairs of feline lungs were trimmed, by slicing transversally (cross sections) at right angles to the main bronchi (Fig 1). A third study was done slicing the lungs parallel to a plastic probe previously introduced into the major airways of the fixed lung (Fig 2)

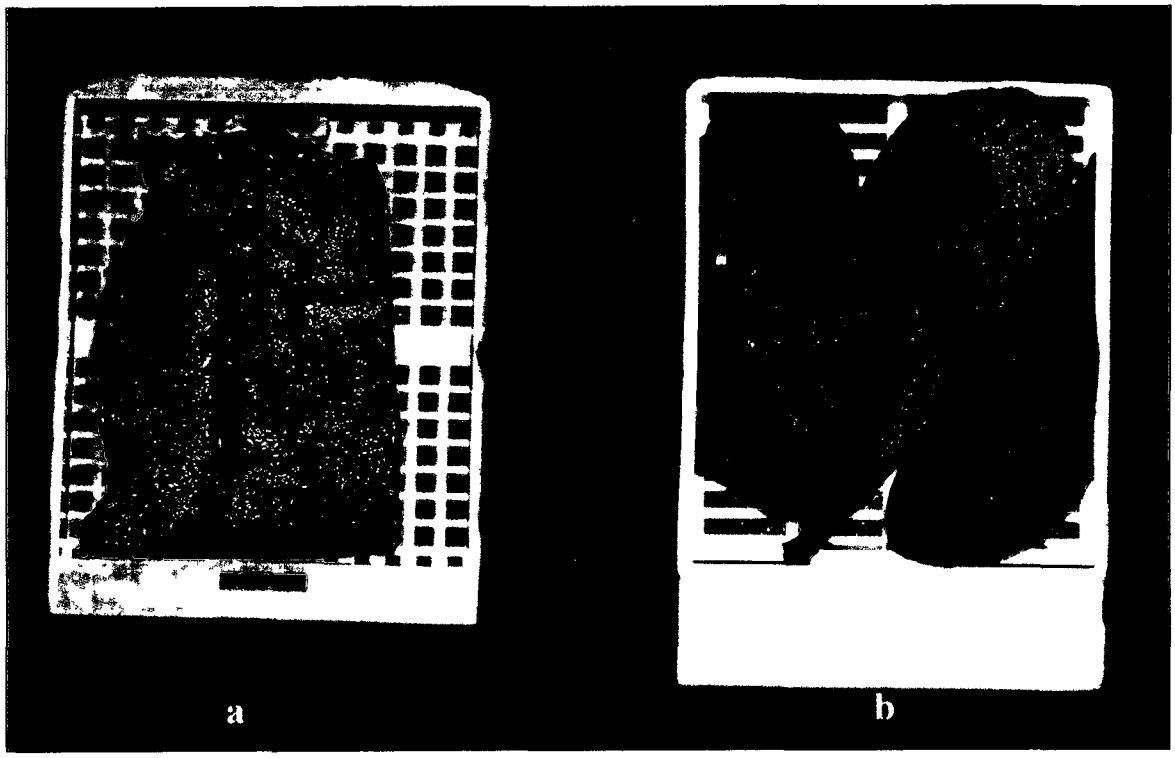



Figure 1. Sections of the lung lobes. (a) Lung lobe sliced longitudinally (saggital section). (b) Lung lobe sliced transversally (cross section).

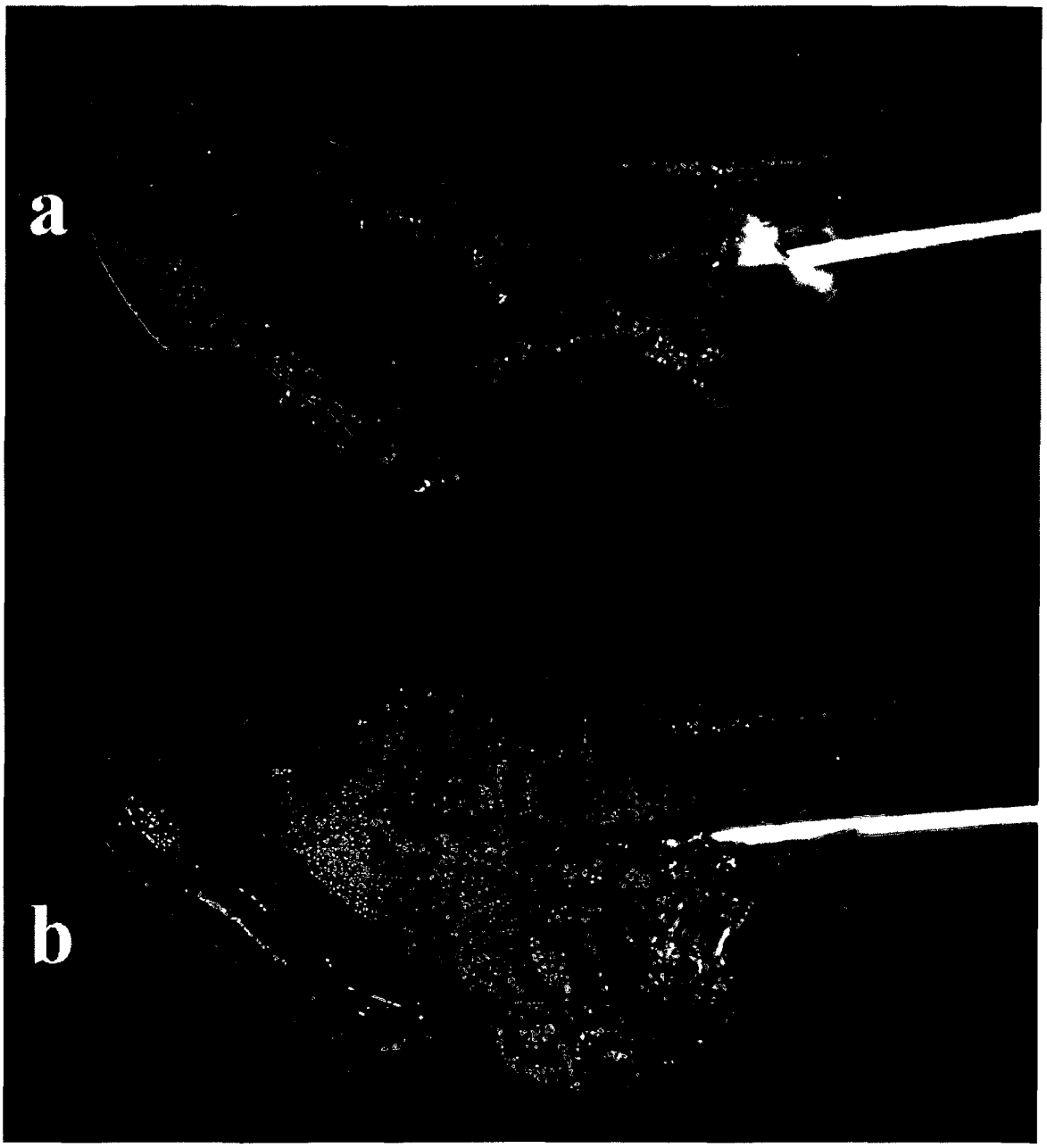



Figure 2. Slicing the lung lobe longitudinally using a plastic probe. (a) Introduction of the plastic probe to the major airway of the lobe. (b) Slicing the major airway parallel to the plastic probe.

as recommended for gross examination of the bronchial tree in human pathology (7). Lung lobes from seven cats were used for this study, these cats were also used in the study of longitudinally sectioned lung. The plastic probe was introduced to pieces of these lungs and the rest of the same lungs were used for longitudinal sectioning (without probe n = 27). The total number of cats in these studies is 41.

**2.1.4.1.** Cross sections of lung lobes

Samples of six lung lobes were taken from 14 cats as follows: right apical (R-AP), right intermediate (R-IN), right accessory (R-AC), right diaphragmatic (R-D), left apical (L-AP), and left diaphragmatic (L-D). The first transverse section was done at the entrance of the main bronchi into the lung (Fig 3). The second section was taken half way from the margin of the first section to the terminal airway at the opposite margin of the lung. The third and last sample was taken at the middle of the remaining lung lobe. All three sections were placed in individual cassettes and labeled with the cat number, lung lobe, and side of the lung (right or left).

**2.1.4.2.** Longitudinal sections of lung lobes

The pulmonary lobes from 27 cats were cut longitudinally starting at the origin of the main intrapulmonary bronchus to ensure that most of the bronchial airway was present in one slide. These longitudinal sections were obtained from the lung lobes as follows: right apical (R-AP), right intermediate (R-IN), right accessory (R-AC), right diaphragmatic (R-D), left apical (L-AP), and left diaphragmatic (L-D). Each section was placed individually in a plastic cassette, labeled and submitted for tissues processing, sectioning and staining.

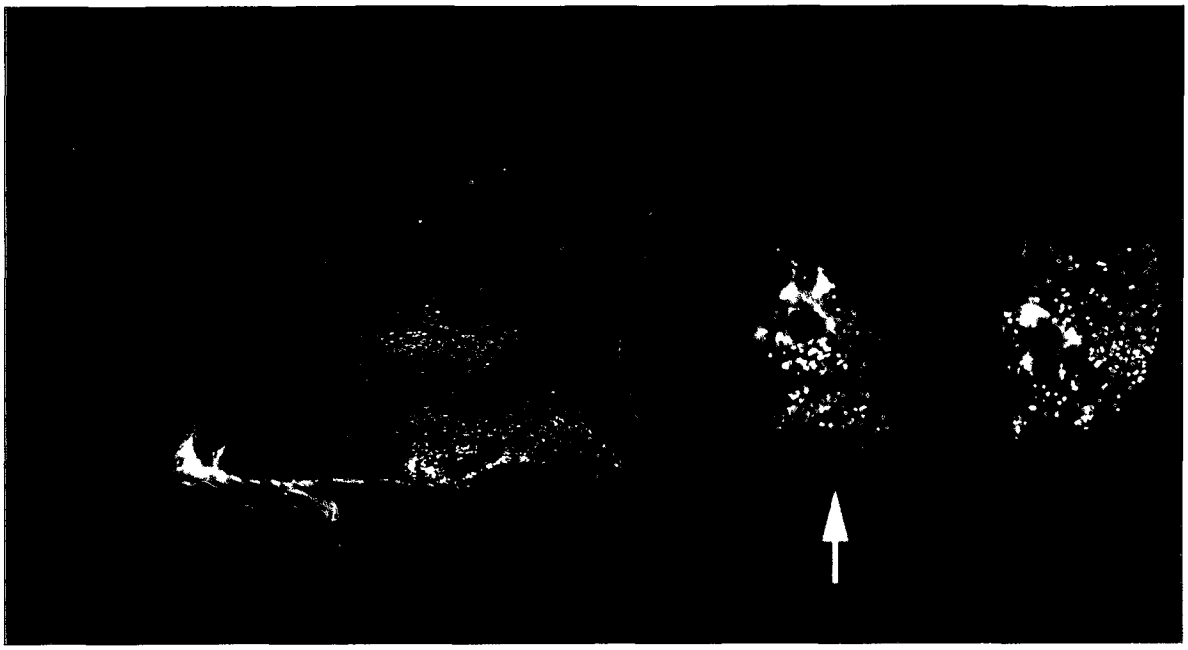


Figure 3. Slicing the lung lobe transversally. The first transverse section of the lobe close to the entrance of the main bronchi into the lung (arrow).

**2.1.5.** Tissue processing

Fixed lung samples, whether cross or longitudinally sectioned, were processed routinely, embedded in paraffin, cut at 5-6 μm-thick and stained with hematoxylin and eosin (H&E) (81) at the diagnostic laboratory of AVC. Periodic acid-Schiff (PAS) stain was used in selected lung sections for the detection and confirmation of mucus in the bronchial lumen (82).

**2.1.6.** Selection criteria for histologic slides of the lungs

Although all the fifty eight cats submitted to AVC by the PEI Humane Society had been found to be clinically healthy prior to euthanasia, a microscopic screening of all lungs was conducted to exclude seventeen cats that showed evidence of lung diseases such as chronic pneumonia, alveolitis, bronchial diseases or congestive heart failure.

The lungs selected for this study were microscopically classified in two groups: 1- Lungs with unremarkable bronchi, bronchioles, and alveoli here after referred to as lungs with no lesions (n = 28); and 2- lungs with minor bronchial or bronchiolar changes characterized by the presence of PAS-positive material in the lumen here after referred to as lungs with minor (incidental) lesions (n = 13). The lungs with mucus, pulmonary edema, and foci of inflammatory cells in airways were lungs with one single bronchus affected or only a few bronchi affected. The normal heart: body weight in cats ranges from 0.28% to 0.88%, and cats with abnormal heart: body weight ratios suggesting compensatory heart failure or feline cardiomyopathy were excluded (83).

**2.1.7.** Digital images of bronchi

Images of the bronchi from all cats were taken using a digital microscope at magnification of 20 x with a scale bar of 100μm embedded in the picture (Carl Zeiss

axioscope A1, modular microscope, LTD, Canada). Images were individually coded according to cat number, lobe and side of the lung (left or right). For subsequent morphometric analyses, images were stored digitally as JPG format without any digital cropping or manipulation (Fig 4). There were approximately 8 to 146 images per lobe for cross section cassettes and 6 to 35 images per lobe for longitudinal section cassettes. Two methods of measurements (linear and two-dimensional measurements) were used per image to calculate Reid Index(RI).

