Epithelial Migration on the Canine Tympanic Membrane

THESIS

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Abstract

The tympanic membrane and external auditory canal serve auditory and non-auditory functions. Both the luminal surface of the external auditory canal and the lateral surface of the tympanic membrane are composed of a stratified squamous, keratinizing epithelium. Epithelial migration is a process that serves as a self-cleaning and repair mechanism for the external auditory canal and tympanic membrane. Epithelial migration has been evaluated in humans and several other species, but not in dogs. The majority of these studies employ an ink drop method in which discrete markers of ink are placed on the lateral surface of the tympanic membrane and the pattern and rate of these markers are monitored over time. Failure or abnormal epithelial migration has been implicated as a potential cause of some otic diseases in humans and has been assessed in otitis externa, keratosis obturans, external auditory canal cholesteatomas, retraction pockets, and middle ear cholesteatomas.

The objectives of this study were to determine the rate and pattern of epithelial migration on the tympanic in clinically normal laboratory dogs and to describe a technique for ink drop placement on the canine tympanic membrane to be used for future studies. Eighteen dogs were anesthetized, and three drops of waterproof drawing ink were placed on two sites of the pars tensa and one on the pars flaccida. Images were recorded

with a video otoscope and digital capture system. Each dog was evaluated and images recorded every six to eight days for four evaluations. Migration pattern analysis and epithelial migration rate calculation were performed with image processing software. Descriptive statistics for epithelial migration rate (mean, standard deviation, 95% confidence interval) were calculated for all ink drop locations on the tympanic membrane (pars tensa 1 [PT1], pars tensa 2 [PT2], and pars flaccida [PF]) at each time point. Eight fox hounds had digital images from both ears that met the criteria for image analysis, while all beagles and two fox hounds only had images from one ear that met the criteria for image analysis. No significant differences in the mean epithelial migration rates were identified between right and left ears of the eight fox hound dogs, between breeds (beagle, fox hound), or between locations PT1 and PT2. The mean overall epithelial migration rates ($\pm$ standard deviation) were 96.4 ($\pm$ 43.1) and 225.4 ($\pm$ 128.1) micrometers per day for the pars tensa and pars flaccida, respectively. All ink drops moved outwards, the majority (48 of 53) in a radial direction, from the original location to the periphery of the tympanic membrane. Migration of the ink drops off the tympanic membrane was observed during the study period for all three locations. The ink drop placement method used in this study can be used in future studies to determine the epithelial migration rate of the canine tympanic membrane. Future investigations of epithelial migration should focus on dogs of different ages, dogs predisposed to otic diseases, as well as dogs with existing otic conditions.

Dedicated to my family, most importantly my husband Chris

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Table of Contents

Abstract.....	ii
Dedication	iv
Acknowledgement	v
Vita	vi
List of Tables	x
List of Figures	xi
CHAPTER 1 Introduction	1
1.1 List of references	3
CHAPTER 2 Literature review	5
2.1 Structure and function of the human and canine external auditory canal and tympanic membrane	5
2.1.1 Structure of the external auditory canal and tympanic membrane	5
2.1.2 Function of the external auditory canal and tympanic membrane	10
2.2 Epithelial migration, desquamation, and generation center	11
2.2.1 Roles for epithelial migration on the external auditory canal and tympanic membrane.....	11
2.2.2 Ink drop technique to assess epithelial migration on the external auditory canal and tympanic membrane.....	12
2.2.3 Normal epithelial migration on the human tympanic membrane and external auditory canal	14
2.2.3.1 Early history of epithelial migration on the human tympanic membrane	14

2.2.3.2 Later 20 th century assessments of epithelial migration on the human tympanic membrane	15
2.2.3.3 Modern assessments of epithelial migration on the human tympanic membrane	18
2.2.3.4 Effect of age on epithelial migration on the human tympanic membrane	19
2.2.3.5 Epithelial migration on the human external auditory canal	20
2.2.4 Normal epithelial migration on the tympanic membrane and external auditory canal in other species	21
2.2.4.1 Normal epithelial migration on the tympanic membrane and external auditory canal in guinea pigs.....	22
2.2.4.2 Normal epithelial migration on the tympanic membrane and external auditory canal in gerbils, rats, and mice	23
2.2.5 Desquamation of the external auditory canal and tympanic membrane epidermis	24
2.2.6 Epithelial migration on the tympanic membrane and external auditory canal in selected pathologic conditions	26
2.2.6.1 Epithelial migration on the tympanic membrane and external auditory canal in otitis externa and abnormal appearing tympanic membranes	26
2.2.6.2 Epithelial migration on the tympanic membrane and external auditory canal in keratosis obturans	29
2.2.6.3 Epithelial migration on the tympanic membrane and external auditory canal in external auditory canal cholesteatomas	31
2.2.6.4 Epithelial migration on the tympanic membrane and external auditory canal in retraction pockets	33
2.2.6.5 Epithelial migration on the tympanic membrane and external auditory canal in middle ear cholesteatomas.....	34
2.2.6.5.1 Acquired middle ear cholesteatomas ...	34
2.2.6.5.2 Animal models of middle ear cholesteatoma	35
2.2.6.5.3 Middle ear cholesteatomas in the canine	36
2.2.6.5.4 Epithelial migration on the tympanic membrane in humans with middle ear cholesteatoma	38
2.2.7 Generation center of the tympanic membrane	38
2.2.8 Epithelial migration on the canine tympanic membrane....	43
2.3 List of References	46
CHAPTER 3 Epithelial migration on the canine tympanic membrane	55

3.1 Abstract	55
3.2 Introduction	56
3.3 Materials and methods	59
3.3.1 Subjects	59
3.3.2 Sample size estimation	60
3.3.3 Ink drop placement (day 0)	61
3.3.4 Reevaluation (days 6-32)	62
3.3.5 Image selection	63
3.3.6 Image analysis	64
3.3.7 Calibration	66
3.3.8 Statistical analyses	66
3.4 Results	67
3.4.1 Subjects	67
3.4.2 Calibration	67
3.4.3 Ink drop placement and image analysis	68
3.4.4 Preliminary data analysis	68
3.4.5 Epithelial migration rate and pattern	69
3.5 Discussion	70
3.6 List of references	83
CHAPTER 4 Conclusions and Future Directions	86
4.1 List of references	89
BIBLIOGRAPHY	91

List of Tables

Table 3.1	Means and 95% Confidence Intervals of Epithelial Migration Rates for All Ink Drop Locations at All Time Points for Right and Left Ears in Eight Fox Hound Dogs	80
Table 3.2	Means and 95% Confidence Intervals of Epithelial Migration Rates for All Ink Drop Locations at All Time Points for Fox Hound Dogs and Beagle Dogs in all 18 Dogs	81
Table 3.3	Means and 95% Confidence Intervals of Epithelial Migration Rates for Ink Drops Located on the Pars Tensa at All Time Points in All 18 Dogs	82

List of Figures

Figure 2.1	Left Canine Tympanic Membrane	51
Figure 3.1	Ink Drop Placement Instrument	76
Figure 3.2	Locations for Ink Drop Placement on the Canine Tympanic Membrane	77
Figure 3.3	Radial Path of Epithelial Migration on the Canine Tympanic Membrane.....	78
Figure 3.4	Centrifugal Path of Epithelial Migration on the Canine Tympanic Membrane.....	79

Chapter 1

Introduction

The tympanic membrane and external auditory canal serve auditory and non-auditory functions.^{1, 2} Both the luminal surface of the external auditory canal and the lateral surface of the tympanic membrane are composed of a stratified squamous, keratinizing epithelium.¹⁻³ Cerumen, composed of glandular secretions and desquamated keratinocytes, accumulates within the external auditory canal lumen.^{4, 5} Epithelial migration on the tympanic membrane and the external auditory canal is necessary to remove this cerumen and debris from the external auditory canal and to maintain a clear passageway for sound to travel to the tympanic membrane.¹ Epithelial migration also functions in the repair of spontaneous tympanic membrane perforations⁶⁻¹⁰ and in the healing of post-operative tympanic membrane incisions.¹¹

Epithelial migration on the normal tympanic membrane has been evaluated in adults, children, guinea pigs, gerbils, and rats.¹²⁻²² Epithelial migration on the tympanic membrane and external auditory canal has also been evaluated in a number of otic diseases in humans, such as otitis externa^{9, 13} and middle ear cholesteatomas.²³ Studies of epithelial migration rely on a standardized method of placing ink on the tympanic membrane to create dynamic markers that are monitored over time.^{13, 15} Epithelial

migration on the healthy or diseased canine tympanic membrane has not been evaluated. Epithelial migration on the canine tympanic membrane must be evaluated in order to determine its role in the healthy canine ear. Furthermore, numerous otic diseases exist in dogs³ and normal canine epithelial migration rates and patterns are necessary for comparative purposes in future pathophysiologic, diagnostic, and therapeutic studies in dogs with otic diseases. In this study, we aimed to determine the rate and pattern of epithelial migration on the tympanic membrane in clinically normal laboratory dogs. Additionally, we aimed to describe an ink drop technique that could be used for future studies of epithelial migration on the canine tympanic membrane.

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Chapter 2

Literature Review

2.1 Structure and function of the human and canine external auditory canal and tympanic membrane

2.1.1 Structure of the external auditory canal and tympanic membrane

In humans and in dogs, the external auditory canal consists of cartilaginous and bony portions which are both covered with epidermis.¹⁻⁴ Dermis, densely packed with elastic and collagen fibers, and subcutis are located beneath the epidermis of the external auditory canal lumen.^{3, 5}

The canine cartilaginous external auditory canal consists of vertical and horizontal portions, creating an “L” shaped structure,^{1, 3, 6} whereas the human cartilaginous external auditory canal is composed only of a horizontal portion with a slight “S” shaped curve.^{1, 6} The elastic cartilage of the external auditory canal is located below the epidermis, dermis and subcutis.^{3, 5} The canine cartilaginous external auditory canal is further divided into auricular and annular sections of elastic cartilage. The auricular portion of cartilage forms the vertical external auditory canal and the distal portion of the horizontal external auditory canal.^{3, 4, 7} The annular portion of cartilage, an incomplete cylinder, forms the

proximal horizontal external auditory canal.^{3, 4, 7, 8} In both species, the most proximal portion of the cartilaginous external auditory canal overlaps with the bony portion to create one functional unit.^{1, 3, 4}

The length of the canine external auditory canal averages 5.3 (range 3.0-7.0) centimeters (cm) with 1.2 cm as the average length of the annular cartilage and 4.1 cm the average length of the auricular cartilage. The diameter of the canine external auditory canal narrows as it approaches the tympanic membrane, with the most distal portion measuring an average of 5.8 cm diameter and the most proximal portion measuring only 0.5 cm.⁹ The size of the adult human external auditory canal is about 2.5 cm in length and 0.8 cm in diameter.⁶

In humans and in dogs, the epidermis of the external auditory canal is extremely thin,^{1, 10-12} measuring only 15 to 30 micrometers (μm) in humans.¹ The exact thickness of the canine epidermis of the external auditory canal has not been reported, except to be noted as only a few layers thick.^{11, 13} The cellular structure of the lumen of the external auditory canal is comparable to stratified squamous keratinizing epithelium found at other body sites, as it is an epidermis composed of four layers: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. The stratum corneum, the most superficial layer of the epidermis, is composed of anucleated, cornified cells. The continuous production of cells from the basal layer (stratum basale) maintains the integrity of the stratum corneum, because the stratum corneum keratinocytes are terminally differentiated and cannot undergo further replication. The cells of the stratum basale divide, producing daughter cells that undergo differentiation as they migrate

upward through the stratum spinosum. When these migrating cells reach the stratum granulosum, the layer subtending the stratum corneum, they start the process of keratinization and ultimately become fully keratinized as they are incorporated into the surface of the epidermis.^{14, 15}

Adnexal structures, such as hair follicles, sebaceous glands, and ceruminous glands, have been identified in the dermis of both human and canine external auditory canals.^{1, 6, 8-13} Sebaceous glands use holocrine secretion to exude their lipid contents into the sebaceous gland duct which opens into the hair follicle.¹⁵ Ceruminous glands are modified apocrine sweat glands of the external auditory canal and also secrete a lipid substance through their ducts. Ceruminous glands can be found that are either epitrichial, with ducts terminating in the hair follicle, or atrichial, with ducts emptying directly on the epidermis and lacking a follicular connection.^{1, 16, 17} In veterinary studies, variable results have been published regarding the density of adnexal structures and it appears these differences in density may be related to the length of the hair coat as well as breed, specifically between dog breeds that are and are not predisposed to otitis externa.⁸⁻¹¹

The desquamated cells of the tympanic membrane and external auditory canal epidermis combine with ceruminous and sebaceous gland secretions within the external auditory canal lumen to form cerumen. The primary constituents of cerumen are keratinized, squamous epithelial cells¹⁸ and the glandular secretions.¹⁹ Additional components of cerumen may include dust, bacteria, fungi, and other foreign debris.¹⁹