**2.1.7.1.** The Reid Index Calculated by linear measurement (Image J)

The Reid Index (RI) or gland to wall ratio was determined using linear measurement in both cross and longitudinal sections of the bronchi using a commercial software package called Image J which was developed by the National Institute of Health (NIH) <rsb.info.nih.gov/ij/>. Similar to what has been previously indicated, the lungs showing inflammation were excluded. The lungs in which the mucosa had been completely separated and lost from the lamina propria were also excluded from the study.

The wall and gland dimensions for bronchi cut on both cross and longitudinal sections were calculated using one single digital line that intersected both bronchial wall and bronchial glands. The gland thickness was measured on the widest view of the bronchial gland, and the linear measurement was calculated as the percentage of bronchial wall occupied by the gland.

The linear measurement in sections of bronchi was calculated using three different methods: 1. In images/ areas with a view of the cartilage the measurements were taken from the epithelial basement membrane to the inner edge of cartilage (Fig 5, a). In other words, the inner edge of the cartilage or perichondrium was only used as the

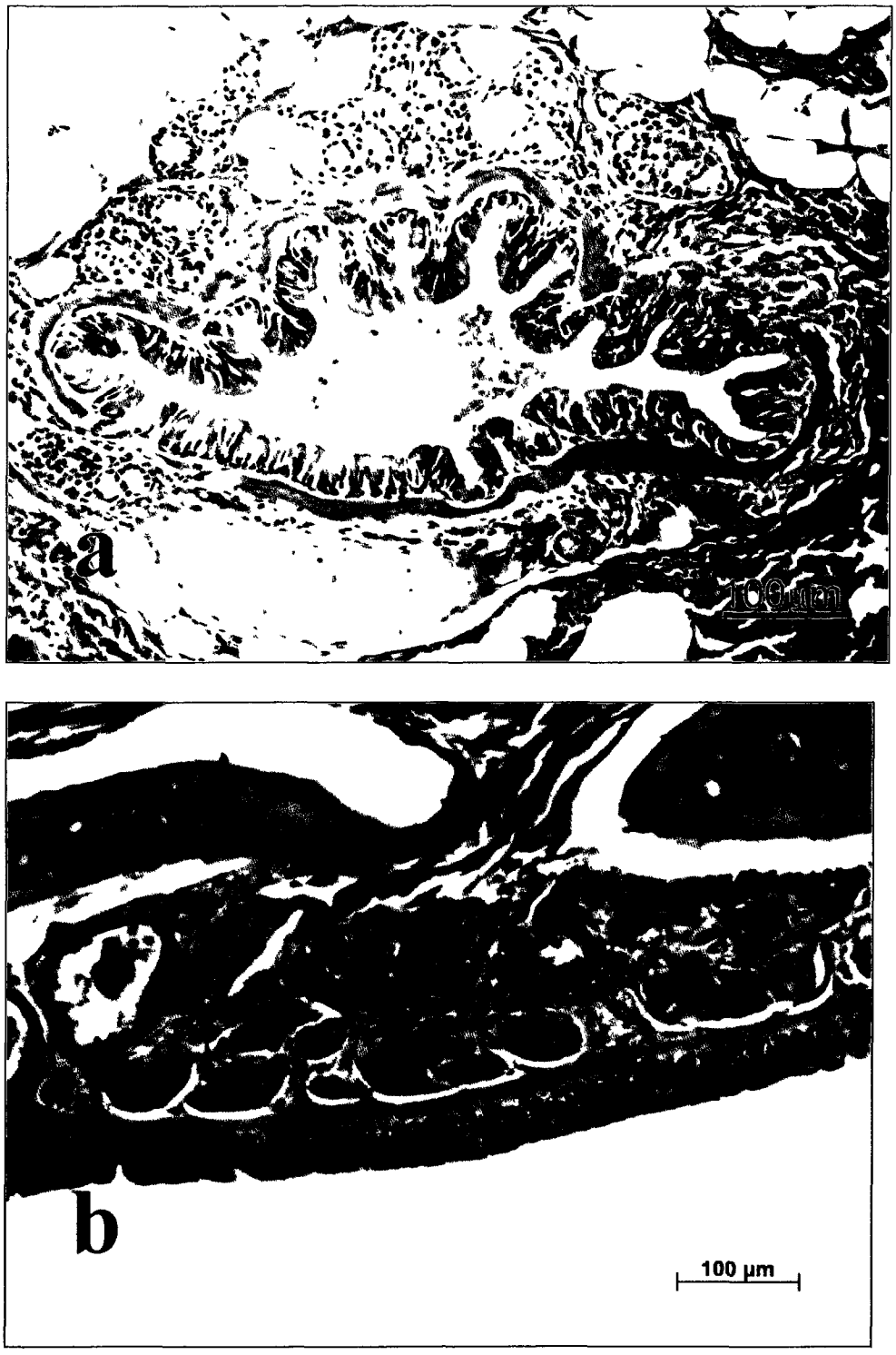



Figure 4. Photographs of bronchi. (a) Cross section of feline bronchus.

(b) Longitudinal section of feline bronchus.

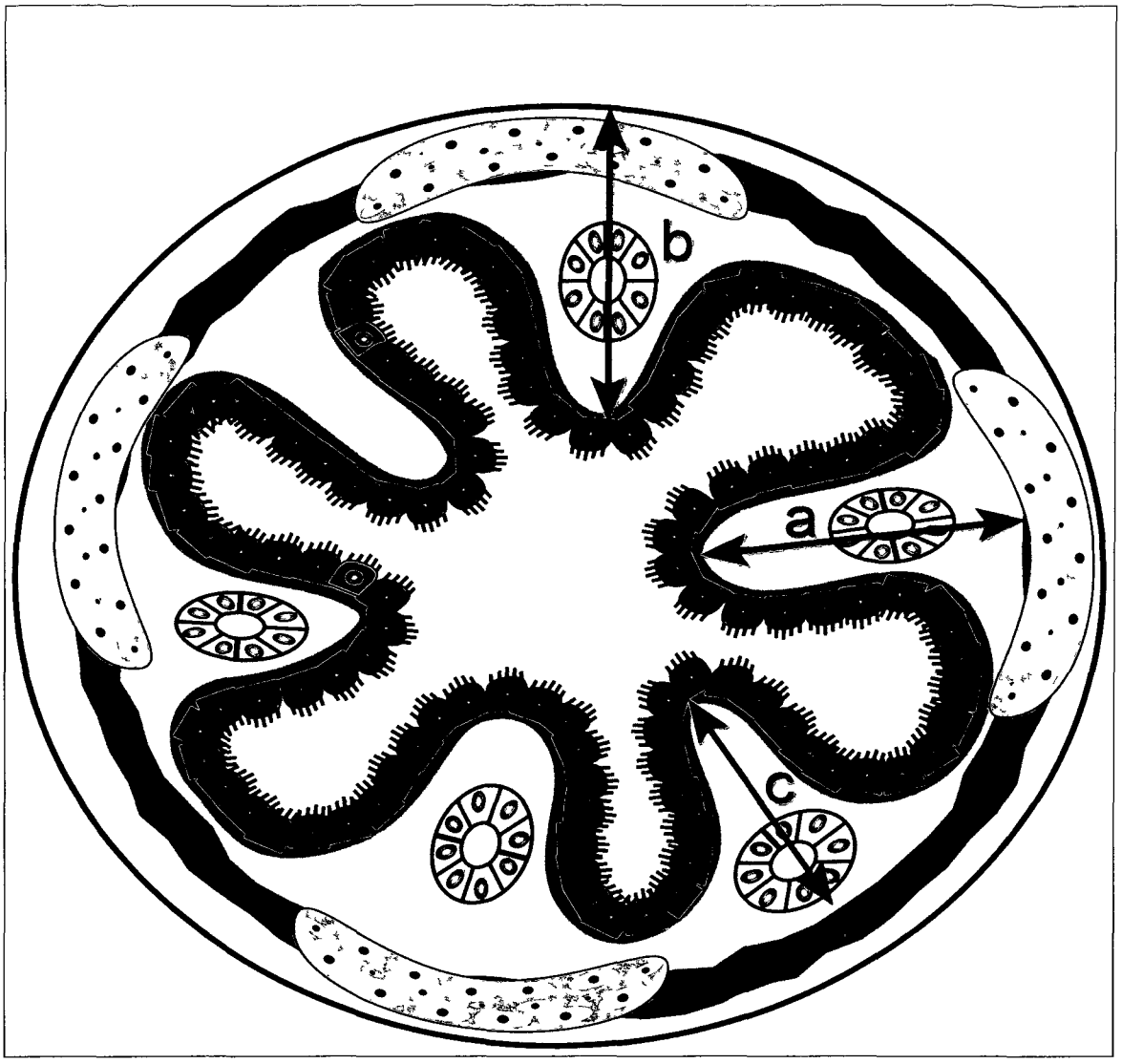



Figure 5. Diagram of a bronchus showing the gland/wall ratio taken by different types of measurements. (a) Areas of bronchi with cartilage, cartilage excluded from calculation. (b) The cartilage included. (c) Areas of bronchi without cartilage.

starting point for thickness measurements of the bronchial wall (80). 2. In images/ areas with a view of the cartilage the measurements were taken from the epithelial basement membrane to the outer edge the cartilage. In other words, the cartilage was included as part of the thickness of the bronchial wall (Fig 5, b). This measurement was excluded from the study when the statistical analysis revealed its inaccuracy, because adding cartilage introduced more variation to the RI. 3. In images/ areas without cartilage, the measurements were calculated from the epithelial basement membrane to the outer edge of the gland (at the widest point in the linear measurement). In other words, the external edge of the gland was the starting point for wall thickness calculations (Fig 5, c).

**2.1.7.2.** The Reid Index Calculated by 2-D measurement (Point Count Stereology (PCS))

The wall to gland relationship of the feline bronchi was also calculated by 2-D measurement using point count stereology (PCS) for both cross and longitudinal sections of bronchi. The relationship between the wall and glands was expressed as a bronchial gland proportion using commercial computer software (Bioquant nova prime image analysis software BQ-NVP-XP, Nashville, TN <www.Bioquant.com>). Digital images of bronchi taken at a magnification of 20 x were measured using a multipurpose test grid with 112 lines each measuring 1.00 cm in length and 224 test points. The grid was digitally superimposed over the image and the number of points falling in the bronchial wall and bronchial glands were counted as illustrated in Figure (6) and (7) (67). The bronchial gland proportion was analyzed by manual counts in the bronchi cut in cross section and by bioquant PCS computer program for

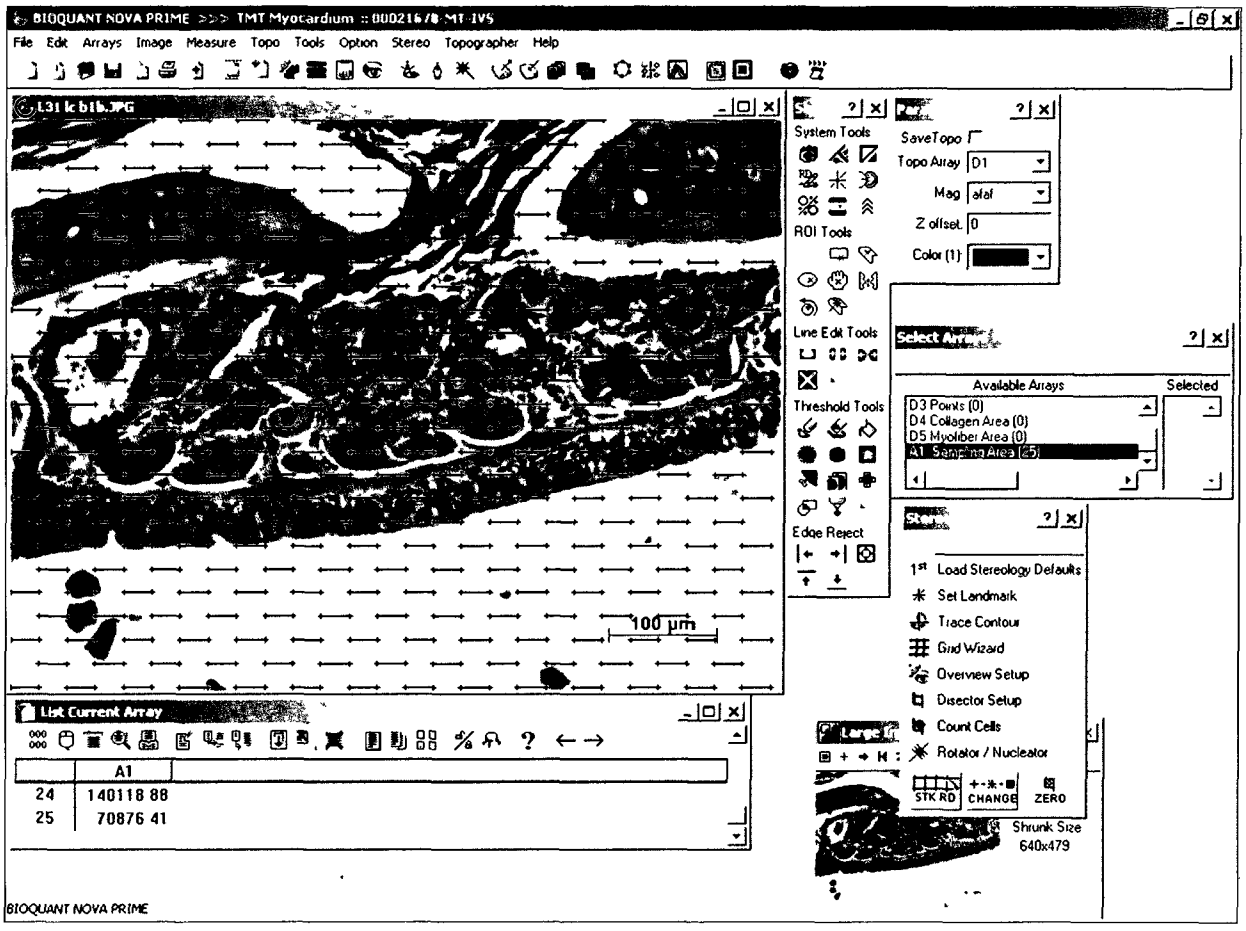



Figure 6. Computer screen illustrating a typical image in the Bioquant view to determine the proportion of the bronchial glands using 2-D measurement.

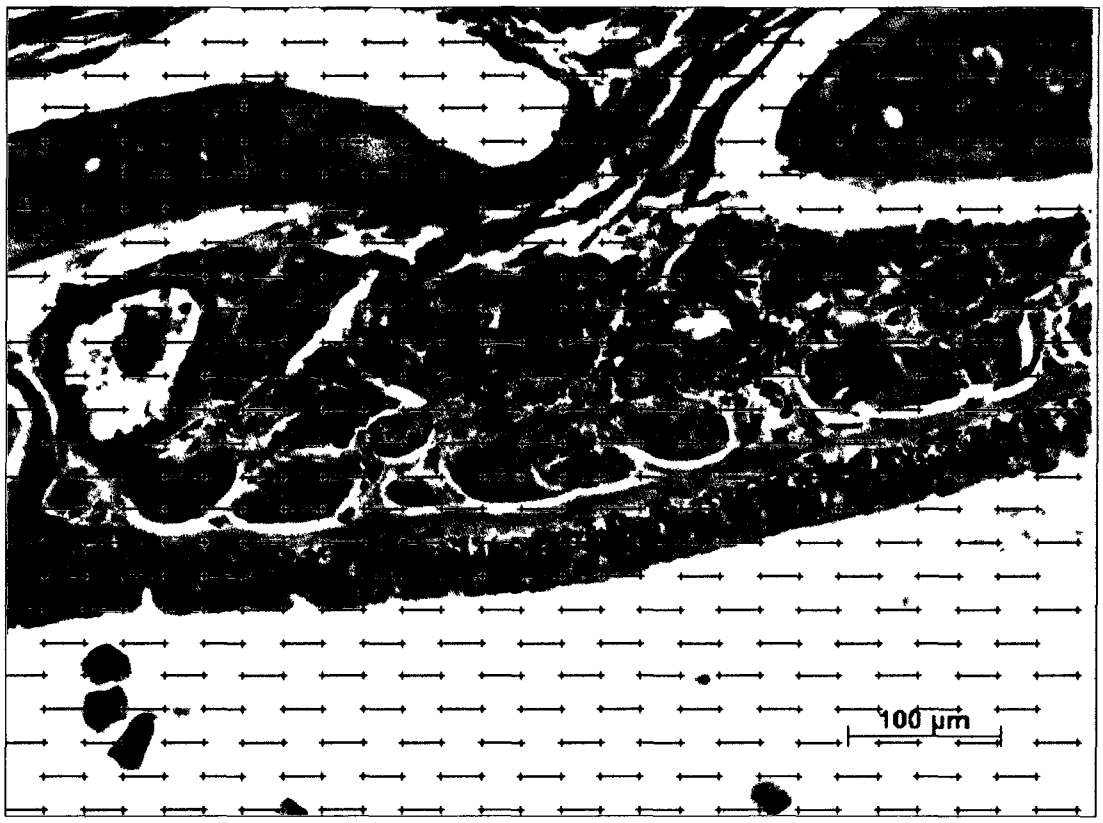



Figure 7. Computer screen showing test grid used to determine the proportion of the bronchial glands using 2-D measurement.

the bronchi cut longitudinally. The proportion of the glands relative to the bronchial wall was calculated also by the three different methods described in section 2.1.7.1 for the Reid index calculated by linear measurement (image J).

**2.2.** Statistical analyses

The linear measurement and 2-D measurement methods of the bronchi of lungs were expressed as means (the sample group mean is calculated from a list of the means of the measurements (bronchial gland to wall ratios) in each lobe) and standard deviations (SD), correlation between both methods were calculated by Pearson's correlation coefficient.

When the type of measurement was made in bronchial view without cartilage, the difference between the four sub-groups: cross cut with linear measurement, cross cut with 2-D measurement, longitudinal cut with linear measurement, and longitudinal cut with 2-D measurement in all lungs with and without microscopic lesions was evaluated by using generalized linear mixed model in STATA 10 as follows:

$Yijk = \mu + Ci + Caj + Ai + Bj + Eijk$

Where:

Yijk = Reid index (RI) on the *j* cartilage of the *i*-th lesion

$\mu$ = general population mean in each sub-group

C*i* = Effect of the *i-th* lesion (i = 1, 2 e.g. lungs with and without microscopic lesions)

Ca*j* = Effect of the *j-th* cartilage (*j* = 1, 2 e.g. measurements to the inner edge of cartilage and measurements without cartilage)

A*i* = additional variation of each individual cat

B*j* = additional variation of each lobe

E*ijk* =Random error associated with each observation ~ N (0,σ)

The differences between lobes in both groups with and without microscopic lesion, were examined with generalized linear mixed model

Yijk = μ + C*i* +L*j* + CL*ij* + Cu*i* + M*j* + CuM *ij* + Ca*k* + (C) *K* + E*ijk*

Yijk = Reid index (RI) on the *j* lobe of the *i*-th lesion and the *j* method and the *i* cut on the *k* cartilage

μ = general population mean

C*i* = Effect of the *i-th* lesion (i = 1, 2 e.g. lungs with and without microscopic lesions)

L*j* = Effect of the *j-th* lobe (*j* = 1, 2, 3, 4, 5, 6 e.g. six different lobes)

Cu*i* = Effect of the *i-th* cut (i = 1, 2 e.g. cross and longitudinal)

M*j* = Effect of the *j-th* method (*j* = 1, 2 e.g. linear measurement and 2-D measurement)

Ca*k* = Effect of the *k-th* cartilage (*k* = 1,2 e.g. measurements to the inner edge of cartilage and measurements without cartilage)

CL *ij* = interaction of lobe and lesion status

CuM *ij* = interaction of cut and method

(C)*k* = additional variation of each individual cat ( *k* = 1, 2,…, 83 e.g. cat number)

E*ijk* =Random error associated with each observation ~ N (0,σ)

The test was followed by Bonferroni P-values adjustment.

The differences between the two wall measurements; not including the cartilage (from basal lamina to the perichondrium) and including the cartilage (from the basal lamina to the outer edge of the cartilage) were statistically evaluated by Analysis of Variance (GLM-ANOVA) using Minitab according to the following model:

Yijk = μ + C*i* +L*j* + (C) k +E*ijk*

Where:

Yijk = the ratio of Reid index (RI) on the *j* lobe of the *i*-th lesion

$\mu$ = general population mean

C*i* = Effect of the *i-th* lesion (i = 1, 2)

L*j* = Effect of the *j-th* lobe (*j* = 1, 2, 3, 4, 5, 6)

(C)*k* = additional variation of each individual cat

E*ijk* =Random error associated with each observation ~ N (0,$\sigma$)

The number of repetitions needed for a meaningful estimate of the ratio was statistically analyzed using sampling simulation. A fixed number of measurements were randomly sampled for each cat, and the variability between measures (variance) was estimated. The sampling and analysis were rerun 1,000 times which provided a distribution of the inter-measurements variability for a set number of samples (i.e. M). The number of sampled measurements was changed for different value and the simulation rerun: n=2, 4, 6, 8, 10, 12, 14, 16, 18, and 20. When the number of measurements was lower than n, all measurements were included.

## 3. Results

### 3.1. Heart weights

The heart weights ranged from 6.8- 26.6 gm with an average of 14.3 ± 4.5 gm and the heart to body weight ratios ranged from 0.3 %-1.8 % with an average ratio of 0.5 % ± 0.2 (Table 1). All heart to body weight ratios were within the normal range (0.28 %-0.88 %) except one cat (1.77 %).

### 3.2. Microscopic findings of the lungs

Microscopic observation in this study demonstrates that the distribution and shape of the feline bronchial glands was notably inconsistent. In some sections the glandular acini appear closely-packed in a single glandular cluster, while in other cases, the glands appear as an irregularly branching cluster partially encircling the bronchial cartilage.

Sample selection criteria dictated that the feline lungs were essentially normal on histologic examination. The lungs of one third of the cats had one or more minor incidental microscopic lesions such as small amounts of free mucus in the bronchial lumen, pulmonary edema likely as a result of the barbiturate used for euthanasia, and some sporadic foci of inflammatory cells in the airways. For the purpose of this study, the cats were divided to two groups: 1- cats with lungs showing no microscopic lesions (n = 28), and 2- cats with lungs showing minor microscopic or incidental lesions (n = 13). The bronchi that were cut on cross section often showed artifacts such as exfoliation of the epithelial cells, excessive folding of the bronchial mucosa, formation of artifactual spaces between the submucosa and cartilage, and cartilages misaligned

Table 1. Body weights, heart weight and heart to body ratio in the lungs with no microscopic lesions and lungs with minor lesions. The lungs of cats with numbers 10 – 24 were cut in cross section, and those with numbers 51 – 83 were cut longitudinally.

| Cat ID | No lesions | | | Cat ID | Minor lesions | | |
|---|---|---|---|---|---|---|---|
| | Body(g) | Heart(g) | Ratio(%) | | Body(g) | Heart(g) | Ratio(%) |
| 10 | 2298.2 | 11.1 | 0.48 | 17 | 2326.1 | 13.3 | 0.57 |
| 11 | 2715.4 | 10.9 | 0.40 | 19 | 1324.4 | 6.8 | 0.51 |
| 12 | 2929.7 | 15.3 | 0.52 | 22 | 3717.2 | 14.8 | 0.40 |
| 14 | 3175.0 | 9.5 | 0.30 | 23 | 4503.2 | 19.9 | 0.44 |
| 15 | 2546.2 | 13.2 | 0.52 | 51 | 3127.0 | 14.2 | 0.45 |
| 16 | 2277.8 | 8.3 | 0.36 | 52 | 2870.0 | 16.4 | 0.57 |
| 18 | 1314.3 | 7.44 | 0.57 | 53 | 4626.0 | 26.6 | 0.58 |
| 20 | 3997.0 | 15.4 | 0.39 | 57 | 1952.0 | 9.0 | 0.46 |
| 21 | 6841.8 | 17.2 | 0.25 | 60 | 1079.0 | 19.1 | 1.77 |
| 24 | 5080.2 | 13.5 | 0.27 | 62 | 3484.0 | 17.4 | 0.50 |
| 54 | 3041.0 | 12.0 | 0.39 | 71 | 3336.0 | 9.3 | 0.28 |
| 55 | 2626.0 | 12.0 | 0.46 | 81 | 3061.0 | 14.3 | 0.47 |
| 56 | 3485.0 | 12.4 | 0.36 | 82 | 2704.0 | 11.9 | 0.44 |
| 58 | 2029 | 13.8 | 0.68 | | | | |
| 61 | 1351.0 | 7.9 | 0.58 | | | | |
| 64 | 5926 | 15.4 | 0.26 | | | | |
| 65 | 3584.0 | 18.5 | 0.52 | | | | |
| 68 | 3086 | 11.5 | 0.37 | | | | |
| 72 | 3404.0 | 11.8 | 0.35 | | | | |
| 73 | 3985.0 | 17.7 | 0.44 | | | | |
| 74 | 3640.0 | 10.6 | 0.29 | | | | |
| 75 | 4313.0 | 14.2 | 0.33 | | | | |
| 76 | 5071.0 | 22.1 | 0.44 | | | | |
| 77 | 6547.0 | 18.4 | 0.28 | | | | |
| 78 | 3331.0 | 12.1 | 0.36 | | | | |
| 79 | 4871.0 | 18.5 | 0.38 | | | | |
| 80 | 4617.0 | 14.1 | 0.31 | | | | |
| 83 | 4660.0 | 24.4 | 0.52 | | | | |

with respect to the mucosal surface. All these artifacts, besides the inconsistency in the location of the bronchial glands, caused a difficulty of applying the Reid index measurement on bronchi cut on cross section. In contrast, the bronchi that were cut longitudinally were largely free of sectioning artifact. Many bronchial cultures showed some bacteria, which likely represented postmortem overgrowth as there was no evidence of an inflammatory host response (Table 2). Histological examination of tissue of the third method study (longitudinal sections that were obtained by inserting the plastic probe) revealed extensive artifactual changes characterized by complete separation and translocation of the bronchial mucosa.