The medial aspect of the external auditory canal is closed by the tympanic membrane, a round, semi-transparent structure, only 15.0 μm thick in humans²⁰ and 24 to 38 μm thick

in dogs.²¹ The tympanic membrane is composed of two sections: the pars flaccida and the pars tensa (Figure 2.1).^{1, 4, 22} The pars flaccida is the opaque, pink, semi-circular, dorsal portion of the tympanic membrane. It comprises approximately one-third of the entire tympanic membrane in dogs, but a smaller proportion of the human tympanic membrane.^{1, 4, 7} The remaining tympanic membrane consists of the pars tensa, a transparent, thin, concave structure stretched taut across a fibrocartilaginous ring.^{1, 4, 7} The manubrium of the malleus attaches to the medial surface of the pars tensa, applies tension to the pars tensa, and creates the concave appearance of the pars tensa; the point of greatest depression in this concavity is referred to as the umbo.^{3, 4, 7} The human tympanic membrane is approximately 9 to 10 mm in diameter,^{1, 23} while the canine tympanic membrane has a larger area. Measurements appear to vary based on the study, ranging from reported dimensions of 15 mm by 10 mm³ and 13.5 mm by 10 mm²³ for the entire membrane to 11.2 mm by 7.2 mm for the pars tensa alone.²⁴

The pars tensa lacks any adnexal structures, such as glands and hair follicles,^{25, 26} and is composed of three distinct layers (epidermis, lamina propria, and mucosal epithelium).²² The epidermis, the most lateral layer,²² is composed of four layers like the luminal epidermis of the external auditory canal, which are the stratum corneum, stratum granulosum, stratum spinosum, and the stratum basale. The epidermis has a thickness of 3 to 5 cell layers (15-30 μ m) in humans.¹ However, its width is not uniform, with a thicker portion over the handle of the malleus and at the annulus (peripheral rim of the membrane), and a thinner pars tensa found midway between the umbo and the tympanic annulus.¹ The middle layer of the pars tensa is the lamina propria, with an unusual

composition of thick, tightly packed collagen bundles.^{22, 23} The lamina propria is composed of four layers itself: a subepidermal connective tissue layer, an outer collagenous radial layer, an inner circular collagenous layer, and a submucosal connective tissue layer. The inner most layer of the pars tensa consists of mucosal epithelium^{1, 22} that is continuous with the epithelium of the tympanic cavity.⁶

The canine pars tensa is also thin, measuring 24 to 38 μm thick²¹ and contains more collagen than elastin fibers, no mast cells, and no inflammatory cells.²³ Similar to the human pars tensa, it is reported to be thinner in the center and progressively thicker at its periphery.³ In a histologic study directly comparing human and canine pars tensas, similar amounts of collagen, macrophages and keratin were detected based on subjective assessments. Elastin was subjectively more plentiful in the human samples than the canine samples, and mast cells were only detected in the human samples.²³

In general, the human and canine pars flaccidas tend to be thicker than the pars tensa, with less collagen, more mast cells and more heavily keratinized than the pars tensa.²³ The pars flaccida also lacks adnexal structures^{25, 26} and consists of three distinct layers: the epidermis, the lamina propria, and the mucosal epithelium.²² There is no clear distinction between the external auditory canal wall and the pars flaccida,²⁷ as the pars flaccida is continuous with the external auditory canal epidermis.²²

The epidermis of the human pars flaccida measures 5 to 10 cell layers and the fibrous tissue within the lamina propria of the pars flaccida does not have the same organized pattern as that of the pars tensa.¹ Instead, it is a loose arrangement of collagen

bundles and elastin fibers,^{22, 23} with external and internal networks of vessels and nerves.²² The abundance of elastin fibers in the human pars flaccida may account for its apparent pliable nature.²²

The canine pars flaccida contains collagen and rare mast cells.^{23, 28}

Elastin fibers were found in low numbers in the canine pars flaccida in one study²³ and were not present in another study.²⁸ In a study directly comparing human and canine pars flaccidas, similar subjective numbers of mast cells were noted in both samples, more collagen and elastin fibers were noted in the human samples than the canine samples, and more macrophages and keratin were noted in the canine samples when compared to the human pars flaccidas.²³

2.1.2 Function of the external auditory canal and tympanic membrane

The external auditory canal provides an efficient means for sound transmission from the environment to the tympanic membrane by maintaining a clear passage for the conduction of sound and protects the tympanic membrane, middle, and inner ear structures from injury.^{1, 2} The shape, depth, and rigidity of the external auditory canal provides protection from foreign material accumulating and penetrating the tympanic membrane.^{1, 2, 6}

The epidermis of the external auditory canal and the lateral aspect of the tympanic membrane provide the underlying tissues with a protective barrier from the environment.^{1, 2, 7} The stratum corneum, the most superficial layer of the epidermis, is composed of anucleated, cornified cells. It is these keratinocytes, with their lipid-

depleted, proteinaceous contents, and the surrounding extracellular lipid matrix, that afford the protective functions of the epidermis.¹⁵

Cerumen serves several protective functions: first, by ensuring the epidermis of the external auditory canal and tympanic membrane remain well hydrated.⁶ Secondly, the adherent nature of cerumen traps debris and foreign material, assisting in their removal from the external auditory canal.^{1, 2, 6, 19} Finally, cerumen has been demonstrated to have antibacterial properties in humans; this has not been evaluated in dogs.^{1, 19} The arrangement of hairs in the external auditory canal, with their tips pointed outward, provides further protection by preventing the entry of foreign bodies.^{1, 2}

The lumen of the external auditory canal needs to remain free of blockage to prevent conductive hearing loss.¹ Although the tympanic membrane is thin, it provides an excellent covering for the middle and inner ear structures because of its characteristics of elasticity and toughness, as well as insolubility to water and neutral reagents.^{6, 30} The pars tensa must remain thin to effectively vibrate when stimulated by sound waves^{1, 25} and to remain responsive to small air pressure changes on either side.²⁹ The pars flaccida, because of its elastic nature, also has a role in equalizing pressure between the external and middle ear cavities.⁶

2.2 Epithelial migration, desquamation, and generation center

2.2.1 Roles for epithelial migration on the external auditory canal and tympanic membrane

Epithelial migration has many roles. It is the primary mechanism for the removal of cerumen and maintaining a clear passageway in the external auditory canal for sound transmission.^{31, 32} Without a self-cleaning function for the external auditory canal, accumulation of cerumen within the external auditory canal lumen would prevent the passage of sound to the tympanic membrane, causing conductive hearing loss, preventing the external auditory canal from serving its auditory function.^{1, 2} Secondly, epithelial migration transports stratum corneum cells off of the tympanic membrane toward the opening at the distal end of the external auditory canal,^{1, 22, 33} maintaining the thickness of the tympanic membrane.^{31, 32} Furthermore, epithelial migration is a key factor in the repair of spontaneous tympanic membrane perforations, as has been demonstrated in guinea pigs, rats, cats, and humans.^{29, 34-37} Finally, epithelial migration also functions in repair of post-operative tympanic membrane incisions.³⁸ In summary, epithelial migration serves as a self-cleaning mechanism for the tympanic membrane and external auditory canal and as a repair mechanism for the tympanic membrane.^{29, 39-41}

2.2.2 Ink drop technique to assess epithelial migration on the external auditory canal and tympanic membrane

Studies of epithelial migration in humans (and other species) rely on a method of placing ink on the tympanic membrane to create dynamic markers that are monitored over time to record the pattern and rate of migration.^{41, 42} Various inks have been applied to the tympanic membrane in previous studies, including aqueous non-waterproof drawing ink,³² waterproof India ink,⁴² India ink,^{31, 36, 40, 43-46} waterproof Chinese ink,³⁰

gentian violet,^{39, 42, 47} methylene blue,^{38, 48, 49} Bonney's Blue,⁵⁰ and Sudan black B.⁵¹ A variety of instruments have also been employed for ink application, such as a glass micropipette with a rounded tip,³² a fine cotton tipped metal ear probe,⁵⁰ a cotton tipped applicator,^{30, 36, 39, 42, 45, 47} a metal suction tip,⁴⁸ and a malleable wire.^{38, 39, 47, 49} Most researchers applied drops of ink to the tympanic membrane,^{30-32, 36, 38-42, 44, 46-50, 52} but two studies have applied ink to the entire tympanic membrane.^{45, 51}

Materials selected by investigators appear to be based on previous studies or the investigators' personal preferences.^{31, 36, 38, 40, 41, 43-45} Cotton tipped applicators were replaced by an angled, malleable wire in one study as it was deemed more precise for ink placement.⁴⁷ In fact, Tinling and Chloe designed a customized instrument for ink drop placement.^{32, 53} In Alberti's pilot study, an aqueous solution of two percent gentian violet was applied with a cotton tipped applicator. This product was chosen because it is known to be a nonirritant and was easily visible on the tympanic membrane. However, in most of the fifteen tympanic membranes studied, the gentian violet covered nearly the entire tympanic membrane, presumed to be due to moisture that might have caused a diffusion artifact.⁵⁴ Thus, monitoring of dye clearing was observed, not migration of discrete ink drops. Consequently, waterproof India ink and a cotton tipped applicator were chosen for the final study.⁴² Gentian violet was also selected by another investigator because it was not irritating, sterile, and easily available.⁴⁷ Some investigators hypothesized that dyes containing 70% ethanol were toxic to the cells of the tympanic membrane's stratum basale and stratum spinosum and could affect the observed epithelial migration rate and pattern.³²

2.2.3 Normal epithelial migration on the human tympanic membrane and external auditory canal

2.2.3.1 Early history of epithelial migration on the human tympanic membrane

Epithelial migration on the tympanic membrane has been described for over a hundred years. In 1876 Blake reported to the First International Otological Congress his observations of outward movement of small paper disks, designed to cover small perforations, placed on the tympanic membrane. This prompted him to place paper disks on healthy tympanic membranes and observe their movement. As had been noted in the perforated membranes, the disks placed on healthy membranes also migrated off of the tympanic membrane, onto the wall of the external auditory canal, and desquamated (fell off the external auditory canal wall epidermis) at the junction of the osseous and cartilaginous portions of the external auditory canal.⁵⁵

Stinson, in 1936, performed the first study of epithelial migration on the tympanic membrane using dye markers. Initially, he observed foreign bodies, implanted in human tympanic membranes (pars tensa) during traumatic perforations, migrating off of the tympanic membrane and extruding into the external auditory canal lumen. Stimulated by those observations, he applied drops of India ink on human tympanic membranes (quantity not specified) to describe the rate and pattern of epithelial migration. Migration was non-uniform, more rapid in the posterior-inferior quadrant, and the migratory pathway of the ink drops was from the anterior margin to the posterior wall of the external auditory canal. He then published his “laws of proliferation” to describe the observations he made.⁴⁰

2.2.3.2 Later 20th century assessments of epithelial migration on the human tympanic membrane

Interest in epithelial migration on the tympanic membrane peaked again when skin grafts and prosthetic devices were first attempted for the repair of perforations of the tympanic membrane.⁴² Grafts composed of artificial substrates or harvested from non-auditory epidermis often resulted in unsatisfactory outcome. The graft was carried away from the tympanic membrane perforation, alone or in combination with the *in situ* desquamation of the grafted tympanic membrane, because the transplanted material did not have the migratory properties characteristic of the epithelium of the external auditory canal and tympanic membrane.^{42, 56} The use of canal skin for grafting the tympanic membrane perforation was then attempted and resulted in improved outcomes, with a tympanic membrane that more closely resembled a normal tympanic membrane in its appearance.^{41, 42}

These observations prompted scientists to revisit the migratory properties of the external auditory canal and the tympanic membrane. Simmons³⁶ and Litton⁴¹ both briefly described ink drop placement on human tympanic membranes for the assessment of migration on the membrane's surface, in relation to tympanic membrane perforations and on normal tympanic membranes, respectively. Litton also described that the pattern on the membrane as centrifugal, from the umbo toward the periphery, and the rate of migration as 50 micrometers per day ($\mu\text{m}/\text{d}$), with the rate appearing subjectively slower in the external auditory canal, but without direct measurements. Litton's was the first study to report a rate of migration on the tympanic membrane.⁴¹

In a larger study in 1964, Alberti observed epithelial migration on fifty-three human tympanic membranes, with various numbers of ink drops placed on six regions of the tympanic membrane: four quadrants of the pars tensa, the umbo region of the pars tensa, and the pars flaccida. The anterior superior quadrant of the pars tensa had a predominant migratory direction of anterior-superior; a small portion of the subjects had purely anterior migration in this quadrant. The anterior-inferior quadrant had three patterns observed, with the radial to periphery movement seen most commonly, but about one quarter of the subjects demonstrated anterior-superior migration and another one quarter demonstrated posterior migration. Posterior migration was observed in most of the subjects' posterior inferior quadrant, but a small portion demonstrated posterior-superior migration. In the posterior superior quadrant of the pars tensa, most ink drops moved in a posterior-superior direction, with the remaining drops migrating posteriorly. In this quadrant, a high proportion of ink drops were seen to migrate from the pars tensa onto the pars flaccida.⁴²

Two distinct patterns in all subjects were observed in the region where ink was placed on the umbo and/or the malleus. If the ink was placed on or immediately adjacent to the handle of the malleus, it was carried upwards (superiorly) toward the lateral process of the malleus with very little, if any, fanning out of the ink drop and was associated with the same spiral migratory pattern reported in the two superior quadrants. The second pattern observed was ink migration at right angles away from the handle of the malleus.⁴²

Migration of ink drops on the pars flaccida demonstrated superior, outward migration; these observations were based on ink drops placed directly on the structure as well as ink drops that passed onto this region from the pars tensa. The epithelial migration pattern was essentially the same for both tympanic membranes of the same individual; this was confirmed by marking similar regions in both ears of a subject. The pattern was also constant when the technique was repeated, as six ears of four subjects had repeated ink drop placements.⁴²

The results of the migration patterns of these six regions were combined to develop two main patterns of epithelial migration on the tympanic membranes. The majority of subjects (80%) demonstrated a centrifugal pattern in which ink drops moved in a curved path outward, from the long process of the malleus and umbo. A less common radial movement of ink drops was also reported, with the ink drop paths migrating outward, oriented at right angles to the manubrium. For both patterns observed, migration continued outwards to the periphery of the tympanic membrane, off the membrane, and onto the epithelium of the external auditory canal. When ink drops travelled off the membrane and onto the external auditory canal wall, the rate and direction of migration were not affected. Once on the external auditory canal wall, the ink drops migrated along the shortest route to the exterior. Migration was never seen to occur from the periphery of the tympanic membrane toward the center, nor was it seen from the external auditory canal wall onto the tympanic membrane.⁴²

Alberti determined an overall rate of migration on the pars tensa of 104 $\mu\text{m}/\text{d}$ which accelerated from the umbo to the periphery.⁴² However, when the graphical

information published in the study was assessed, the migration rate appeared more variable, ranging from 24 to 128 $\mu\text{m}/\text{d}$.^{32, 42} The rate of movement on the pars flaccida appeared similar to that seen in the pars tensa, but there was insufficient data to calculate the rate on the pars flaccida.⁴²

2.2.3.3 Modern assessments of epithelial migration on the human tympanic membrane

Investigations of epithelial migration on human tympanic membranes have continued to elaborate on these early observations generated by Litton and Alberti. Michaels and Soucek observed centrifugal migration on the pars tensa region of human tympanic membranes.⁵⁰ The centrifugal and radial patterns reported previously were also observed on human tympanic membranes by Makino and Amatsu, in a study where the entire tympanic membrane had marker dye applied to it. They described three directions of migration (Types A, B, and C), but Types A and B were essentially the same centrifugal pattern, simply separated into clockwise and counterclockwise categories. Although the investigators reported the most rapid migratory rate as 140 $\mu\text{m}/\text{d}$, graphical depiction of the rates identified the fastest rate as only 107 $\mu\text{m}/\text{d}$.⁴⁵ Bonding,⁵⁷ Bahadur,⁵⁸ and Moriarty⁴⁴ all demonstrated a centrifugal pattern of epithelial migration on human tympanic membranes as well. Moriarty specifically commented that the ink drops migrated continuously from the pars tensa onto the attic (pars flaccida) region.⁴⁴ All three investigators also corroborated previously published rates of epithelial migration on the

tympanic membrane by reporting average rates of approximately 100, 70, and a range of 40 to 110 $\mu\text{m}/\text{d}$, respectively.^{44, 57, 58}

More recently, in a study of postmyringoplasty tympanic membranes, epithelial migration rate and pattern of the normal contralateral tympanic membrane, which served as a control, was determined. The migratory patterns of these normal tympanic membranes demonstrated six patterns of migration, with the posterior-superior pattern being the most common (56.5%). In decreasing order of frequency, the anterior, posterior-inferior, anterior-superior, superior, and posterior migratory patterns were also observed. The mean migratory rate on the normal tympanic membranes was 91.7 $\mu\text{m}/\text{d}$, with a slower rate near the umbo region and an average duration 45.32 total migratory days.³⁸ Normal tympanic membranes also served as a control group for the assessment of epithelial migration on atelectatic tympanic membranes. Again, the posterior-superior pattern of migration was the most common pattern (42.5%) observed on normal tympanic membranes, with migration from the umbo towards the annulus and external auditory canal wall. However, the rate of migration from the umbo towards the periphery was reported as constant in these normal tympanic membranes, with a mean rate of 64.7 $\mu\text{m}/\text{d}$ (range 50-80.5 $\mu\text{m}/\text{d}$).⁴⁹

2.2.3.4 Effect of age on epithelial migration on the human tympanic membrane

Age differences for epithelial migration rates have been demonstrated in humans. Epithelial migration was assessed on normal ears of 31 children (61 ears) by marking the tympanic membrane with ink at or near the umbo. The majority of membranes (72%)

demonstrated a posterior-superior migratory pathway, with other patterns such as anterior-superior, observed less frequently. Movement was rarely in a straight line (radial); instead it was described a smooth, curved line. The pars tensa and pars flaccida appeared to function as a unit. The time for the ink drops to move off the membrane averaged 36 days (range 21-50 days).^{47, 59} The overall epithelial migration rates (131 $\mu\text{m}/\text{d}$, range 79-167) reported were faster^{47, 59} than previously reported rates in adults (50-104 $\mu\text{m}/\text{d}$).^{41, 42} The author hypothesized that the more recent embryological development of the external auditory canal contributed to this observation of a more rapid rate in children.⁴⁷ In contrast to that reported by Alberti, the rate of migration was most rapid around the umbo (nearly 200 $\mu\text{m}/\text{d}$) during the first week after ink drop placement, followed by a decreased rate over the following four weeks, and then a slight increase in rate as the ink drops approached the annulus. The patterns were concordant in most (26 of 30) pairs of ears studied and, even when the patterns were different, the rates were nearly identical. A reproducible pattern was demonstrated on 6 tympanic membranes when the ink placement was repeated. Furthermore, as has been demonstrated on adult tympanic membranes, no significant differences were noted for the mean epithelial migration rates between the left or right ears, nor between the pars tensa and pars flaccida in these children.⁴⁷

2.2.3.5 Epithelial migration on the human external auditory canal

Few studies in humans report on epithelial migration observed on the external auditory canal itself. Most studies only reported outward migration from the tympanic

membrane onto the external auditory canal without a calculated migratory rate. In Alberti's study, rates could also not be calculated for migration on the external auditory canal wall, as no fixed landmarks were present, but 89-157 $\mu\text{m}/\text{d}$ was provided as an estimated rate of migration for this region.^{42, 54} Although Makino and Amatsu reference Albert's methods, they were apparently able to calculate a migratory rate on the human external auditory canal of 142 $\mu\text{m}/\text{d}$.⁴⁵

Revadi et al designed their study to specifically assess migration on the external auditory canal wall by applying the ink drops at the annulus of the tympanic membrane. A malleable wire with markings on it was used to measure the distance between the ink drops, the annulus, and the bony-cartilaginous junction of the external auditory canal. In the normal patients enrolled in their study, the migration pattern was lateral and outwards, nearly in a straight line, and migration of the ink drops ceased at the bony cartilaginous junction. It was at this junction that the ink drops were observed to be shed off the wall and combine with cerumen. The mean rate of migration, for the external auditory canal wall, was 94.33 $\mu\text{m}/\text{day}$ (range 42-205 $\mu\text{m}/\text{d}$).⁶⁰

2.2.4 Normal epithelial migration on the tympanic membrane and external auditory canal in other species

In addition to humans, the rate and pattern of epithelial migration on the tympanic membrane has been assessed in guinea pigs,^{32, 39, 43, 59, 61} and gerbils,^{32, 51} while the pattern of epithelial migration and length of time on the tympanic membrane has been examined in guinea pigs^{39, 59} and rats.⁴⁸ Assessment of epithelial migration has also been attempted

in adult hamsters, however the ink markers disappeared rapidly and no patterns could be observed.⁵⁶

2.2.4.1 Normal epithelial migration on the tympanic membrane and external auditory canal in guinea pigs

Epithelial migration on the guinea pig tympanic membrane has been reported to be faster and have a different pattern than what has been reported in humans. In a study of tympanic membrane perforations in guinea pigs, two control tympanic membranes were marked with ink and a rapid rate of migration (nearly 1000 $\mu\text{m}/\text{d}$) was reported.³⁴ Sixty-eight guinea pig tympanic membranes were marked with a total of 115 ink drops to describe the normal epithelial migration pattern and rate. A predominantly superior migratory direction was observed. Ink drops were noted to migrate off the tympanic membrane three to five days after placement with a mean daily migratory rate of 486 $\mu\text{m}/\text{d}$ (range 119-1124 $\mu\text{m}/\text{d}$) and the rate remained constant, regardless of the location of the migrating ink drops.^{39, 59} Other investigators have reported similar results on fourteen guinea pigs tympanic membranes, with a superior path of migration and a mean rate of 790 $\mu\text{m}/\text{d}$ (standard deviation ± 410 $\mu\text{m}/\text{d}$),³² on ten guinea pig tympanic membranes, with an inferior to superior migratory path with a mean rate of 670 $\mu\text{m}/\text{d}$ (range 100-980 $\mu\text{m}/\text{d}$),³¹ on eight guinea pig ears (16 tympanic membranes), with a superior path of migration and ink drops migrating off the tympanic membrane in an average of six days;⁵² and on sixty-four guinea pig tympanic membranes, with a pattern of superior and lateral migration and a mean migration rate of 500 $\mu\text{m}/\text{d}$.⁴³ Additionally, no significant

differences were noted for mean epithelial migration rates between the left or right ears, nor between the pars tensa and pars flaccida in guinea pigs.^{32, 43} O'Donoghue placed ink drops on the external auditory canal wall in addition to on the tympanic membrane in his study of guinea pigs and always observed outward migration of ink drops on the external auditory canal wall.³⁹

Employing an alternate method, Johnson and Hawke labeled all layers of guinea pig membranes by wounding them with a fine needle dipped in ink. The investigators sought to identify in which level of the epidermis migration occurs within the tympanic membrane. The authors concluded the results of their study provided further evidence that epithelial migration occurs in the deeper layers of the tympanic membrane epidermis. However, their conclusion was based on behavior of an injured tympanic membrane with a localized inflammatory response. Ink was located, on histology, in dermal macrophages as well as in epidermal (basal and suprabasal) cells.⁶¹

2.2.4.2 Normal epithelial migration on the tympanic membrane and external auditory canal in gerbils, rats, and mice

In contrast to guinea pigs, the patterns of epithelial migration on the gerbilline and rodent tympanic membranes were similar to humans, with an outward, centrifugal migratory path. One study of fifteen gerbil tympanic membranes reported a faster overall migration rate (320 $\mu\text{m}/\text{d}$, standard deviation $\pm 160 \mu\text{m}/\text{d}$) than reported in humans.³² However, other investigators have reported migration rates in gerbils similar to the rates reported in humans. Yi et al reported tympanic membrane migration rates of 86 to 116

$\mu\text{m/d}$ for thirty-three Mongolian gerbils ranging in age from three to 30 months.⁵¹ As has been demonstrated in humans and guinea pigs, no significant differences were noted for mean epithelial migration rates between the left or right ears, nor between the pars tensa and pars flaccida in gerbils.³² Additionally, age differences for rates of migration on the tympanic membrane have been demonstrated in the Mongolian gerbils, as reported in humans. The epithelial migration rate was significantly faster in Mongolian gerbils aged three to six months ($116 \mu\text{m/d}$) compared to those aged 24 to 30 months ($86 \mu\text{m/d}$).⁵¹

No migratory rate has been reported for the rat tympanic membrane, but most markers traveled off the tympanic membrane 14 to 21 days after placement. When ink drops were placed at the border between the pars flaccida and the external auditory canal in rats, all markers migrated outward to the cartilaginous portion of the external auditory canal and disappeared within a week.⁴⁸

2.2.5 Desquamation of the external auditory canal and tympanic membrane epidermis

Unlike other body locations, the stratum corneum of the external auditory canal and tympanic membrane are not usually subject to the friction of everyday activities; it is this friction that normally removes desquamated stratum corneum cells from the epidermal surface into the local environment.^{1, 15, 62} Thus, alternative mechanisms must exist for removal of the stratum corneum from the deep portion of the external auditory canal and the tympanic membrane. It has been demonstrated that tympanic membrane stratum corneum cells migrate not only in a vertical plane during differentiation, but also

in a horizontal plane, prior to desquamation.^{1, 22} This horizontal migration (epithelial migration) of the epidermis has not been observed at any other locations on the body.^{1, 54} Evaluation, by electron microscopy, of the stratum corneum of the tympanic membrane in humans and other species has not demonstrated any morphologic changes that would account for the differences in observed behavior of the tympanic membrane compared to epidermis elsewhere.⁶³