**3.3.** Gland to wall ratio in bronchi cut on cross section

**3.3.1.** Group of lungs with no microscopic lesions

The gland to wall ratios, taken from bronchial view without cartilage, in the group of lungs with no microscopic lesions and cut in cross section and measured by linear measurement (using only the individual measurements) ranged from 27.8 % to 97.9 % with an average ratio of 63 % ± 12 (n = 1851), while those measured by 2-D measurement ranged from 19 % to 92 % with an average ratio of 49.76 % ± 11.6 (n = 595). In this group, where the measurements were taken in bronchi that had no cartilage, the correlation between values obtained by linear measurement and by 2-D measurement was 0.530 ($p = 0.000$).

The correlation between linear measurement and 2-D measurement in the same group where the measurements were taken in areas of the bronchi that had cartilage was 0.347($p = 0.065$). Microscopic examination showed that there was

Table 2. Microbiological findings in bronchial swabs taken from feline lungs cut in cross section (n = 14).

| Cat ID | B. growth | Bacterial identification | Minor lesions |
|---|---|---|---|
| 10 | ± | Mixed flora | No |
| 11 | - | No growth | No |
| 12 | ++, + | *Bordetella brochiseptica* + *Pasteurella multocida* | No |
| 14 | + | *Pasteurella multocida* | No |
| 15 | + | Mixed flora | No |
| 16 | ± | Mixed flora | No |
| 17 | +, ++ | Mixed flora + *Bordetella brochiseptica* | Yes |
| 18 | +, +, ++ | Mixed flora + *Pasteurella multocida* + *Bordetella brochiseptica* | No |
| 19 | ±, ++ | Mixed flora + *Bordetella brochiseptica* | Yes |
| 20 | ±, ± | *Pasteurella multocida* + mixed flora | No |
| 21 | ± | *Pasteurella multocida* | No |
| 22 | +++ | G ram positive bacilli | Yes |
| 23 | +, ±, + | *Pasteurella multocida* + *Klebsiella pneumonia* + *Alpha hemolytic strep.* | Yes |
| 24 | + | *Pasteurella multocida* | No |

- = No growth. ± = scant growth. + = light growth. ++ = moderate growth. +++ = heavy growth.

B. growth = Bacteria growth

notable lung lobe variation in the number of bronchi that had cartilage.

**3.3.2.** Group of lungs with minor microscopic lesions

The gland to wall ratio, taken from bronchial view without cartilage, for the group of lungs with minor microscopic lesions and cut on cross section measured by linear measurement (using only the individual measurements) ranged from 32 % to 96.8 % with an average ratio of 67 % ± 12 (n = 1099), and those measured by 2-D measurement ranged from 29 % to 87 % with an average ratio of 54 % ± 9.6 (n = 298). In this group, where the measurements were taken in bronchi that had no cartilage, the correlation between linear measurement and 2-D measurement was 0.569 ($p$ = 0.004), when these data were put in a scatter plot graph, the lines confirmed a good correlation (Fig. 8).

The correlation between linear measurement and 2-D measurement was - 0.354 ($p$ = 0.179) where the measurements were taken in areas of the bronchi that had cartilage. The lines in the scatter plot graph confirmed a weak correlation (Fig. 9).

**3.4.** Gland to wall ratio in bronchi cut on longitudinal section

3.4.1. Group of lungs with no microscopic lesions

The gland to wall ratio, taken from bronchial view without cartilage, in the group of lungs with no microscopic lesions, cut longitudinally and measured by linear measurement (using only the individual measurements) ranged from 28 % to 81.85% with an average ratio of 50.9 % ± 9 (n = 2725), and those measured by 2-D measurement ranged from 29 % to 86.9 % with a average ratio of 44.49 % ± 7.8 (n = 855). In this group, the correlation between the linear measurement and 2-D measurement methods was 0.502 ($p$ = 0.000) when the measurements were made in

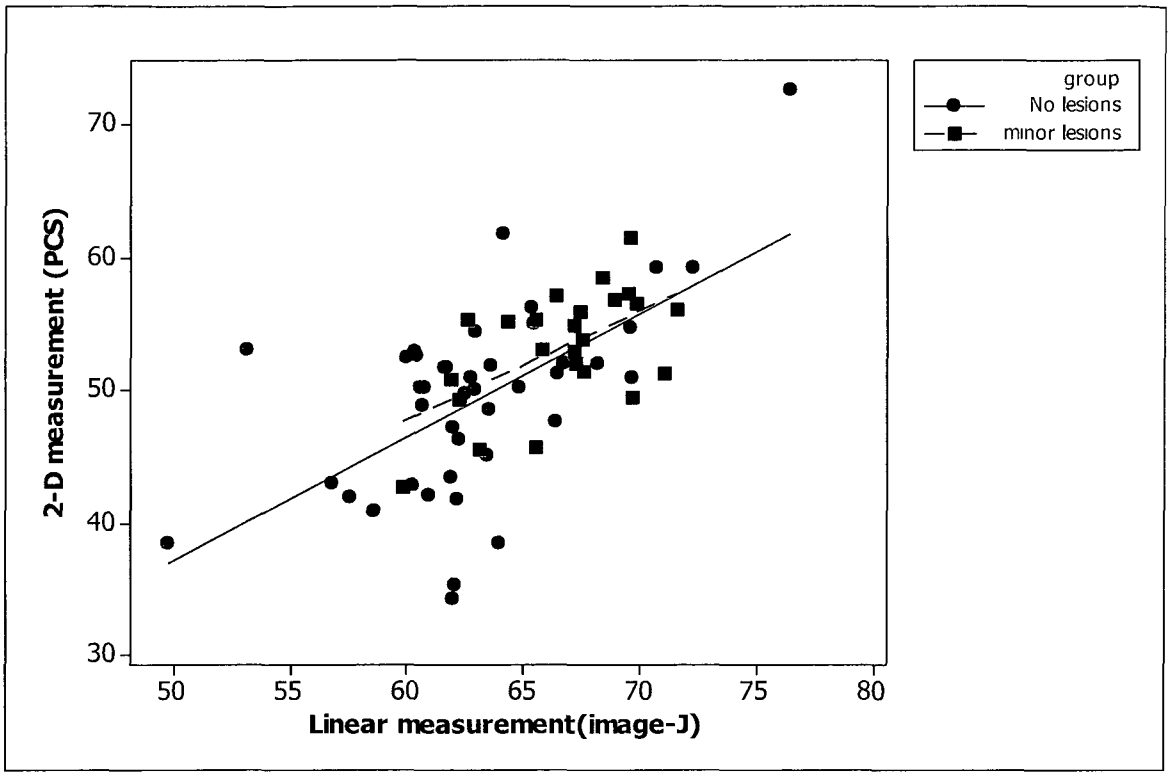



Figure 8. Relationship between 2-D measurement (PCS) and linear measurement of the ratio of samples without cartilage in the cross section of both groups lungs with no microscopic lesions and lungs with minor microscopic lesions.

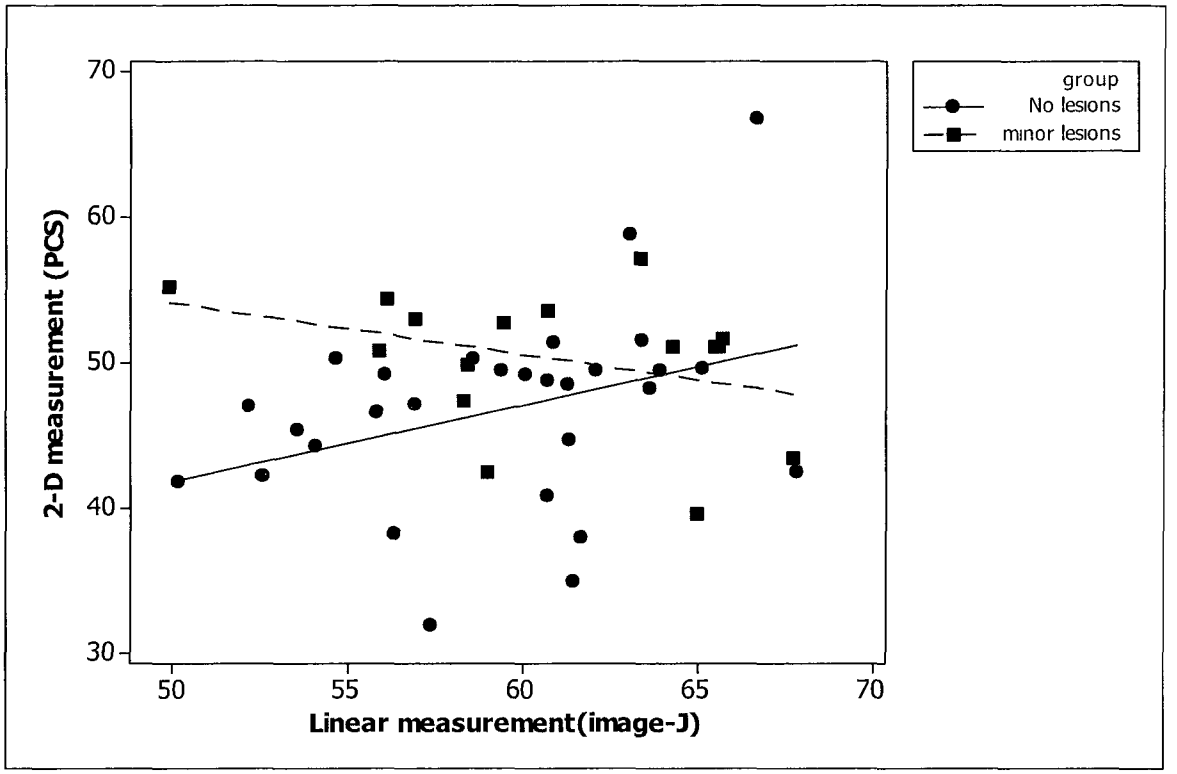



Figure 9. Relationship between of 2-D measurement (PCS) and linear measurement (image-J) of the ratio of samples with cartilage in the cross section of both groups of lungs with no microscopic lesions and lungs with minor microscopic lesions.

areas of the bronchi that lack cartilage. The correlation between the two methods was not analyzed in areas that have cartilage because of small data set (one cat with no microscopic lesions and two cats with minor lesions).

3.4.2. Group of lungs with minor Microscopic lesions

The gland to wall ratio, taken from bronchial view without cartilage, in the group of lungs with minor microscopic lesions, cut longitudinally and measured by linear measurements (using only the individual measurements) ranged from 28 % to 99 % with a average ratio of 52.47 % ± 9.9 (n = 1010), while those measured by 2-D measurement ranged from 30 % to 68.5 % with a average ratio of 43.46 % ± 8 (n = 347). In this group, the correlation between the linear measurement and 2-D measurement methods was -0.041($p$ = 0.788) when the measurements were made in areas of the bronchi that lack cartilage.

**3.5.** The bronchial sectioning and the methods of measurement

The data of measurements (bronchial gland to wall ratio) made on the bronchial views without cartilage was divided to four sub-groups according to the lung sectioning and the method of measurement in all cats (lungs with and without microscopic lesions):

1. cross section with linear measurement sub-group (Figure 10 and 11).
2. cross section with 2-D measurement sub-group (Figure 12 and 13).
3. longitudinal section with linear measurement sub-group (Figure 14 and 15).
4. longitudinal section with 2-D measurement sub-group (Figure16 and 17).

The inter-measurements variability between cats, between lobes, and within each lobe was analyzed. The variance of the bronchial gland to wall ratio was the

Figure 10. Bronchial gland to bronchial wall ratio: linear measurement (image-J) in cross section of lungs of cats without microscopic lesions in bronchial view without cartilage using the means of lobes (mean, SE).

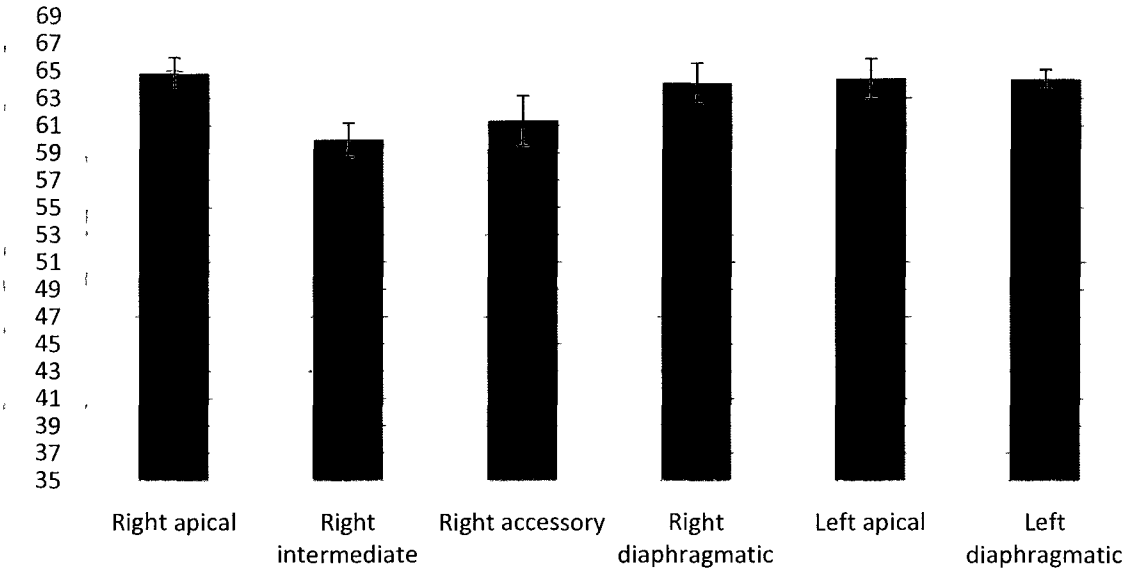


Figure 11. Bronchial gland to bronchial wall ratio: linear measurement (image-J) in cross section of lungs of cats with minor lesions in bronchial view without cartilage using the means of lobes (mean, SE).