A histopathologic study of external auditory canal skin from human cadaveric specimens has been performed to identify the site of desquamation. The bony-cartilaginous junction was identified as the site where the stratum corneum separated from the underlying, deeper epidermis and desquamated into the external auditory canal. The authors proposed that the stationary adnexal structures of the superficial external auditory canal, hair follicles and ceruminous glands, obstruct the outward movement of the stratum corneum. The oblique angle of the deepest hairs lift the migrating stratum corneum off the underlying cells and the adhesive cerumen kept the corneocytes in the superficial external auditory canal, preventing them from falling backward into the deeper portions of the external auditory canal.⁶⁴

Few ink drop studies specifically assess the site of desquamation. In Alberti's study, eighty percent of the tympanic membranes studied had little or no desquamation of the ink drops while they were located on the membrane itself. The site of desquamation within the external auditory canal could not be definitively determined, but when an ink drop could be followed from the tympanic membrane and onto the external auditory canal epithelium, it could be seen to move from the bony to the cartilaginous portion of

the external auditory canal where it was then taken up into the cerumen.⁵⁴ Others claimed later that Alberti reported the site of desquamation to be on the external auditory canal epithelium, at the junction of the deep and superficial parts of the external auditory canal.²⁰

In Revadi's study, migration of ink drops, placed on the external auditory canal epithelium in human patients, ceased at the bony cartilaginous junction. It was at this junction that the ink drops were observed to combine with cerumen and shed off.⁶⁰ A small percentage (16.7%) of human ears demonstrated desquamation in Makino and Amatsu's study, but it was not clear where the desquamation occurred.⁴⁵ O'Donoghue merely commented that little or no desquamation of ink was seen on the tympanic membrane itself in the children's ears he evaluated.⁴⁷ One investigator reported the site of desquamation of ink drops that migrated from the tympanic membrane onto the external auditory canal wall to be the chondro-osseous junction of the guinea pig's external auditory canal.⁴³ Finally, in an ink impregnation experiment, Indian ink was used but it was applied with a sterile needle to create a wound in the tympanic membrane. Desquamation of the "inky scab" was noted at the deep-superficial junction of the external auditory canal in the guinea pigs studied.⁶¹

2.2.6 Epithelial migration on the tympanic membrane and external auditory canal in selected pathologic conditions

2.2.6.1 Epithelial migration on the tympanic membrane and external auditory canal in otitis externa and abnormal appearing tympanic membranes.

Otitis externa, uncommon to rare in humans with an annual incidence of 0.4 to one percent, is an infection of the external auditory canal.⁶⁵⁻⁶⁷ Minor local trauma and exposure to moisture are the most common predisposing factors for and bacterial organisms (*Pseudomonas aeruginosa*, *Staphylococcus aureus*) are usually the cause of otitis externa.^{68, 69} On otic examination, erythema and edema of the external auditory canal is noted and cerumen is often absent.^{67, 68} If the tympanic membrane can be visualized, it usually appears normal except for a small amount of exudate on its lateral surface. Occasionally, otitis externa develops secondary to otitis media with tympanic membrane rupture. In this instance, the external auditory canal is infected with the purulent discharge from the middle ear,⁶⁷ the tympanic membrane is typically bulging, erythematous, and a perforation in the tympanic membrane may be visualized.^{67, 68}

In contrast to humans, otitis externa is a common condition in dogs, affecting 20-40% of dogs presenting for veterinary care.^{2, 70} Based on histopathologic examination of sixteen canine tympanic membranes from ears with otitis externa and media, excised during total ear canal ablation with lateral bulla osteotomy surgery, it is not uncommon to find abnormal tympanic membranes as well.⁷¹

Altered epithelial migration has been reported in humans with otitis externa, and/or tympanic membrane pathology. Litton evaluated epithelial migration on both normal and abnormal tympanic membranes of two patients with recurrent otitis externa. He demonstrated a much slower rate (10 $\mu\text{m}/\text{d}$) of epithelial migration on the tympanic membrane in the patients with otitis externa.⁴¹ Saad placed drops of waterproof ink on the tympanic membranes of humans with recurrent otitis externa or abnormal tympanic

membranes to evaluate epithelial migration. These patients all had unilateral ear disease and the normal, opposite ear was used as a control. In patients with unilateral otitis externa, the ink drops migrated much slower than on the normal tympanic membrane in the same patient. No comments were made regarding the patterns of epithelial migration.³⁰

In central-type perforations of the human tympanic membrane, variations in the pattern of epithelial migration on the tympanic membrane may indicate whether epithelium has invaded the middle ear, requiring surgical removal via tympanotomy.³⁶ Simmons evaluated two humans with perforations in their tympanic membranes and placed India ink markers on each tympanic membrane distal to the perforation site. In one patient, he observed ink drop migration around the perforation and toward the periphery of the tympanic membrane; in this ear, no epithelium was identified in the middle ear at the time of surgery. In the second patient, he observed abnormal epithelial migration on the tympanic membrane and identified a thin sheet of epithelium, along with the India ink drop, in the patient's middle ear at the time of exploratory tympanotomy.³⁶ This early observation by Simmons supports the immigration theory of middle ear cholesteatoma formation in people.⁷²

In a study by Makino et al, epithelial migration was evaluated in 15 people with pathologic (atrophied, cloudy, or calcified) tympanic membranes. India ink was applied to the entire tympanic membrane and all pathologic tympanic membranes demonstrated a slower migration rate and desquamation. Some of these tympanic membranes (four atrophied, two cloudy) also demonstrated an abnormal pattern of epithelial migration.⁴⁵

2.2.6.2 Epithelial migration on tympanic membrane and external auditory canal in keratosis obturans

Historically, the condition of keratosis obturans was considered a variation of external auditory canal cholesteatoma, but these two conditions have been considered separate diseases for at least twenty years.^{73, 74} Keratosis obturans is a rare disease in which the bony external acoustic meatus becomes occluded with a plug of desquamated keratin, leading to conductive hearing loss. In mild cases of keratosis obturans, only the tympanic membrane and not the external auditory canal epithelium may be affected.⁷⁵ Localized bony destruction of the osseous external auditory canal is not noted, unlike in external auditory canal cholesteatomas,⁷³ however rare cases of circumferential bone erosion have been described.⁷⁵ Following removal of the keratin plug, the tympanic membrane may be normal but more commonly appears thickened or retracted.⁷⁴

The underlying etiology of keratosis obturans has not been elucidated, but it has been generally accepted that it results from chronic hyperemia leading to a chronic desquamative process of the external auditory canal, but the initiating cause is not known.⁷⁴ However, others have questioned whether the hyperemia causes excess desquamation and accumulation of keratin or if the hyperemia is a consequence of the keratin plug.⁷⁵

Faulty epithelial migration has been demonstrated in keratosis obturans in two small studies.^{76, 77} Soucek discovered the complete absence of epithelial migration in two of the patients evaluated and an abnormal pattern of epithelial migration in the other two patients.⁷⁷ An additional study corroborated these results by demonstrating abnormal

epithelial migration in two people with unilateral keratosis obturans; both patients had normal epithelial migration on the unaffected tympanic membrane. One patient had a significantly delayed epithelial migration rate, with ink drops still located on the tympanic membrane eight weeks after placement, compared to the normal contralateral ear in which the ink drops migrated off the tympanic membrane within four weeks. In this patient, an abnormal epithelial migration pattern was also observed, with both anteriorly and posteriorly placed ink drops both migrating anteriorly. The second patient evaluated also had delayed epithelial migration and an abnormal pattern, with all ink drops moving downward across the tympanic membrane, including the ink drops placed on the pars flaccida.⁷⁶ In the most recent study, only one patient (two ears) with keratosis obturans had results reported because the other two patients failed to complete the study. In this individual, the EM pattern (linear) and rate (88 to 140 $\mu\text{m}/\text{d}$) was normal in the deeper part of the canal for the original four ink drop locations but migration of the ink drops initially placed in the inferior quadrant of the annulus stopped migration in the area desquamation (bony cartilaginous junction). The ink drops were still observed on the surface of accumulating layers of keratin in the external auditory canal six months after placement.⁶⁰

Cerumen plugs are mass-like accumulations of desquamated keratinocytes that have been identified deep in the horizontal ear external auditory canal in dogs and can develop into hard concretions, termed ceruminoliths. These conditions could be considered similar to keratosis obturans in humans. One author hypothesized that failure

of epithelial migration in the dog leads to the formation of cerumen plugs and ceruminoliths; this theory remains unproven.⁷

2.2.6.3 Epithelial migration on the tympanic membrane and external auditory canal in external auditory canal cholesteatomas

A cholesteatoma is a destructive, but non-neoplastic, cystic lesion, derived from keratinizing stratified squamous epithelium, with a cavity containing layers of keratin.⁷² An external auditory canal cholesteatoma is a rare condition reported in humans.⁷⁸ It is characterized by localized osteonecrosis or bony sequestration of the posterior-inferior aspect of the osseous external auditory canal with focal loss of epithelium and epithelial invasion into this localized area of periostitis.^{73, 75} In this condition, the tympanic membrane is usually normal.⁷³ External auditory canal cholesteatomas may be categorized based on pathogenic theories as congenital or acquired; acquired external auditory canal cholesteatomas are further classified as primary or secondary.^{79, 80} Congenital external auditory canal cholesteatomas form as a result of external auditory canal stenosis.^{79, 80} The etiologies of acquired primary and secondary external auditory canal cholesteatomas, however, are unclear.⁸¹ Most cases can be considered secondary, attributed to surgery or trauma of the external auditory canal.^{74, 79} Stenosis of the external auditory canal has also been considered to cause secondary acquired external auditory canal cholesteatomas.^{80, 82} Development of external auditory canal cholesteatomas in elderly patients, without a history of surgery or trauma, has been hypothesized to be a

result of the loss of normal epithelial migration with age and would be classified as acquired primary external auditory canal cholesteatoma.^{78, 79}

Abnormal epithelial migration has been observed in external auditory canal cholesteatomas. A decreased rate of epithelial migration was noted on tympanic membranes of 17 patients with external auditory canal cholesteatomas. In this study, all tympanic membranes had associated pathology, such as atrophy, clouding, or retraction,⁴⁵ even though the tympanic membrane usually appears normal in external auditory canal cholesteatomas.⁷³ India ink was applied to the entire tympanic membrane in this study and all subjects demonstrated a slower migration on the tympanic membrane and external auditory canal; slower desquamation was also observed in all cases. In three cases, absence of migration was reported and an abnormal pattern of migration was noted in one ear; this pattern was not described.⁴⁵ Interestingly, a similar study attempted to reproduce these findings by applying discrete drops of ink to the tympanic membrane instead of covering the entire tympanic membrane with ink.⁸³ However, the investigators demonstrated that the patients with abnormal tympanic membranes in the presence of external auditory canal cholesteatomas actually had qualitatively and quantitatively similar epithelial migration⁸³ to normal ears the investigators had studied previously.⁵⁷ They speculated that the conflicting results were due to differences in experimental methods.⁸³ The previous study (in 1986) did not specify the location, but may have applied ink on defective skin or the crust of the external auditory canal cholesteatoma,⁴⁵ whereas the more recent investigators specifically stated ink was applied to healed or unaffected skin.⁸³

2.2.6.4 Epithelial migration on the tympanic membrane and external auditory canal in retraction pockets

Retraction pockets of the tympanic membrane can be formed from localized invagination of either the pars tensa or the pars flaccida, but are usually located in the posterior-superior portion of the pars tensa.^{84, 85} Their formation is a result of dysfunction of the Eustachian tube^{85, 86} and/or weakening of the tympanic membrane from otitis media with effusion.⁸⁵ Retraction pockets are commonly observed in previously diseased ears⁸⁵ and are considered the most common trigger for middle ear cholesteatoma formation.^{84, 87, 88}

Epithelial migration on human tympanic membranes with retraction pockets has been reported in several studies. Saad demonstrated failure of ink migration in three of five retraction pockets marked.³⁰ Bahadur evaluated posterior-superior retraction pockets in twenty ears. In 16 of the 20 ears, ink drops migrated toward the retraction pocket, but the migration was still outward as the ink drop was placed at the umbo and the retraction pocket was located lateral to the umbo. The initial average rate of migration for the ink drops was 320 $\mu\text{m}/\text{d}$ (range 120-700) and the rate decreased as they approached the annulus and were observed to migrate onto the external auditory canal wall.⁵⁸ Finally, Tang applied methylene blue ink drops on TMs in the vicinity of the umbo and demonstrated no difference in epithelial migration rate and patterns when comparing atelectatic (secondary to any otic pathology) and normal tympanic membranes within the same forty individuals.⁴⁹

Retraction pockets and atelectasis have only rarely described in the canine otologic literature.^{2, 23} “False middle ears” reported in dogs^{12, 89-91} may be analogous to retraction pockets and/or atelectatic tympanic membranes.