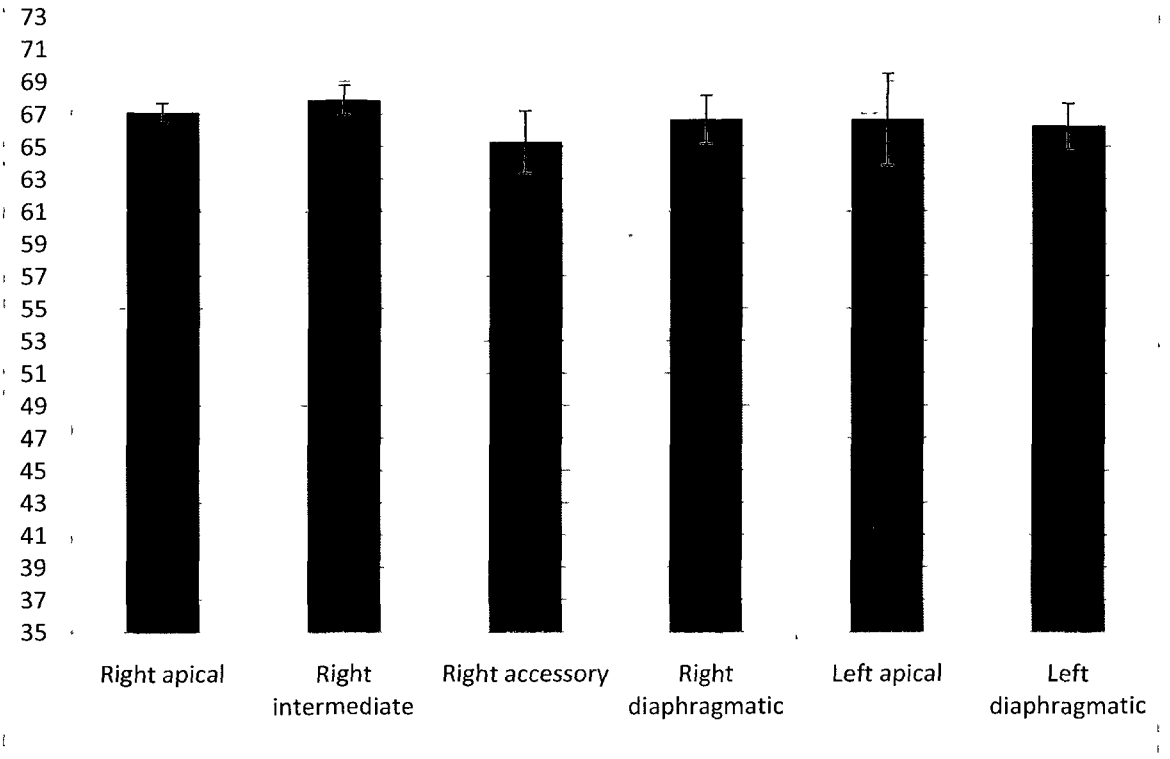


Figure 12. Bronchial gland to bronchial wall ratio: 2-D measurement (PCS) in cross section of lungs of cats without microscopic lesions in bronchial view without cartilage using the means of lobes (mean, SE).

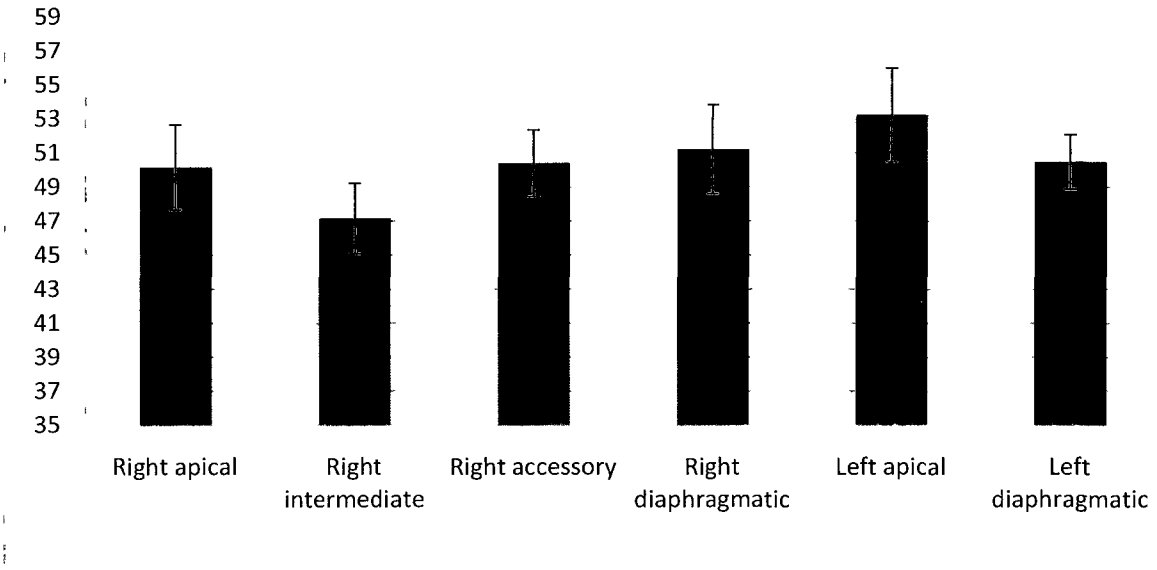


Figure 13. Bronchial gland to bronchial wall ratio: 2-D measurement (PCS) in cross section of of lungs of cats with minor lesions in bronchial view without cartilage using the means of lobes (mean, SE).

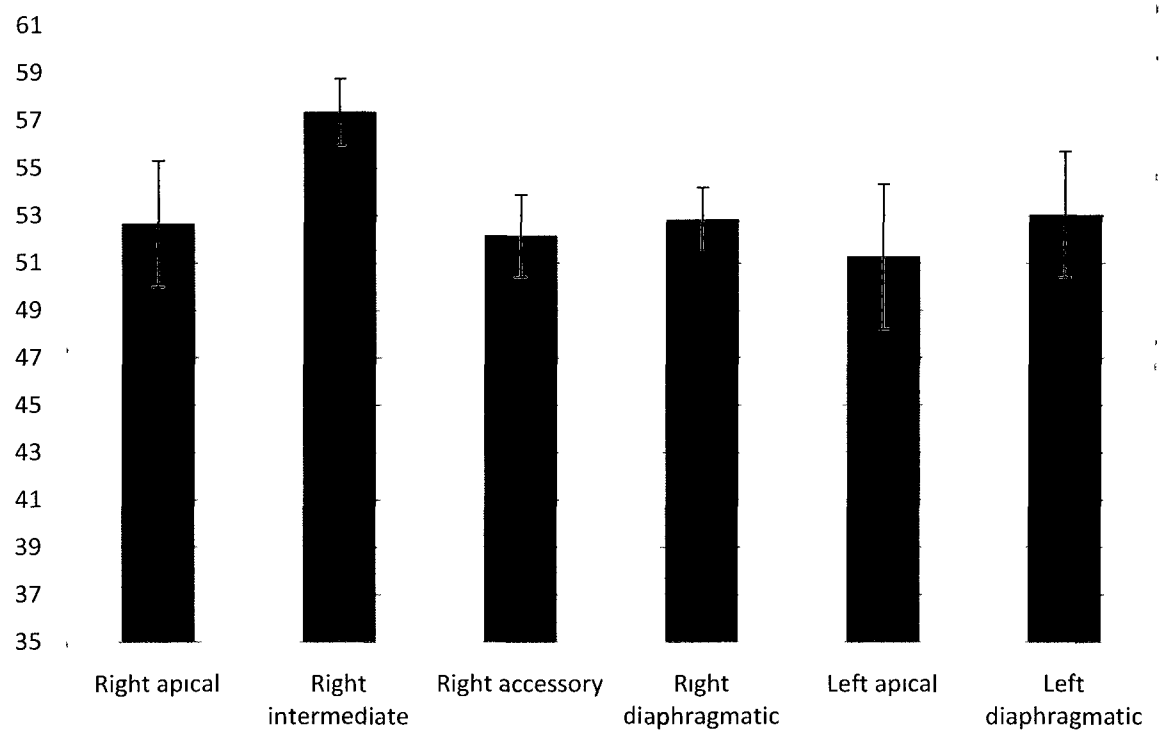


Figure 14. Bronchial gland to bronchial wall ratio: linear measurement (image-J) in longitudinal section of lungs of cats without microscopic lesions in bronchial view without cartilage using the means of lobes (mean, SE).

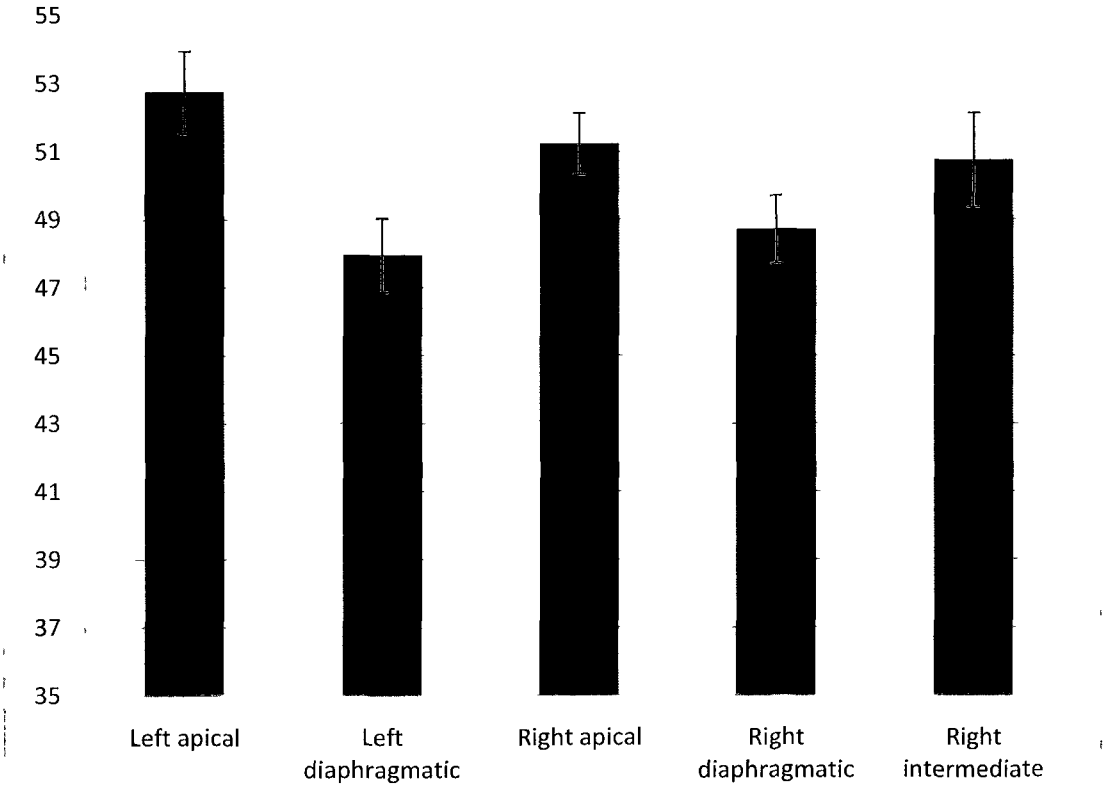


Figure 15. Bronchial gland to bronchial wall ratio: linear measurement (image-J) in longitudinal section of lungs of cats with minor lesions in bronchial view without cartilage using the means of lobes (mean, SE).

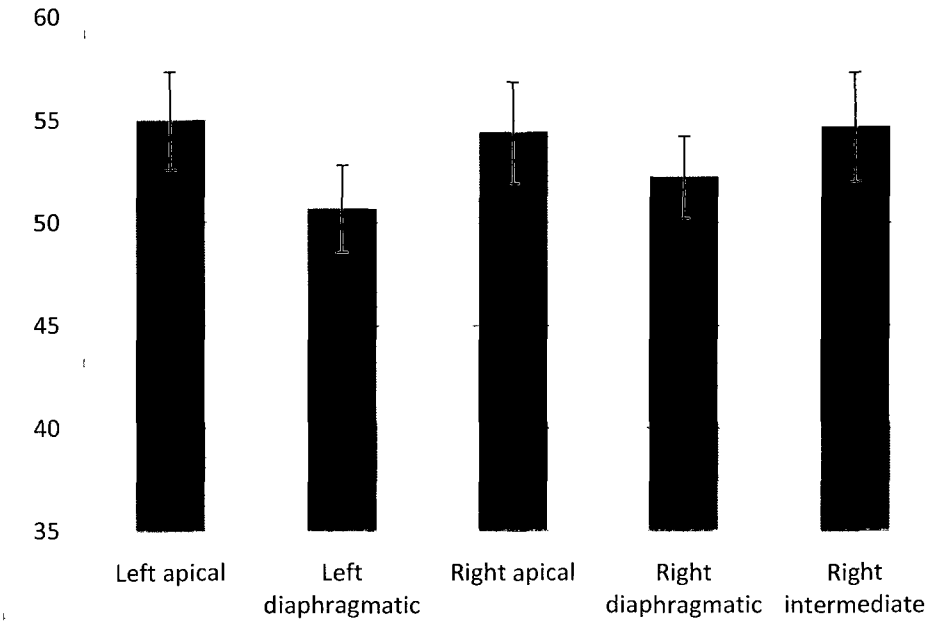


Figure 16. Bronchial gland to bronchial wall ratio: 2-D measurement (PCS) in longitudinal section of lungs of cats without microscopic lesions in bronchial view without cartilage using the means of lobes (mean, SE).