2.2.6.5 Epithelial migration on the tympanic membrane and external auditory canal in middle ear cholesteatomas

2.2.6.5.1 Acquired middle ear cholesteatomas

Cholesteatomas in humans are more commonly found in the middle ear than in the external auditory canal, with the incidence of middle ear cholesteatomas being 60 times that of external auditory canal cholesteatomas.⁸¹ Of these middle ear cholesteatomas, nearly all (98%) are presumed to be acquired; rare cases of congenital (primary) middle ear cholesteatomas have been reported.⁹² Congenital middle ear cholesteatomas tend to be associated with normal tympanic membranes and external auditory canals, whereas acquired cholesteatomas are associated with a defect in the tympanic membrane.⁷²

Similar to keratosis obturans and external auditory canal cholesteatomas, the pathogenesis of acquired middle ear cholesteatomas remains unidentified,⁹³ but numerous mechanisms have been hypothesized.^{72, 86} The most accepted theories include immigration, basal cell hyperplasia, retraction pocket, and iatrogenic theories of development.^{72, 86} The immigration theory states that the epidermis from the margin of a perforated tympanic membrane migrates into the middle ear forming a cholesteatoma.^{72, 88, 94} In the basal cell hyperplasia theory, chronic inflammation of the

middle ear cavity stimulates basal cells of the tympanic membrane epidermis to form papillary ingrowths, breaking through the basement membrane, and invading the middle ear cavity.^{72, 95} The retraction pocket theory states that Eustachian tube dysfunction causes retraction of the pars flaccida toward the middle ear prior to cholesteatoma formation.^{72, 86, 87} Papillary ingrowth secondary to basal cell hyperplasia within retraction pockets has also been observed, combining the ideas identified in the basal cell hyperplasia and retraction pocket theories.^{72, 84, 87} Finally, iatrogenic placement of epidermis in the middle ear cavity during surgery for non-cholesteatomatous conditions can trigger cholesteatoma development.^{72, 94}

The most recent research identified the origin of the epithelial cells of the middle ear cholesteatoma to be from the tympanic membrane using a modified method of the well described animal model of cholesteatoma formation in Mongolian gerbils. The investigators created a hybrid tympanic membrane by repairing an iatrogenic perforation in male gerbils with grafts harvested from female gerbils' tympanic membranes. After ensuring the grafts would take, the external auditory canals of the male gerbils were then ligated to induce cholesteatoma formation. Two years after ligation, the gerbils were sacrificed and genetic analysis confirmed the gender of the cholesteatomas. In the gerbils with hybrid tympanic membranes, the tissue gender of the cholesteatomas was confirmed to be female in origin.⁹³

2.2.6.5.2 Animal models of middle ear cholesteatoma

Spontaneous development of middle ear cholesteatomas arising from the tympanic membrane and proximal external auditory canal has been documented in Mongolian gerbils. These middle ear cholesteatomas closely resemble the middle ear cholesteatomas in humans and the Mongolian gerbil is now used as an animal model for studying middle ear cholesteatomas. Anatomic differences in tympanic membrane histology between Mongolian gerbils and humans do not explain the propensity for the Mongolian gerbil to develop cholesteatomas.²³

Prior to this discovery, experimental animal models used procedures to induce the formation of middle ear cholesteatomas not found in the spontaneous development of human middle ear cholesteatomas. One such procedure involved the surgical implantation of keratinizing epithelium in the middle ear, with or without additional inflammatory or infectious agents.⁹⁶ A second procedure utilized the surgical ligation of the external auditory canal to induce middle ear cholesteatoma formation. This manipulation produces middle ear cholesteatomas in Mongolian gerbils that appear histologically similar to the spontaneous middle ear cholesteatomas in this species.^{95, 97} Ligation did not result in cholesteatoma formation in cats, guinea pigs, hamsters, mice, or rats. The authors reported the difference to be due to an unusually high rate of keratin production in the gerbil's external ear.⁹⁷

2.2.6.5.3 Middle ear cholesteatomas in the canine

Middle ear cholesteatomas have been reported in dogs and most are considered acquired as they are identified in the presence of chronic otitis externa and/or media.⁹⁸⁻¹⁰⁰

A single case has been published describing a 13 month old German Shepherd dog with a middle ear cholesteatoma in the absence of an ear infection.¹⁰¹ Little identified middle ear cholesteatomas in seven of 62 (11.3%) ears with chronic otitis externa and concurrent otitis media.⁷¹ In general, dogs may have unilateral or bilateral middle ear cholesteatomas, range in age between two to twelve years old, and have a previous history of otitis externa. Symptoms of middle ear cholesteatoma may include head shaking, otic discharge, otic pain, head tilt, facial nerve paralysis, pain on opening mouth, inability to open mouth fully, ataxia, nystagmus, circling, unilateral temporalis and masseter muscle atrophy, increased respiratory noise with dyspnea, and/or hearing loss.^{99,101} Definitive diagnosis is made based on radiographic imaging (i.e. plain radiographs, computed tomographic imaging). Abnormalities identified on imaging may include soft tissue density in the bulla, sclerosis, lysis, osteoproliferation, expansion of the tympanic bulla, lysis of the temporal bone, and/or contrast enhancement of the middle ear tissue.^{99,100} Surgical intervention is the recommended treatment to address the middle ear cholesteatomas, with total ear canal ablation and lateral bulla osteotomy being the most commonly performed technique.⁹⁹ However, in one report, a caudal auricular approach to the caudodorsal quadrant of the tympanic bulla was used to remove the abnormal tissue and reconstruct the tympanic membrane and auditory ossicles.¹⁰¹ Resolution of middle ear disease after surgery has been reported, however the clinical signs and disease may recur. Reported recurrence rates range from 36 to 53 percent.^{99,100} In one study, based on the histopathology obtained postmortem or surgically the authors concluded that these cholesteatomas formed from retraction pockets of the tympanic

membrane, with the external auditory canal stenosis being analogous to the surgical ligation of the external auditory canal performed in the experimental animal models.⁹⁸

2.2.6.5.4 Epithelial migration on the tympanic membrane in humans with middle ear cholesteatomas

Abnormal epithelial migration on the tympanic membrane has been hypothesized as a cause for some patients to develop middle ear cholesteatomas. Since studies have indicated that the pattern of epithelial migration is symmetrical in both ears of an individual patient,^{42, 47} Moriarty et al measured epithelial migration on the unaffected tympanic membrane in patients with a history of unilateral middle ear cholesteatomas to determine if the epithelial migration on the tympanic membrane was abnormal.

Unfortunately, epithelial migration was not assessed on the tympanic membranes of the ears affected with middle ear cholesteatomas. In their study, 15 patients with unilateral cholesteatomas had a normal, centrifugal, migratory pattern and a normal rate (40-110 $\mu\text{m}/\text{d}$) in the unaffected ear, suggesting that there is some other factor other than abnormal epithelial migration in these patients leading to the development of middle ear cholesteatomas.⁴⁴

2.2.7 Generation center of the tympanic membrane

In addition to describing the rate and pattern of epithelial migration on the tympanic membrane, investigators have sought to identify the source of mitotic activity or “generation center” which contains the stem cells of the tympanic membrane needed to

replenish migrating and desquamating cells. Stem cells are defined as those cells in a tissue that under normal circumstances are able to maintain their population and furnish the daughter (progenitor) cells to provide the new cells of that tissue.¹⁰² In regards to the tympanic membrane, it has been hypothesized that the stem cells located in the generation center produce progenitor cells which then are the cells involved in epithelial migration. Numerous techniques have been utilized to identify a generation center on the tympanic membrane, including histopathologic identification of mitotic activity,^{54, 103, 104} radioactive labeling of actively dividing cells with a molecule with subsequent exposure to X-ray film,^{31, 46, 56, 105-107} immunohistochemical labeling studies of cell proliferation,^{108,109} and immunohistochemistry and immunofluorescence of progenitor / stem cell markers.¹⁰²

In addition to his ink drop experiments on human tympanic membranes, Alberti performed a small study on mitosis in feline tympanic membranes and external auditory canal epithelium. Colchicine, used to arrest mitosis in metaphase, was administered to the cats, they were sacrificed, and their tympanic membranes and external auditory canal epithelia were dissected for study. Mitotic figures were identified in the basal layer and the lowest row of spinous cells in the epidermis of the tympanic membrane, in all regions of the tympanic membrane (central, peripheral and at the umbo). The direction of mitosis was usually parallel to the surface of the basal cells and mitotic figures were mostly found in groups of three to five. Mitotic figures were also located in the basal layers and rarely in the spinous layers of the epidermis of the deep external auditory canal, but the direction of mitosis could not be determined. Based on these findings, Alberti

hypothesized that random additions of cells and not a generation center caused outward movement of the cells of the tympanic membrane.⁵⁴

Several additional researchers have reported on the presence of mitosis in the epidermis of the tympanic membrane. McMinn and Taylor noted a mitotic index of 0.15% on adult guinea pig tympanic membranes when studying the entire lateral surface of the tympanic membrane.¹⁰³ Likewise, a second investigator harvested normal guinea pig tympanic membranes, counted mitotic figures present in the epidermis, and reported the values as a percentage of the total number of cells. In the region between the annulus and the midpoint of the tympanic membrane, the average mitotic index was 0.19 percent (range 0.11-0.31).¹⁰⁴ Both measurements were considered higher than expected, because epidermis found elsewhere has a lower mitotic index of only 0.02%.¹⁰³

Autoradiography uses X-ray film to detect cells that have been radioactively labeled with a molecule (i.e. tritiated thymidine, bromodeoxyuridine [BrdU]) that is only taken into the cell during synthesis of DNA (S phase of cell cycle).³¹ This material remains with the cell indefinitely and the strength of labeling diminishes as the cells continue to divide.¹⁰⁵ Litton employed this technique with tritiated thymidine to localize the generation center in guinea pig tympanic membranes to the junction of the annulus of the tympanic membrane and the external auditory canal. He then tracked the outward migration of labeled cells through the deeper layers of the epidermis of the external auditory canal. He demonstrated that the basal cells moved at a similar rate and in a similar direction to ink drops placed on the tympanic membrane's surface and took this as

evidence that epithelial migration originated in the deeper layers of the tympanic membrane's epidermis.^{31, 105}

Similar autoradiographic techniques have been used to identify the generation center on the murine tympanic membrane. In the study by Brown, labeled cells were distributed randomly across the entire tympanic membrane in mice and no specific generation center was identified,¹⁰⁶ which is in agreement with the results found in Alberti's study on feline tympanic membranes.⁵⁴ Two different groups studying murine tympanic membranes have identified the highest mitotic activity in the basal cell layer of the pars tensa, located at the annulus and the handle of the malleus. In these studies, labeling was equally distributed across the entire pars flaccida.^{46, 56, 107} Unlike Litton, Boedts and Kuijper detected essentially no migration of labeled basal cells even after 18 days, the longest interval between isotope administration and tympanic membrane harvest. The investigators explained the discordant findings as a result of the difference in thickness of tympanic membranes between the two species. Due to the thickness of the guinea pig tympanic membrane (20-40 μm), the labeled cells in the basal layer were unable to activate the film, and the observed blackening in the emulsion originated from the superficial layers of the tympanic membrane epithelium.⁵⁶ Thus, the authors concluded that the rapid migration, observed by Litton in the stratum corneum of the tympanic membrane with ink drop and autoradiographic experiments, was the physiological self-cleaning mechanism of epithelial migration, but the mitotic activity identified in the basal cell in their study indicated this was the location of the generation center.^{31, 56}

Immunohistochemistry using BrdU staining of tympanic membranes has also been performed on tympanic membranes of a number of species, including mice and gerbils. In a study by Kakoi and Anniko in mice, similar generation centers of the tympanic membrane were identified with this technique as were with autoradiography which were the handle of the malleus and the annular regions of the pars tensa and scattered without a pattern in the pars flaccida. In normal gerbil tympanic membranes, intense BrdU labeling was noted, primarily of basal keratinocytes, at the handle of the malleus and the annular regions of both the pars tensa and pars flaccida. Additionally, labeling was also denser in the deep bony portion of the external auditory canal epidermis, compared to the more lateral cartilaginous portion of the external auditory canal.¹⁰⁹ Unfortunately, similar autoradiographic or immunohistochemical studies are not available for human or canine tympanic membranes.

Most recently, additional immunohistochemical studies on rodent and human tympanic membranes have been performed. Since the epidermis of the skin and the lateral aspect of the tympanic membrane are histologically similar, authors have proposed that epidermal stem cells of the human tympanic membrane would most likely share characteristics with interfollicular epidermal stem cells.¹⁰² Although no definitive stem cell markers exist for the interfollicular epidermal stem cells, alpha-6 integrin appears to be the best marker available. Beta-1 integrin and cytokeratin 19 have also been evaluated, but the specificity for these two molecules to identify only stem cells remains questionable. Alpha-6 integrin was identified in the basal keratinocytes of the umbo, in the annular region, and along the malleus of human tympanic membranes. Beta-1 integrin

and cytokeratin 19 were also identified in these regions of the human tympanic membrane, but were also detected in the suprabasal layers of the epidermis, so the investigators concluded the beta-1 integrin and cytokeratin 19 staining was not specific for stem cells. The investigators believe they identified stem cells and progenitor cells, as both types of cells are positive for alpha-6 integrin. However, because stem cells are the origin of progenitor cells, they concluded this region also harbors stem cells.¹⁰²

Although the exact site(s) of generation centers may differ between species, mitotic activity, based on most of these labeling studies of the tympanic membrane, occurs at a slow rate in the cells of the basal layer of the epidermis. This slow rate of migration is in contrast to the rapid migration rate of the cells in the upper layer of the stratum corneum reported from ink drop studies, which represent epithelial migration.^{108,110}

2.2.8 Epithelial migration on the canine tympanic membrane.

To the author's knowledge, there are no published reports describing epithelial migration on the canine tympanic membrane in the normal ear or in ears with otic pathology, as well as no published techniques for marking the canine tympanic membrane with drops of ink. It has been assumed that the pattern of epithelial migration in the dog is similar to the pattern in humans.