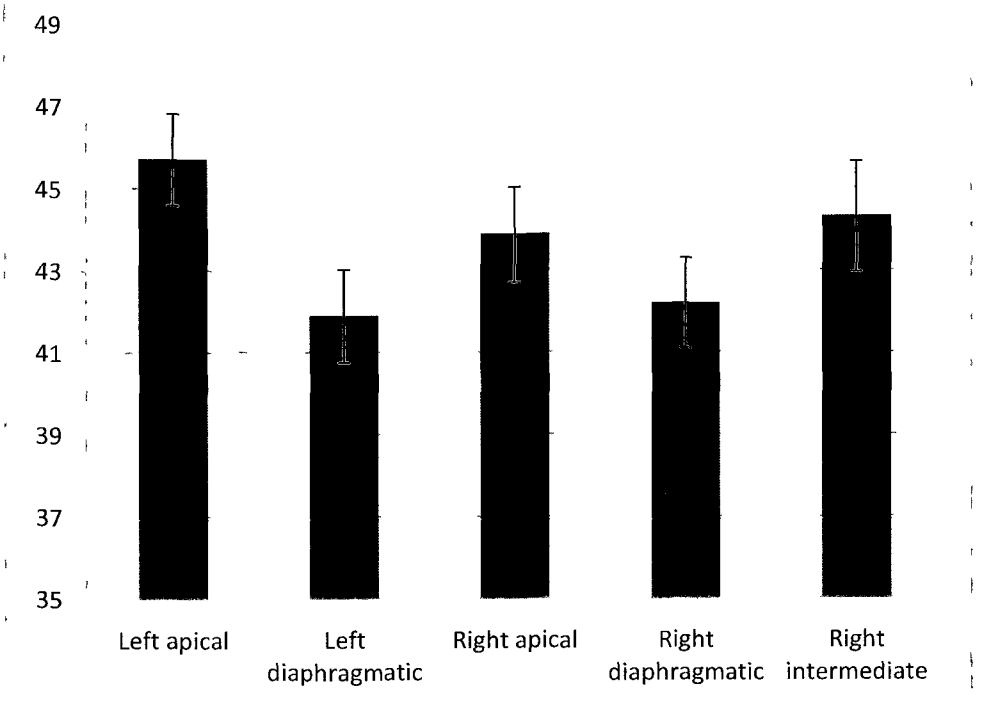
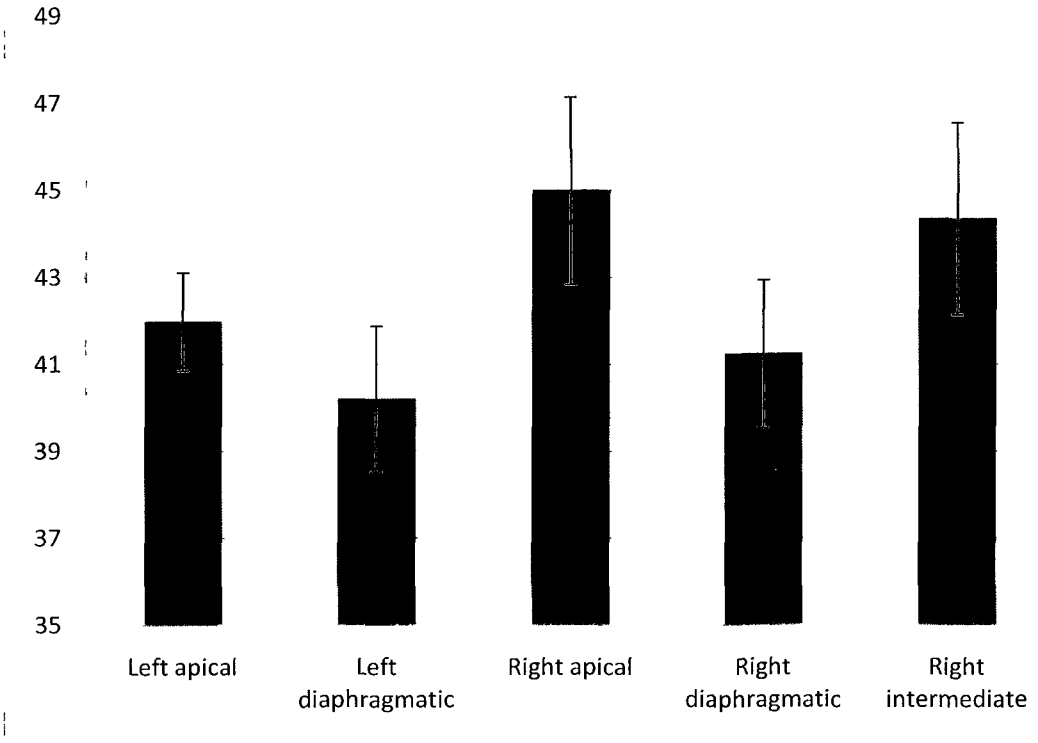


Figure 17. Bronchial gland to bronchial wall ratio: 2-D measurement (PCS) in longitudinal section of lungs of cats with minor lesions in bronchial view without cartilage using the means of lobes (mean, SE).



lowest in the longitudinal section with 2-D measurement sub-group, while it was the highest in the cross section cut with linear measurement sub-group (Table 3).

**3.6.** Bronchial gland to wall ratio in bronchi in the different lung lobes

The mean bronchial gland to wall ratio in the right intermediate lung lobe (RI N) was the highest of all lobes in the lungs with minor lesions while the same ratio was the lowest in the RIN in the group of lungs with no microscopic lesions (Table 4). There was a significant difference between the right intermediate lobe (RIN) in lungs with no microscopic lesions and the RIN in lungs with minor lesions ($p = 0.029$). Moreover, there was also a significant difference between RIN and the rest of the lobes in each group, i.e. those with no microscopic lesions ($p = 0.034$) and those with minor microscopic lesions ($p < 0.001$). Further analyses showed that the interaction between lung lobes and groups (lungs with no microscopic lesions and lungs with minor microscopic lesions) was not significant ($p > 0.05$).

In the group of lungs with no microscopic lesions cut longitudinally and measured by linear measurement, the average of the bronchial gland to wall ratio ( the means of measurements of each lobe) in lung lobes (except RIN lobe) was $50.44 \pm 0.359$ (SE) and the ratio had slightly decreased less than 1% (0.764) in the RIN, while the average of the ratio in lung lobes of the group with minor microscopic lesions (except RIN lobe) was $52.29 \pm 0.54$ (SE) and the ratio slightly increased 2% (2.02) in RIN. Overall, there was no significant difference in the mean bronchial gland to wall ratio when comparing between all lung lobes in group of cats without microscopic lesions to all lung lobes in the group of cats with microscopic lesion ($p < 0.946$). Even though the right accessory lobe (RAC) had the highest standard error (SE) of all lobes in both groups of lungs with

Table 3. Variance of the ratio in the longitudinal cut with linear measurement sub-group, longitudinal cut with 2-D measurement sub-group, cross cut with linear measurement sub-group, and cross cut with 2-D measurement sub-group : lungs of cats with and without minor lesions in two bronchial views (excluded cartilage and without cartilage) using the individual measurements.

.

| Variance | Long+IJ | Long+PCS | Cross+ IJ | Cross+PCS |
|---|---|---|---|---|
| N = | 3775 | 1219 | 3183 | 1017 |
| Var.cats | 21.0 | 15.28 | 4.36 | 23.15 |
| Var. lobes | 7.67 | 8.27 | 4.29 | 6.73 |
| Var. error | 62.60 | 42.05 | 141.2 | 94.58 |
| Total | 91.28 | 65.60 | 149.87 | 124.46 |
| ICC for cat | 0.23 | 0.23 | 0.03 | 0.19 |
| ICC for lobe | 0.31 | 0.36 | 0.06 | 0.24 |
| r | 22.38 | 18.34 | 33.61 | 27.51 |
| R | 23.7 | 20 | 34 | 28.47 |

Var.cats = variance within cat. Var. lobes = variance within lobe. Var.erro r= variance within image. ICC = interclass correlation coefficient. r = repeatability. R = reproducibility. No.obs = number of observations. Long+IJ = longitudinal cut with linear measurement sub-group. Long+PCS = longitudinal cut with 2-D measurement sub-group. Cross+IJ = cross cut with linear measurement sub-group. Cross+PCS = cross cut with 2-D measurement sub-group.

Table 4. Mean of bronchial gland to bronchial wall ratio (linear measurement (image-J)) of lobes of longitudinal section: cats without microscopic lesions and cats with minor lesions in bronchial view without cartilage.

| Lobe | Name | lungs with no lesions | lung with minor lesions |
|---|---|---|---|
| LAP | Left apical | 51.65 (0.83) | 52.86 (1.11) |
| LD | Left diaphragmatic | 49.66 (0.83) | 51.27 (1.1) |
| RAC | Right accessory | 50.15 (0.86) | 51.45 (1.18) |
| RAP | Right apical | 50.96 (0.83) | 53.67 (1.116) |
| RD | Right diaphragmatic | 49.80 (0.827) | 52.18 (1.11) |
| RIN | Right intermediate | 49.68 (0.84) | 54.31 (1.137) |

If cross section with IJ add 13.75 (1.16)

If cross section with PCS add 0.35 (1.19)

If longitudinal section with PCS subtract 7.14 (0.33). (SE)

or without microscopic lesions (Table 4), statistical analysis revealed that there was no difference between the RAC in the lungs with no microscopic lesions and the RAC in the lungs with minor lesions ($p = 0.101$).

**3.7.** The bronchial cartilage

The gland to wall ratio in both groups of lungs with and without minor lesions, cut either longitudinally or transversely on bronchial view that have cartilage and cartilage was excluded and measured by linear measurement ranged from 33.18 % to 97.69 % (using the individual measurements) with an average ratio of 61.5 % ± 11.8 (n = 273), and those measured by 2-D measurement ranged from 25.0 % to 77.78 % with a average ratio of 49.38 % ± 10.4 (n = 141). The gland to wall ratio in both groups of lungs with and without minor lesions, cut either longitudinally or transversely on bronchial view that have cartilage and cartilage was included and measured by linear measurement ranged from 22.95 % to 74.07 % (using the individual measurements) with a average ratio of 42.36 % ± 8.6 (n = 273), and those measured by 2-D measurement ranged from 13.86 % to 64.58 % with a average ratio of 38.0 % ± 9.8 (n = 141).
In areas of bronchi that have cartilage, the correlation between the ratio in the measurement that excluded cartilage and the ratio in the measurement that included cartilage was 0.724 ($p = 0.000$) for all groups, lung sections, and morphometric methods. The gland to wall ratio when cartilage was included had lower variation than the ratio excluding cartilage (Table 5). The correlation between linear measurement and 2-D measurement was 0.030 ($p = 0.725$) when the measurement was taken in the bronchi that included cartilage. The correlation between linear measurement and 2-D measurement, was 0.131 ($p = 0.122$) when the measurement was taken in the bronchi that excluded

Table 5. Variance of the bronchial gland to wall ratio in lungs of cats with both measurements including cartilage and not including cartilage in all sections and methods.

| Variance | cartilage in all data | | IJ | | PCS | |
|---|---|---|---|---|---|---|
| | Ratio-c | Ratio+c | Ratio-c | Ratio+c | Ratio-c | Ratio+c |
| Var. cats | 19.73 | 3.84 | 30.47 | 8.20 | 25.69 | 18.79 |
| Var. error | 148.23 | 83.52 | 118.33 | 68.732 | 87.99 | 79.86 |
| Total | 167.96 | 87.37 | 148.8 | 76.93 | 113.68 | 98.65 |

Ratio+c = Measurement including cartilage.

Ratio-c = Measurement excluding the cartilage.

IJ = Linear measurement.

PCS = 2-D measurement

cartilage; when these data were put in a scatter plot graph, the lines confirmed the absence of correlation (Fig.18 and 19). The mean and standard deviation for the three types of measurements are displayed (Table 6).

**3.8.** The number of repetitions needed for a meaningful estimate of the ratio

The number of observations per cat in the group of lungs with no microscopic lesions when the measurements applied on area of bronchi lacking cartilage ranged from 65-477 with an average of 224.2. The number of observations per lobe ranges from 6-146 with an average of 39.5. The simulation showed that the minimum number of measurements per lobe and per cat needed to obtain meaningful values was 12 and 15, respectively (Fig. 20 and Fig. 21). The minimum number of measurements that were needed per sub-group to obtain low variation for meaningful bronchial gland to wall ratio values was analyzed (SE (mean) = range of the ratio (2.5% -97.5 %) /8, (Var (y ) = $\sigma$2 of lobe/6 + $\sigma$2 of measurements/N)). The results revealed that 8- 10 measurements are required in all sub-groups except the sub-group of cross section with linear measurement (IJ) in which 28 measurements are needed (Table 7).