Considering the proven importance of epithelial migration for the tympanic membrane and external auditory canal in humans and other species, documenting epithelial migration rates and patterns on normal canine tympanic membranes may lead

to additional studies to help improve our understanding of otitis and other pathologic otic conditions in the dog.

Figure 2.1 - Left Canine Tympanic Membrane

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Chapter 3

Epithelial Migration on the Canine Tympanic Membrane

3.1 Abstract

Epithelial migration is a process that serves as a self-cleaning and repair mechanism for the external auditory canal and tympanic membrane. Epithelial migration has been evaluated in humans and several other species, but not in dogs. The objective of this study was to determine the rate and pattern of epithelial migration on the tympanic membrane in clinically normal laboratory dogs. Eighteen dogs were anesthetized, and three drops of waterproof drawing ink were placed on two sites of the pars tensa and one on the pars flaccida. Images were recorded with a video otoscope and digital capture system. Each dog was evaluated and images recorded every six to eight days for four evaluations. Migration pattern analysis and epithelial migration rate calculation were performed with image processing software. Descriptive statistics for epithelial migration rate (mean, standard deviation, 95% confidence interval) were calculated for all ink drop locations on the tympanic membrane (PT1, PT2, PF) at each time point. No significant differences in the mean epithelial migration rates were identified between right and left ears of the fox hound dogs, breeds (beagle, fox hound), or locations PT1 and PT2. The mean overall epithelial migration rates ($\pm$ standard deviation) were 96.4 ($\pm$ 43.1) and 225.4

(± 128.1) micrometers per day for the pars tensa and pars flaccida, respectively. All ink drops moved outwards, the majority in a radial direction, from the original location to the periphery of the tympanic membrane. The ink drop placement method used in this study can be used in future studies to determine the epithelial migration rate of the canine tympanic membrane.

3.2 Introduction

The external auditory canal provides an efficient means for sound transmission from the environment to the tympanic membrane, allowing protection of the tympanic membrane and inner ear structures from damage as well as maintenance of a clear passage for the conduction of sound.¹ The external auditory canal and tympanic membrane are composed of a continually renewing, keratinizing, squamous epithelium. A mechanism for the removal of these desquamating epithelial cells is necessary to prevent their accumulation from impeding sound transmission to the tympanic membrane. Epithelial migration provides a mechanism for both removal of debris and repair of injury to the tympanic membrane. As the epithelial cells migrate, they eventually desquamate, similar to corneocytes. These desquamated epithelial cells combine with apocrine and sebaceous gland secretions to form cerumen within the external auditory canal.² Epithelial migration transports cerumen away from the tympanic membrane and toward the opening at the distal end of the external auditory canal. Furthermore, the migratory process is a key factor in the repair of spontaneous tympanic membrane perforations³⁻⁷ and post-operative tympanic membrane incisions.⁸ The role of

epithelial migration in healing tympanic membrane perforations has been demonstrated in guinea pigs,^{3,7} rats,⁴ cats,⁶ and humans.⁸ In summary, epithelial migration serves as a self-cleaning mechanism for the tympanic membrane and external auditory canal and as a repair mechanism for the tympanic membrane.^{7,9-11}

In 1882, Blake noted outward movement of small paper disks placed on the tympanic membrane, which migrated onto the wall of the external auditory canal, and desquamated at the junction of the osseous and cartilaginous portions of the canal.¹² Stinson applied drops of India ink on human tympanic membranes to describe the rate and pattern of epithelial migration. Migration was non-uniform, more rapid in the posterior-inferior quadrant, and the migratory pathway of the ink drops was from the anterior margin to the posterior wall of the canal.¹⁰ In 1963, Litton applied single ink drops in various locations on human tympanic membranes and demonstrated that the epithelial migration was centrifugal from the umbo and the rate of migration was 50 micrometers per day ($\mu\text{m}/\text{d}$).¹¹ In a larger study in 1964, Alberti observed two main patterns of epithelial migration on the human tympanic membrane: a centrifugal pattern in which ink drops moved from the long process of the malleus and umbo outward and a less common radial movement of ink drops at right angles from the manubrium outward. This migration continued outwards to the periphery of the tympanic membrane, off the tympanic membrane, and onto the epithelium of the external auditory canal. The overall rate of migration was 104 $\mu\text{m}/\text{d}$ and accelerated from the umbo to the periphery.¹³

More recently, the rate and pattern of epithelial migration on the tympanic membrane has been assessed in children,^{14,15} guinea pigs,¹⁶⁻¹⁸ and gerbils,^{16,19} while the

pattern of epithelial migration and length of time on the tympanic membrane has been examined in guinea pigs¹⁵ and rats.²⁰ Epithelial migration patterns on the tympanic membrane may differ between species. For example, epithelial migration patterns differ considerably between humans (centrifugal) and guinea pigs (inferior to superior) while migration patterns in humans, gerbils, and rats are similar.^{15,16,20} Age differences for epithelial migration rates also occur within species. In children, epithelial migration rates (131 $\mu\text{m/d}$) are faster^{14,15} than in adults (50-104 $\mu\text{m/d}$)^{11,13} and, likewise, the epithelial migration rate is significantly faster in Mongolian gerbils aged three to six months (116 $\mu\text{m/d}$) compared with those aged 24 to 30 months (86 $\mu\text{m/d}$).¹⁹ These results indicate the importance of considering the impact of age and species when studying epithelial migration on the tympanic membrane.

Studies of epithelial migration in humans (and other species) rely on a method of placing ink on the tympanic membrane to create dynamic markers that are monitored over time to record the pattern and rate of migration.^{11,13} Various inks have been applied to the tympanic membrane in previous studies, including aqueous non-waterproof drawing ink,¹⁶ waterproof India ink,¹³ India ink,^{5,10,18,21-24} waterproof Chinese ink,²⁵ gentian violet,^{9,13,14} methylene blue,^{8,20,26} Bonney's Blue,²⁷ and Sudan black B.¹⁹ A variety of instruments have also been employed for ink application, such as a glass micropipette with a rounded tip,¹⁶ a fine cotton tipped metal ear probe,²⁷ a cotton tipped applicator,^{5,9,13,14,22,25} a metal suction tip,²⁰ and a malleable wire.^{8,9,14,26} Most researchers applied drops of ink to the tympanic membrane,^{5,8,10,11,13,16,20,25-28} but two studies have applied ink to the entire tympanic membrane.^{19,22}

To the authors' knowledge, there are no published reports to describe normal or abnormal epithelial migration in the dog, nor a technique in dogs to mark the tympanic membrane with ink drops. The primary objectives of this study were to characterize and quantify epithelial migration on the tympanic membrane in clinically normal laboratory dogs without otitis and to develop an ink drop method to be used for future research. It was hypothesized that the rate and pattern of epithelial migration on the canine tympanic membrane would be similar to that demonstrated in humans.

3.3 Material and Methods

3.3.1 Subjects

The experimental protocol adopted was approved by the Institutional Animal Care and Use Committee (IACUC). This study was conducted using laboratory dogs (beagles and fox hounds). Dogs included were between one and four years of age. Dogs selected had normal physical examinations, including normal dermatologic examinations; normal findings on hand-held otoscopic examination, including normal tympanic membranes in both ears; and unremarkable complete blood counts and serum biochemical profiles no more than 60 days prior to study enrolment. Dogs were housed in the laboratory research facility at a veterinary college and were under the care of the University Laboratory Animal Resources (ULAR) staff. The animals were housed indoors, in individual concrete runs, in a temperature and humidity controlled environment. They were maintained on a diet of Iams Mini Chunks (The Iams Company, Cincinnati, OH, USA) or Teklad 25% Lab Dog Diet (Harlan Laboratories, Indianapolis, IN, USA), fed once a day,

with occasional Iams dog treats, and water *ad libitum*. They were socialized and exercised indoors daily. Dogs were not permitted to be bathed nor have any products applied on or into their ears during the study period. Concurrent use of topical and oral medications, except flea control and heartworm prophylaxis, was prohibited during the study period. Prior drug history for these dogs was unknown, but all dogs had at least a fourteen day acclimation period under the investigators' care and not yet enrolled in the study. During this acclimation period, prohibited products were not used. The ULAR staff visually monitored the dogs daily for adverse effects associated with study procedures which included, but were not limited to, otic pruritus (e. g. head shaking, scratching at pinnae) and neurologic symptoms (e. g. head tilt, circling, ataxia, facial droop).

3.3.2 Sample size estimation

Sample size estimate was based on preliminary measurements taken during a pilot study of epithelial migration in a canine ear performed by the investigators, previous measurements of the canine tympanic membrane,²⁹ and values from previous epithelial migration studies involving other species.^{11,16} For the present study, it was calculated that 18 canine ears were needed to achieve the expected precision for measurement of an epithelial migration rate of approximately 200 $\mu\text{m}/\text{d}$ (power of 0.80, significance level $\alpha = 0.05$). Sample size was calculated with statistical software (STATA10; StatCorp, College Station, TX, USA).

3.3.3 Ink Drop Placement (day 0)

Dogs were premedicated with acepromazine maleate (generic; Vedco, Saint Joseph, MO, USA) intramuscularly (IM) or subcutaneously (SQ), at a dose of less than or equal to 0.15 mg/kg but not to exceed 3 mg per dog in total. An intravenous catheter was placed in one forelimb and anesthesia was induced using intravenously administered ketamine hydrochloride (KetaVed; Vedco) 2.5 mg/kg and diazepam (generic; Hospiria, Lake Forest, IL, USA) 0.13 mg/kg. Endotracheal intubation was performed and general anesthesia was maintained using inhaled isoflurane (1-3%, IsoSol; Vedco) in 100% oxygen.

A customized instrument (Figure 3.1) for applying the ink was created by gluing a 75 mm glass hematocrit tube (Drummond Scientific Company, Broomall, PA, USA) to a metal probe (Harbor Freight Tools, Columbus, OH, USA). Prior to gluing the hematocrit tube to the probe, the tip of the probe was bent to a 115° angle to horizontal. The open end of the hematocrit tube was rounded and closed using a propane flame. Three different colored drops of waterproof drawing ink (Universal 3080-F; Koh-I-Noor, Bloomsbury, NJ, USA) were used to mark the tympanic membrane. Two sites were marked the pars tensa caudal to the stria mallearis (PT1 and PT2) and one on the center of the pars flaccida, as close as possible to standardized locations, with PT1 always being dorsal to PT2 (Figure 3.2).

Dogs were placed in lateral recumbency, a four millimeter otoscopic cone (Welch Allyn, Inc., Skaneateles Falls, NY, USA) was placed in the vertical external auditory canal, and an operating microscope (Op-Mi 1; Carl Zeiss, Inc., Oberkochen, Germany)

was positioned above the dog for visualization of the tympanic membrane. Marking of the tympanic membrane was performed as follows. Using the operating microscope, the above instrument was inserted through the otoscopic cone after first dipping it in ink. The tympanic membrane was marked by gently touching the tip of the instrument to the surface. The instrument was wiped with a wet gauze sponge, dipped in another color of ink, and the process was repeated. The otoscopic cone was removed, and the otoendoscope of a video otoscope (Karl Storz Veterinary Endoscopy America; Goleta, CA, USA) was inserted into the dog's ear. Multiple digital images of the tympanic membrane were obtained using the AIDA-vet video capture system (Karl Storz Veterinary Endoscopy America). The dog was placed in lateral recumbency on the contralateral side and the above procedure was repeated on the other tympanic membrane. The dog was recovered from general anesthesia.

Only one ear was needed for the analysis; however, ink drops were applied to both tympanic membranes for all dogs to ensure that the data from at least one ear would be acceptable for inclusion in the analysis. A previous study found only one ear suitable, although did not specify why, for epithelial migration evaluation despite initially applying ink drops to both tympanic membranes.¹⁴

3.3.4 Reevaluation (days 6-32)

Each dog was re-evaluated every six to eight days following ink placement for a total of four re-evaluation examinations or over a total of 24 to 32 days. Depending on the disposition, the dogs were restrained during examination using no sedative or

anesthesia; sedation with acepromazine, at a dose of less than or equal to 0.3 mg/kg but not to exceed 3 mg total, I.M. or S.Q; sedation with acepromazine and hydromorphone hydrochloride (generic; Baxter Healthcare Corp., Deerfield, IL, USA), 0.15 mg/kg, I.M. or S.Q., alone or in combination with mask delivery of isoflurane (1-5%) in 100% oxygen. For data collection, the dog was placed in lateral recumbency on the same examination table, in the same position, with the investigator in the same location relative to the dog and the examination table. The otoendoscope and video capture system were used to photograph the tympanic membrane. During re-evaluation examinations, the Day 0 digital images for each dog's ear were simultaneously displayed on a laptop computer (Powerbook G4; Apple, Inc., Cupertino, CA, USA). These Day 0 digital images were used to ensure the same positioning of the tympanic membrane was obtained on the digital image of each re-evaluation as was collected on Day 0. Consistency with regard to depth and angle of the otoendoscope was confirmed based on comparison of images, with adjustment of the otoendoscope depth and angle performed to achieve identical images as judged by the investigators. Data collection was repeated on the contralateral ear in a similar manner.

3.3.5 Image Selection

All digital images were reviewed after each acquisition. The digital image selected for image analysis was the one that most resembled the Day 0 digital image and met the following criteria: positioning of the stria mallearis and pars tensa, depth of the otoendoscope of the video otoscope in the external auditory canal, and angle of the

tympanic membrane. Ten digital images for each dog were selected (five for each ear studied: ink placement and four re-evaluations) for the image analysis.

3.3.6 Image analysis

All selected digital images were loaded into image analysis software (ImagePro Analyzer[®] version 6.3; Media Cybernetics, Inc., Bethesda, MD, USA) for ink drop tracking. In some cases, even though all efforts were made to select digital images for each evaluation that most resembled the Day 0 digital images, the tracking feature was not able to align digital images properly to mark and track the ink drops. Consecutive chronological pairs of digital images for each ear were aligned and scaled in the program, using the manual adjustment feature in the align images function of ImagePro Analyzer[®]. This resulted in four pairs of aligned digital images created for each ear studied: ink drop placement and first re-evaluation (0-1); first and second re-evaluation (1-2); second and third re-evaluation (2-3); and third and fourth re-evaluation (3-4). This produced pairs of digital images for tracking analysis. The pairs of digital images were calibrated using the spatial calibration wizard feature in the image analysis software to record the tracking distance (in micrometers). Calibration was based on the width of the stria mallearis (refer to “Calibration” subsection below).

The manual tracking feature was used to mark the three ink drops on each digital image in a pair. The program measured the distance traveled between time points for each ink drop and reported the value in micrometers. Tracking for each ink drop was completed when all chronological pairs of digital images were evaluated or when the ink

drop was no longer located on the tympanic membrane, whichever event occurred first. Each ear studied had three ink drop locations to monitor (PT1, PT2, PF) during four different time periods (0-1, 1-2, 2-3, 3-4). All distances were exported to Excel spreadsheets (Microsoft Excel 2007; Microsoft Corp., Redman, WA, USA) from ImagePro Analyzer[®] for further analysis. The epithelial migration rate for each dog at each ink drop location, at all four time periods, was calculated by dividing the distance traveled between each time point (μm) by the days between each time point. Finally, the overall epithelial migration rate for each dog at each ink drop location was calculated by dividing the sum of the distance traveled (μm) by the ink drop on the tympanic membrane by the total number of days that ink drop was observed on the tympanic membrane.

The direction of movement for each ink drop was recorded as outward (toward the annulus and external auditory canal) or inward (toward the center of the tympanic membrane). The overall pattern of epithelial migration for each ink drop on each dog's tympanic membrane was also assessed by plotting the path of the ink drops at each time point. The estimated center of the ink drop was marked, using the image analysis software, at each time point and connected by a line to determine the migration path. If the path created was a straight line, the pattern would be categorized as radial. If the path created was not a straight line but was curved outward from the original location, it would be categorized as centrifugal. If the path created was not straight or curved, it would be categorized as other.

3.3.7 Calibration

A calibration standard was necessary for image analysis to record tracking distance in micrometers rather than pixels. The calibration standard was based on measurements of the dog's malleus. A subset of the enrolled laboratory dogs were euthanized after completion of this study, for reasons unrelated to this study, according to ULAR protocol. Dissection of the tympanic membrane was performed, following the techniques described by Rose.³⁰ This exposed the most proximal portion of the annular cartilage, the bony external acoustic meatus, the intact tympanic membrane with associated auditory ossicles, and portions of the tympanic bulla, zygomatic arch, and temporal bone. Hand held calipers, with the smallest increments marked as 1.0 mm, were used to measure the width of the manubrium of the malleus at the midpoint of the pars tensa on the medial aspect of the tympanic membrane.

3.3.8 Statistical Analyses

Descriptive statistics (mean, median, standard deviation [SD], range) were calculated for the age of all dogs, the epithelial migration rate for each ink drop location (PT1, PT2, PF) during each time period (0-1, 1-2, 2-3, 3-4), the overall epithelial migration rate for each ink drop location, and the time until migration off the tympanic membrane for those ink drops that were observed to migrate off the tympanic membrane. Normality of data was assessed using the Kolmogorov-Smirnov test. Mann-Whitney U-tests were used to compare non parametric data.

The means and 95% confidence intervals for epithelial migration rate were calculated for each ink drop location during each time period. The 95% confidence intervals were then compared between ears (right and left), breeds (beagles and hounds), and ink drop locations (PT1, PT2). If the 95% confidence intervals overlapped for any of these comparisons, then no difference existed between the means, at $\alpha = 0.05$ significance, and values for the right ear would be used, values for both breeds would be pooled, and values for PT1 and PT2 would be pooled for further analysis. Descriptive statistics, confidence intervals, and statistical analyses were performed in GraphPad Prism[®] version 5.02 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

3.4 Results

3.4.1 Subjects

Eighteen laboratory dogs completed the study and consisted of eight beagles (five intact females, three intact males) and ten fox hounds (two intact females, eight intact males). The age of dogs at the time of study enrolment ranged from 1.3 to 3.2 years, with a median of two years. The length of time each dog was enrolled in the study ranged from 24 to 32 days after ink drop placement.

3.4.2 Calibration

The width of the manubrium of the malleus was measured in five dogs (four beagles and one fox hound). The manubrium of the malleus at the midpoint of the pars

tensa on the medial aspect of the tympanic membrane of all five dogs measured 1.0 mm wide using the hand held caliper; there was no range of values for the measurements.

3.4.3 Ink drop placement and image analysis

All dogs recovered uneventfully from the procedures and had no evidence of adverse effects from ink drop placement. All rates for each time point for ink drops PT1 and PT2, and rates for time periods 0-1 and 1-2 for ink drop PF were normally distributed. Too few data points were available for testing normality with PF 2-3 and PF 3-4 because many ink drops had already migrated off the pars flaccida. A total of 53 ink drops (18 PT1, 18 PT2, 17 PF) were included in final analyses, one ink drop for each location in one ear of each dog. The PF ink drop in one beagle dog could not be tracked because it was not visible on any re-evaluation digital images at the time of image analysis.

Eight fox hound dogs had digital images from both ears that met the criteria for image analysis, while all eight beagle dogs (five right ears, three left ears) and two fox hound dogs (one right ear, one left ear) had only one ear that met the criteria.

3.4.4 Preliminary data analysis

In the eight fox hound dogs, epithelial migration rates were calculated for both ears to determine whether differences in epithelial migration rates existed between right and left ears of the same dog. Based on the overlapping 95% confidence interval values, no significant differences ($P > 0.05$) were demonstrated between both ears for the

epithelial migration rates at each ink drop location at each time period (Table 1). The data for only the right ear in these eight fox hound dogs were used for further analysis.

Likewise, no statistical differences ($P > 0.05$) were demonstrated between dog breeds at any time point or any location (Table 2), so epithelial migration rates for each ink drop for each time period were pooled. Finally, no statistical differences ($P > 0.05$) between epithelial migration rates for ink drop location PT1 and ink drop location PT2 were found (Table 3), so these values were pooled into PT-total (PTT) for calculation of epithelial migration rate.

3.4.5 Epithelial migration rate and pattern

Migration of the ink drops off the tympanic membrane was observed during the study period in 12 of 18 (66.7%), seven of 18 (38.9%), and 16 of 17 (94.1%) PT1, PT2, and PF ink drops, respectively. The mean overall epithelial migration rate ($\pm$ SD) was 96.4 ($\pm$ 43.1) $\mu\text{m/d}$ for the PTT and 225.4 ($\pm$ 128.1) $\mu\text{m/d}$ for the PF.

All ink drops (53 of 53) moved in an outward direction from the stria mallearis toward the annulus and external auditory canal epithelium. No ink drops were seen to migrate inward toward the umbo. Additionally, no ink drops migrated from the pars tensa to the pars flaccida. Forty-eight of 53 ink drops (90.6%) moved in an outward radial direction; the ink drops moved in a straight line from the original location outwards, like spokes on a bicycle (Figure 3.3). The radial pattern was observed in at least one ink drop location in all 18 ears. The less common centrifugal pattern, in which the ink drop took a curved course outwards from the original location (Figure 3.4), was observed in three

ears (16.7%). In these three ears, a total of five of 53 ink drops (9.4%) demonstrated the centrifugal pattern: PT1 and PT2 ink drops in two fox hound dog and PF ink drop in a third fox hound.

3.5 Discussion

The rate and pattern of epithelial migration on the tympanic membrane has been demonstrated in humans^{11,13} and research animals (e.g. guinea pigs, gerbils)¹⁵⁻¹⁹ by applying ink drops to various locations on the tympanic membrane and tracking the migration of these ink drops. However, to the authors' knowledge, this is the first study to report the epithelial migration rate and pattern on normal laboratory canine tympanic membrane s. In our study, the mean overall epithelial migration rate ($\pm$ SD) on the tympanic membrane for fox hounds and beagles was 96.4 ($\pm$ 43.1) μ m/d for the PTT and 225.4 ($\pm$ 128.1) μ m/d for the PF. All ink drops moved outward from the original ink drop location to the periphery of the tympanic membrane and the majority moved in a radial pattern, not centrifugal.

To the authors' knowledge, this is the first study to describe a technique in dogs with which to assess epithelial migration on the tympanic membrane. The ink drop placement technique described here is based on previously described techniques^{13,16} with several modifications. By inserting an otoscopic cone into the external auditory canal, visualization of the tympanic membrane through the operating microscope was achieved, but difficulty was encountered in accessing the rostral portion of the pars tensa in our study, so ink drops were not applied to this region. Other investigators have reported

similar difficulty in applying ink drops to this region of the pars tensa in human and guinea pig ears.^{9,13,23} In fact, to improve visualization of the tympanic membrane, as well as to allow for ink drop placement rostrally on the tympanic membrane, one investigator performed surgical ablation of the external auditory canal of guinea pigs and gerbils prior to ink drop placement.¹⁶ We did not perform external auditory canal surgery on the dogs in our study because we wanted to develop a clinically applicable technique that could be used in a variety of different breeds of dogs as well as in client-owned dogs with otitis. Surgical manipulation of the external auditory canal in client-owned dogs for assessing epithelial migration would not be justifiable.

One of the more difficult aspects of this study was measuring the distance traveled by the ink drops. In previous studies, the migratory rate was estimated by assessing the distance between ink drops or marker dyes and various landmarks on the tympanic membrane on recorded photographs and line drawings or with a graticule in the eye-piece of the microscope.^{9,13,14,19,20,22,26} Some investigators were only able to estimate the migratory rate visually.^{10,11} Alberti noted that rate measurements were not completely accurate since it was not possible to photograph exactly comparable fields of the same ear from week to week, and therefore a correction factor was used to measure the migration.³¹ In one study, serial photography was attempted, but soon relinquished due to many technical difficulties with the procedure.¹⁴ However, to our knowledge, ours is the first study to report the use of a computerized image analysis program for calculating epithelial migration distances. Utilization of the program does require sequential images to be aligned, which is why the images obtained at the evaluations were chosen with

reference to the previously obtained image on Day 0. Although a great deal of time was spent aligning the images, and in some ears alignment was not possible, the use of digital images and computer software was beneficial. The manual tracking feature was used to mark the ink drops on each digital image in a pair and the software program then measured the distances.

Epithelial migration rate was evaluated for both ears in a subset of the dogs in our study (eight fox hounds) and found to be similar, confirming that either ear could be used for determining epithelial migration rates in this as well as in future studies. This finding is consistent with previous investigators, who also demonstrated that the epithelial migration rate is similar when comparing both ears of a single subject.^{14,16,18,24,29}

Epithelial migration rates in our study ranged from 10.2 to 236.8 and 9.2 to 522.5 $\mu\text{m}/\text{d}$, for the PTT and PF, respectively. The rates for both of these locations had large ranges as well as standard deviations in our study, which correlated well with our subjective observations of wide between-dog variability in ink drop migration speed. In the few other studies that reported ranges of values for tympanic membrane epithelial migration, wide ranges were also noted. Standard deviations in one study of gerbils and guinea pigs reported standard deviations from 110 to 460 $\mu\text{m}/\text{d}$.¹⁶ The epithelial migration rates we determined for the pars tensa region of normal canine tympanic membranes more closely approximate the rates of a child (131 $\mu\text{m}/\text{d}$) or young gerbil tympanic membrane (113 $\mu\text{m}/\text{d}$),^{14,19} than an adult human (50 $\mu\text{m}/\text{d}$).^{11,16} As the dogs chosen for this study were all young adult dogs, age related changes in epithelial migration rate in very young or very old dogs cannot be ruled out with the present study.