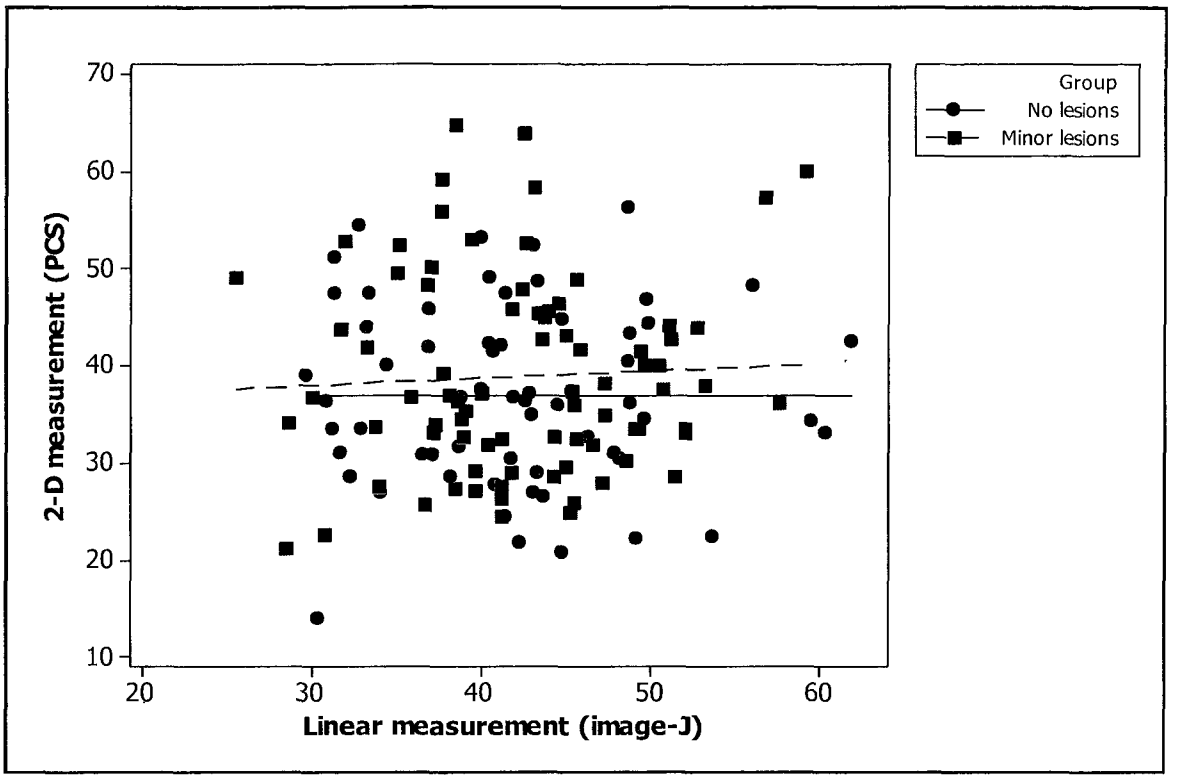



Figure 18. Relationship between 2-D measurement (PCS) and linear measurement (image-J) of the ratio of samples with included cartilage in the cross and longitudinal sections of both groups of lungs with no microscopic lesions and lungs with minor microscopic lesions.

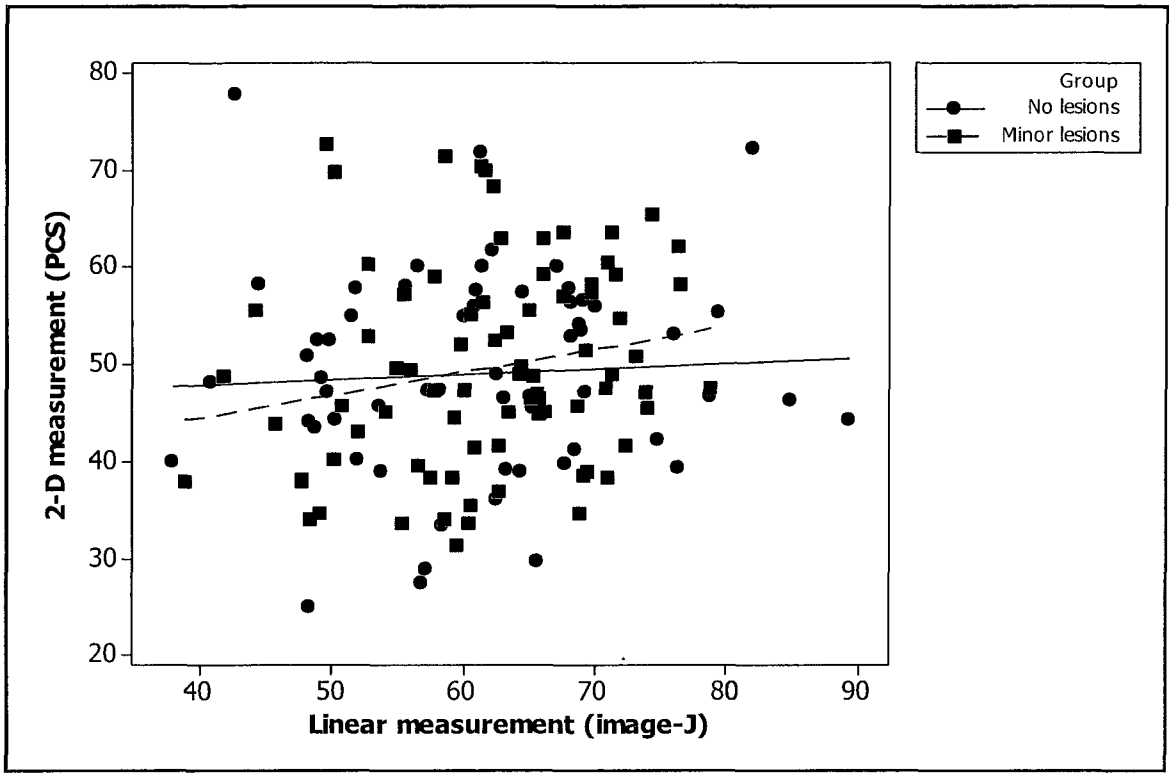



Figure 19. Relationship between 2-D measurement (PCS) and linear measurement (image-J) of the ratio of samples with excluded cartilage in the cross and longitudinal sections of both groups of lungs with no microscopic lesions and lungs with minor micróscopic lesions.

Table 6. Bronchial gland to wall ratios: areas having no cartilage, areas having cartilage but excluded, and areas including cartilage (mean, SD) for all groups, sections, and methods.

| Type of measurement | Mean±SD |
|---|---|
| With no cartilage | 54.85±12.84 |
| Excluding cartilage | 57.41±12.75 |
| Including cartilage | 40.89±9.32 |

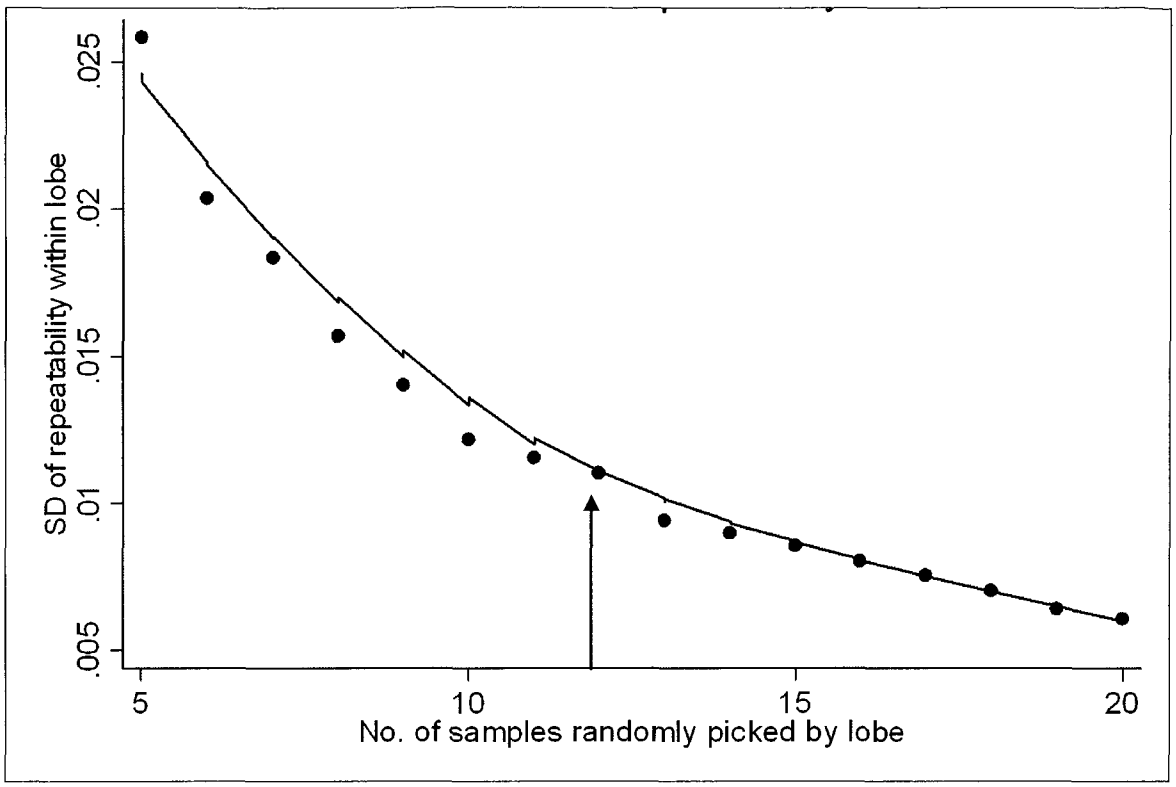



Figure 20. Repeatability of the bronchial gland to wall ratio within the lobes: lungs of cats with and without minor lesions measured by both methods linear and 2-dimensional measurements in bronchial view without cartilage cut either transversely or longitudinally using the individual measurements.

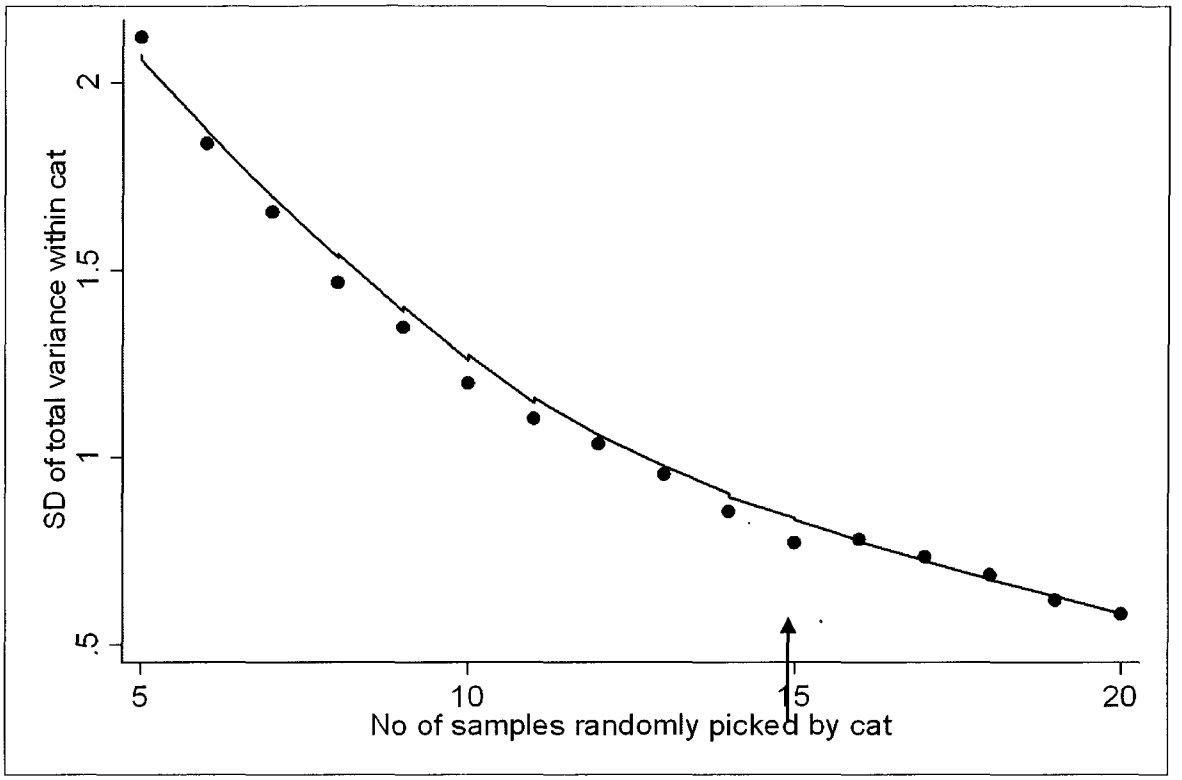



Figure 21. Variance of the bronchial gland to wall ratio within cats: lungs of cats with and without minor lesions measured by both methods linear and 2-dimensional measurements in bronchial view without cartilage cut either transversely or longitudinally using the individual measurements.