Age related changes have been identified in previous studies.^{14,15,19} For example, children had faster tympanic membrane epithelial migration rates than adults,¹⁴ and young Mongolian gerbils had faster rates than older Mongolian gerbils.¹⁹

Previous ink drop studies have not demonstrated a difference between the epithelial migration rate for the pars tensa and the pars flaccida.^{13,16} However, our findings show that the mean epithelial migration rate for the PF (225.4 $\mu\text{m}/\text{d}$) is over two times faster than that calculated for the PTT (96.4 $\mu\text{m}/\text{d}$) in normal laboratory dogs. This is in contrast with identical rates observed in humans, gerbils and guinea pigs. The reason for this difference in the dog is not known.

Outward movement of all ink drops occurred in our study and this finding is similar to all other species studied.^{10,11,13,16,20,21,23} In our study, no ink drops were noted to move from the pars tensa to the pars flaccida. This is in contrast to the findings of other investigators, who observed ink drop movement from the pars tensa to the pars flaccida on adult human and guinea pig tympanic membranes.^{13,16,24}

The length of time for which ink drops remained on the tympanic membrane was variable in previous studies, ranging from one to five days in the guinea pig,^{9,15,16} from 14 to 21 days in the rat,²⁰ and from 21 to 50 days in children.¹⁴ The time that the ink drops spent on the tympanic membrane was most likely to be dependent on drop location and migratory rate and pattern. In the study reported here, because the tympanic membranes were not observed daily, the exact day after placement on which the ink drop migrated off the tympanic membrane could not be determined. However, the majority (94.1%) of PF ink drops had migrated off the tympanic membrane by study conclusion, compared to

a smaller percentage of PT1 and PT2 ink drops (66.7% and 38.9%, respectively). This information will be useful for future epithelial migration studies for determining the time interval for re-evaluations, based on ink drop location.

Both radial and centrifugal patterns of epithelial migration have been described in humans^{11,13,21,22} and rats,²⁰ whereas in other species, only a single pattern (either radial or centrifugal or other) has been reported.^{16,23} In our study, two epithelial migration patterns were found on the tympanic membrane of laboratory dogs: the majority moved in a radial pattern, while a centrifugal pattern was identified in three ears. Additionally, ink drop migration was seen to travel off the tympanic membrane onto the external auditory canal epithelium in the dogs in our study, an observation that has been reported in all other species examined.^{10,11,13,15,16,20,23}

Previous epithelial migration ink drop studies have demonstrated consistent patterns and rates if the technique is repeated in the same ear of an individual. Boedts repeated his experiments on six human tympanic membranes,²⁴ Cecire and Gibson on 16 mouse ears,²⁸ and O'Donoghue on six tympanic membranes of children,¹⁴ all found consistent, reproducible results. Financial limitations and repeated general anesthesia events precluded repetition of ink drop placement on the same dogs in our study.

The dogs enrolled in this study were breeds not known to be predisposed to otitis externa.³²⁻³⁴ Limited history was available on the enrolled dogs, but they were observed at least daily by ULAR staff. Enrolled dogs did not have otic cytology performed at any time during the study, including at the time of enrolment. However, we deemed it not necessary for any of the dogs enrolled, because at the time of initial examination, any dog

with clinical evidence of otitis and/or excess cerumen in the external auditory canal identified on hand held otoscopic examination was excluded. One might argue that this study would have been best performed on client-owned, clinically normal dogs, where an accurate history could be obtained. However, owing to restrictions imposed by IACUC on clinical trials, we were not permitted to use normal client-owned animals for this study. Perhaps future studies can be designed using client-owned dogs, now that the ink drop technique has been refined for use and demonstrated to be effective and safe in dogs.

In conclusion, epithelial migration is primarily radial and similar in rate on the pars tensa to children and young gerbils in the clinically normal laboratory dogs studied. Further studies using a wide variety of breeds and ages of dogs should be performed to further corroborate the results of this study. These normal epithelial migration rates and patterns can be used for comparative purposes in future pathophysiologic, diagnostic and therapeutic studies in dogs with clinical otitis externa. It has been speculated that in dogs with acute otitis externa, impedance of epithelial migration results in histological epidermal changes,³⁵ while in chronic otitis externa, the continued hyperplasia and stenosis further impede epithelial migration^{35, 36} and may result in reversal of epithelial migration.³⁵ Research on human epithelial migration indicated that migration may be altered in pathologic conditions most notably by increased³⁷ or decreased migration rates^{11,22,24,25} and increased desquamation *in situ*.^{24,25} Whether these changes in tympanic membrane epithelial migration occur in dogs with chronic otitis externa remains to be seen.

Figure 3.1 - Ink Drop Placement Instrument. Instrument used to place ink drops on the tympanic membrane.

Figure 3.2 - Locations for Ink Drop Placement on the Canine Tympanic Membrane. Right tympanic membrane of a dog. PT1, pars tensa 1; PT2, pars tensa 2; PF, pars flaccida; SM, stria mallearis; D, dorsal; V, ventral; C, caudal; and R, rostral.

Figure 3.3 - Radial Path of Epithelial Migration on the Canine Tympanic Membrane. Radial path of epithelial migration, seen in the majority of dogs. The path for each ink drop is indicated by progressively darker shades of the same color as follows: green, pars tensa 1; blue, pars tensa 2; violet, pars flaccida; D, dorsal; V, ventral; C, caudal; and R, rostral.

Figure 3.4 Centrifugal Path of Epithelial Migration on the Canine Tympanic Membrane. Centrifugal path of epithelial migration, seen in only three dogs. The path for each ink drop is indicated by progressively darker shades of the same color as follows: green, pars tensa 1; blue, pars tensa 2; violet, pars flaccida; D, dorsal; V, ventral; C, caudal; and R, rostral.

Location	Time Period	Ear	Mean	95% CI	Data Points
PT1	0-1	AD	82.8	47.0, 118.7	8
		AS	87.5	43.7, 131.4	8
	1-2	AD	99.7	58.5, 140.8	8
		AS	67.0	29.8, 104.2	7
	2-3	AD	95.3	69.5, 121.1	6
		AS	89.4	36.3, 142.5	6
	3-4	AD	107.6	-140.6, 355.9	3
		AS	74.6	21.9, 127.4	4
PT2	0-1	AD	70.8	40.2, 101.3	8
		AS	78.7	44.8, 112.6	8
	1-2	AD	84.0	40.2, 127.7	8
		AS	61.4	33.9, 89.0	8
	2-3	AD	56.7	17.7, 95.8	6
		AS	75.0	43.3, 106.7	7
	3-4	AD	64.1	12.0, 116.3	6
		AS	60.9	45.5, 76.2	3
PF	0-1	AD	246.3	159.0, 333.6	8
		AS	296.1	202.2, 390.1	8
	1-2	AD	129.9	53.9, 205.9	8
		AS	134.6	84.8, 184.4	4
	2-3	AD	118.1	-175.0, 411.2	3
		AS	404.6	N/A	1
	3-4	AD	38.7	N/A	1
		AS	N/D	N/D	N/D

Table 3.1. - Mean and 95% confidence interval (CI) of the epithelial migration rate (micrometers per day) for all ink drop locations (PT1, PT2, PF) at all time points (0-1, 1-2, 2-3, 3-4) for right (AD) and left (AS) ears in eight fox hound dogs. No CI was calculated when two or fewer data points were available (N/A) and no data points were available for the left ear for ink drop PF at the last time period (N/D).

Location	Time Period	Breed	Mean	95% CI	Data Points
PT1	0-1	H	81.6	52.5, 110.6	10
		B	105.1	49.3, 161.0	8
	1-2	H	114.6	72.2, 157.0	10
		B	81.1	26.4, 135.7	6
	2-3	H	91.8	69.4, 114.2	7
		B	49.0	15.0, 82.9	4
PT2	3-4	H	107.6	-140.6, 355.9	3
		B	111.2	-105.7, 328.0	3
	0-1	H	69.4	45.4, 93.4	10
		B	88.7	35.4, 141.9	8
	1-2	H	89.6	54.6, 124.6	10
		B	73.5	43.7, 103.2	6
PF	2-3	H	74.6	30.9, 118.2	8
		B	99.5	27.1, 171.9	5
	3-4	H	81.7	36.4, 126.9	8
		B	91.5	10.8, 172.2	3
	0-1	H	223.0	148.0, 297.9	10
		B	261.2	109.1, 413.3	7
PF	1-2	H	143.3	80.0, 206.7	10
		B	82.4	N/A	2
	2-3	H	141.3	-28.9, 311.4	4
		B	N/D	N/D	N/D
	3-4	H	38.7	N/A	1
		B	N/D	N/D	N/D

Table 3.2. Mean and 95% confidence interval (CI) of the epithelial migration rate (micrometers per day) for all ink drop locations (PT1, PT2, PF) at all time points (0-1, 1-2, 2-3, 3-4) for fox hound dogs (H) and beagle dogs (B) in all 18 dogs. No CI was calculated when two or fewer data points were available (N/A) and no data points were available for ink drop PF in beagle dogs at the last two time periods (N/D).

Time Period	Location	Mean	95% CI	Data Points
0-1	PT1	92.0	65.5, 118.6	18
	PT2	78.0	53.8, 102.1	18
1-2	PT1	102.0	71.5, 132.6	16
	PT2	83.5	61.1, 105.9	16
2-3	PT1	76.2	55.4, 97.0	11
	PT2	84.2	51.7, 116.6	13
3-4	PT1	109.4	21.3, 197.5	6
	PT2	84.3	52.3, 116.4	11

Table 3.3. - Mean and 95% confidence interval (CI) of the epithelial migration rate (micrometers per day) for ink drops located on the pars tensa (PT1, PT2) at all time points (0-1, 1-2, 2-3, 3-4) in all 18 dogs.

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Chapter 4

Conclusions and Future Directions

In the study reported here, epithelial migration on the tympanic membrane in fox hound and beagle dogs occurs in a predominantly radial pattern at a mean rate of 96.4 $\mu\text{m}/\text{d}$ for the pars tensa and 225.4 $\mu\text{m}/\text{d}$ for the pars flaccida. This was the first study to document a technique for applying ink drops to the canine tympanic membrane as well as the first study in the canine to document epithelial migration on the tympanic membrane.

We established that both the rate and pattern of epithelial migration on the tympanic membrane of fox hounds and beagles was dissimilar to adult humans,¹ which was not what we hypothesized. The rate more closely approximated the rate demonstrated in children² or young Mongolian gerbils³ and the pattern resembled the less common radial pattern observed in humans.^{4, 5} Based on the rapid migration rate of the ink drops on the PF and the fact that 16 of 17 ink drops migrated off the pars flaccida prior to the study end, future studies evaluating epithelial migration on the pars flaccida will need to include more frequent monitoring (i.e. every three days) rather than weekly.

It must be recognized that this study only evaluated epithelial migration on the tympanic membrane in dogs without any history of otic disease. It would be interesting to

perform this study using several different groups of dogs. As an example, epithelial migration rates and patterns could be compared between the following four groups: dogs with a history of infectious otic disease, such as bacterial otitis externa; dogs with a history of otic disease that was non-infectious in nature, such as cerumen plugs/ceruminoliths; dogs predisposed to otic disease but without current disease; and dogs with no current or previous otic disease.

In addition, the effects of topical otic and systemic medications on the epithelial migration rate and pattern on canine tympanic membranes should be studied. For example, after a two week course of an ear drop containing clioquinol and flumethasone pivalate applied to twelve ears in six guinea pigs, a significant decrease in the epithelial migration rate on the tympanic membrane was observed; while five weeks after the course of treatment the epithelial migration rate returned to normal.⁶ Platelet rich plasma,⁷ vascular endothelial growth factor,⁸ hyaluronic acid,⁹ and epidermal growth factor⁹ have been demonstrated to increase the speed at which tympanic membrane perforations heal in rats. In contrast, mitomycin C,^{9, 10} epidermal growth factor inhibitors,¹¹ and dexamethasone^{8, 12} prolong tympanic membrane perforation healing. Since epithelial migration on the tympanic membrane has already been demonstrated to have an important role in repairing perforations to the tympanic membrane,¹³⁻¹⁷ these topical therapies could also influence epithelial migration in the absence of tympanic membrane perforation. If it is determined that differences exist in epithelial migration on the tympanic membrane between the groups of dogs discussed above and epithelial migration rates can be altered with the use of topical or systemic therapies, then further

studies should be pursued to evaluate the utility of these therapies in regulating the epithelial migration rate on the tympanic membrane in dogs with otic disease.

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