Table 7. Range of the bronchial gland to wall ratio in cats with no microscopic lesions in bronchial view without cartilage.

| Sub-group | No. lobes | 2.5% | 97.5% | SE | Obs. with less variation |
|---|---|---|---|---|---|
| Cross/IJ | 60 | 52.65 | 71.89 | 2.4 | 28.22 |
| Cross/PCS | 60 | 35.38 | 63.81 | 3.55 | 8.24 |
| Long/IJ | 101 | 41.97 | 63.52 | 2.69 | 10.51 |
| Long/PCS | 101 | 34.88 | 54.98 | 2.51 | 8.55 |

Long+IJ = longitudinal cut with linear measurement. Long+PCS = longitudinal cut with 2-D measurement. Cross+IJ = the cross cut with linear measurement. Cross+PCS = cross cut with 2-D measurement. SE = standard error of the mean

## 4. Discussion

### 4.1. Reid Index

Results of this investigation showed that the overall bronchial gland to bronchial wall ratio or Reid Index (0.61 or 61% ± 11.8 (n= 414) using the mean of individual measurements in both groups of lungs with and without minor lesions, measured by linear measurement, and cut either longitudinally or transversely on bronchial view that have cartilage and cartilage was excluded) in healthy cats is notably higher than the 0.40 or 40% reported for the healthy human bronchi (66). This higher feline RI was not a surprise since routine diagnostic work typically shows that cats, compared to humans and other domestic animals species, have histologically more prominent bronchial glands (84); Inexperienced pathologists could erroneously interpret larger than average glands in cats as a bronchial hyperplasia, particularly when these feline glands are compared with those of other domestic species. Another interesting peculiarity of feline bronchi is that the glands are not only found in the primary and secondary bronchi, but also in the tertiary bronchi and as far distally as the proximal bronchioles (22).

This study also showed that otherwise clinically healthy cats have minor microscopic findings in the lungs. This finding further supports the view that small microscopic changes such as minute amounts of mucus in airways, sporadic alveolar thickening or presence of occasional inflammatory cells is to be expected in clinically healthy cats and does not have any clinical significance (59). These minor pulmonary lesions likely result from environmental factors related to air quality or from a transient mild respiratory infections that so often go undetected (10). From the diagnostic point of

view, these minor histological changes should be considered incidental and should not be over-interpreted in routine diagnostic histopathology.

The quantitative differences in RI, for both healthy cats and cats with minor lesions, were relatively small (49.7-54.1), particularly when compared with the much wider range of RI reported in human for healthy individuals and patients with chronic bronchitis (21,73). A presence of a few secretions in the bronchial lumen should not be regarded as a sign of chronic bronchitis (84) unless these changes are accompanied by bronchial inflammation and bronchial gland hyperplasia (7).

The overall RI of 0.61 or 61% in the cats of this study is slightly lower than the wall to gland ratio reported for the feline trachea (0.65 or 65%) by Jeffery (74). This difference is small and likely reflects normal variation in glands size in the various segments of the conducting system (22) or may simply reflect methodological differences between studies. Cats have a peculiar morphological difference in the tracheal wall since their smooth muscle fibers attach to the external surface of the cartilaginous rings (9). This peculiarity implies that the trachealis muscles do not take part for the calculations of the RI and therefore their absence results in slightly higher ratio. Overall, the glandular size in all animals and humans tends to decrease distally in the conducting system but the relationship between bronchial diameter and bronchial gland size seems to be less variable (73). Future studies should compare wall to gland ratios for the trachea and bronchi in the same animals.

**4.2.** Effects of lung sectioning on the lung

One objective of this investigation was to determine the most suitable cutting technique to obtain the best quality lung sections for bronchial morphometry in cats.

Results from this investigation revealed that an intraluminal probe technique, as recommended in human pathology (7), makes outstanding bronchial sections but creates unacceptable artifacts in the lining epithelium. These changes are so extensive as to make the bronchial mucosa unsuitable for microscopic examination and morphometry. Bronchial probes should be used only for gross examination of major lesions as those seen with bronchiectasis or pulmonary neoplasia (7) but not for microscopic examination as in case of bronchitis or bronchial gland hyperplasia.

For histological examination, longitudinal sections are much more suitable for the bronchial gland morphometry in cats than cross section. The later method creates excessive postmortem folding of the bronchial mucosal which makes measurement more difficult (22). Longitudinal sections on the other hand, make bronchial glands easier to measure.

**4.3.** The bronchial cartilage

Results of this investigation also revealed that Reid Index in cats varies notably depending on how the measurements were taken and what segment of the bronchial wall was assessed. The presence or absence of cartilage in the calculations in cats yielded different RI and this inconsistency is partially explained by what has been already reported in the human literature (73). There are two basic methods to calculate the RI: one method takes into account the cartilage in the calculation while the other does not. Reid, the creator of the RI calculated this index using bronchial mucosa that contained cartilage but the cartilage itself was excluded from the calculations (66). Other researchers have calculated the RI in segments of the bronchial mucosa that lack cartilage, that is, in areas of the mucosa between two cartilage plates (85). Our results

show that the RI in healthy cats is more consistent and gives better correlations when the index is calculated in areas of bronchial mucosa that have no cartilage. Cartilage makes bronchial glands more difficult to trace morphometrically, particularly because of some glands irregularly curve around the edges of the cartilage (41). By definition, bronchial measurements that include cartilage give lower RI since the cartilage width adds to the overall wall thickness. The present investigation determined that the cartilage should not be included in order to make RI values more consistent and to permit meaningful comparison between studies in cats. When the measurements include cartilage the RI is less sensitive and does not show the difference between PCS and IJ.

**4.4.** Linear measurement vs. two-dimensional measurement

Another objective of this investigation was to compare one-dimensional and two-dimensional methods of morphometry. Results of this investigation showed that the Reid index (RI) in cats is biased by the method used to measure the size of the bronchial wall and bronchial gland. For instance, the average RI for linear measurement was 57% while the 2-D measurement was 47%. Overall, the RI is consistently higher in linear measurement compared to 2-D measurement. The difference between linear measurement and 2-D measurement methods is possibly influenced by two major issues: one relates to the measuring method itself and the other by the uneven distribution of the glands in the feline bronchi. Linear measurement is a one-dimensional value derived from a single measurement taken at the largest width of the gland, while PCS is a two-dimensional value obtained at various points of the gland (72,86).

Other authors have also evaluated morphometric methods for human bronchi and found only minor difference in RI but a major disparity in degree of practicality. For

instance, planimetry, considered a highly accurate biological measurement technique gives the same results as 2-D measurement. 2-D measurement is a much easier and less time consuming method (72).

Microscopic observation in this study also demonstrates that the distribution and shape of the feline bronchial glands was notably inconsistent and unlike the "picture perfect" illustrations often shown in histology textbooks (22,87). In some sections the glandular acini appear closely-packed in a single glandular cluster, while in other cases, the glands appear as an irregularly branching cluster partially encircling the bronchial cartilage. Heterogeneity in glandular shape has been already reported in human as well (41). This heterogeneity in glandular shape explains why one-dimensional measurements using linear measurement yields different values than 2-D measurement (41). The gland thickness in linear measurement is the distance between the epithelial basement membrane and the deepest glandular acini, while 2-D measurement includes all lattice points falling on all glandular acini. In other words, the linear measurement is only influenced by the length while in 2-D measurement the index is not only influenced by the length but also by the width of the gland (67). This difference makes linear ratios higher than 2-D ratios overestimating the actual proportion of glands in RI.

The correlation between linear measurement and 2-D measurement results is also affected by the presence or absence of cartilage in the bronchial wall. Results of this investigation showed a stronger correlation between linear measurement and 2-D measurement when measurements were taken in walls with no cartilage in cats. For instances, the study showed a good correlation between linear measurement and 2-D measurement only when measurements were made in the area of the bronchial wall

lacking cartilage. On the other hand, there was a poor correlation between linear measurement and 2-D measurement when the measurement was taken in bronchial areas with cartilage but the cartilage was excluded from the calculations. This finding in cats disagreed with those reported in humans by Reid (66), Scott (76), and Takizawa and Thurlbeck (78) who had found that the two methods correlated well when RI was calculated, as originally described by Reid. However, other studies such those of Jamal and his colleagues found no correlation between the two methods in humans with severe chronic bronchitis accompanied with airflow obstruction (80). In this study, there was a poor correlation between linear measurement and 2-D measurement when the measurement included cartilage.

Linear measurement can also be affected by artifacts such as separation between the perichondrium and the cartilage (72), spaces between the submucosa and cartilage (41), or cartilage misalignment with respect to the mucosal surface (21,86). Also, changes in the bronchial tissue such as edema or inflammation can affect the ratio (86,88).

The artifactual space between subepithelial mucosa and cartilage can be easily omitted in 2-D measurement when calculating the thickness of the bronchial wall (41,72,86). The most critical issue affecting 2-D measurements is the difficulty in delineating and setting the boundary of the bronchial wall (41). Measuring areas with fewer glandular acini can influence the results and give a lower ratio. The 2-D measurement is a time consuming technique and requires the use of a lattice (78).

**4.5.** The bronchial gland to wall ratio in the different lung lobes

This study shows that there is a significant difference in the Reid Index between lobes in lungs without lesions and similarly in the group of lungs with minor lesions. This difference although statistically significant is perhaps biologically negligible since a difference of only 2% in the Reid Index is still within a normal range of the bronchial gland to bronchial wall ratio in healthy cats. The study also shows that the lung lobes ((right apical (R-AP), right accessory (R-AC), right diaphragmatic (R-D), left apical (L-AP), and left diaphragmatic (L-D)) in group of lungs without lesions have similar RI to the group of lungs with minor lesions. In other words, Minor lesions should not interfere with evaluation of the RI in clinical cases.

There was a significant higher gland to wall ratio in the right intermediate lobe in the lungs of cats with minor lesions while the rest of lobes do not have significantly different RI from each other. This finding is not all that surprising since the right intermediate lobe has unique anatomic features which in other species make it more prone to lesions. This higher susceptibility of the right intermediate lobe could be due to the fact that the lobar bronchus of this lobe is longer than other bronchi (89). Also, the right intermediate lobe has a smaller diameter lumen with angular bifurcation which may predispose to the retention of bronchial secretions. Retention of mucus or exudate leads to partial obstruction in cases of infection and edema of bronchial mucosa (89,90). In animals, the ventral location of the entrance of the right intermediate lobe makes this lobe prone to accumulate the secretion due to flow by gravity (84). In addition, the right intermediate lobe is isolated from the upper and the lower lobes (90,91) because of the presence of complete fissures around the lobe. This isolation causes poor collateral

ventilation and decreases the clearance of mucus or exudate (92,93), especially following atelectasis (94,95). Our findings suggest that pathologists should take measurement from the lung lobes (right apical (R-AP), right accessory (R-AC), right diaphragmatic (R-D), left apical (L-AP), and left diaphragmatic (L-D)) and avoid taking measurement from the right intermediate lobe ( R-IN) in cats. The average of gland to wall ratio (means of measurements of each lobe) in all lobes except RIN lobe in the group of lungs with no microscopic lesions, cut longitudinally and measured by linear measurement on bronchial view that lack cartilage is approximately 50.44 ± 0.359 (mean ± SE).

**4.6.** Sectioning methods and minimum repetitions needed to obtain meaningful Reid index (RI) values

Results showed that the bronchial gland to bronchial wall ratio in cats varies notably depending on how the lung lobes are sliced and what methods are used to do bronchial morphometry. For instances, using 2-D measurement on longitudinal sections has the least amount of total variation when measuring the bronchial gland to wall ratio. On the other hand, linear measurements on cross sections provide the greatest amount of total variation for the same ratio. This increased variation in ratio is most likely influenced by two major issues, the morphometric methodology and the crenation or folding of the bronchial mucosa seen in cross sections.

Statistical analyses also showed that a minimum of 28 measurements are required to insure lower variation and provide meaningful values when the bronchial gland to wall ratios are determined by linear measurement on cross sections. In contrast, only 10 linear measurements are required to obtain low variations and meaningful results in longitudinal sections. For 2-D measurements, a minimum of 8 are needed on bronchi cut either

longitudinal or cross section to obtain meaningful results. All these findings suggest that 2-D measurement results are not much influenced by the plane of sectioning in cross section as it occurs when measurements are taken with linear measurement. Our results agreed with Dunnill *et all* who found that mucosal folding in the bronchi seems to bias linear measurements (21). If there was some standardization of the measurements in the bronchi cut in cross section and measured by linear measurement, in other words avoiding the folding of mucosa while taking measurements, the variance could be reduced significantly. Finally, even though the least variable measurement was obtained by the 2-D method, the equipment and time required to perform this 2-D evaluation may not be available in typical veterinary pathology laboratories. Our results suggest that RI using linear measurements on bronchial view without cartilage and cut longitudinally has only a small amount of increased variability and would be more practical for pathologists to use. Moreover, a minimum of 10 measurements should be taken to evaluate the RI in a clinical case.

**4.7.** Conclusions

Conclusion from this investigation is that the feline bronchial gland has a notably higher RI than human. Diagnostic pathologists should be careful not to overestimate the size of the glands in cats and minor microscopic lesions in the lung should be expected in healthy cats. The right middle lung lobe in cats should be avoided for RI calculation. Two-dimensional measurements should be preferred over linear measurements although it is more time consuming and requires a point count lattice. Longitudinal sections of the bronchial tree are preferred over traditional cross sections of bronchi. The least amount of variation in the bronchial gland to wall ratio is found when two-dimensional

measurement (PCS) is applied to longitudinal sections. Finally, measurements should be made in bronchial areas that do not contain cartilage. If present, cartilage should be excluded from the calculations.

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