# THE EFFECT OF MATERNAL NICOTINE EXPOSURE ON THE ALVEOLAR WALL COMPOSITION DURING THE PHASES OF LUNG DEVELOPMENT

**BY**

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RESPICE PROSPICE

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# ABSTRACT

Cigarette smoking is one of the foremost causes of chronic obstructive pulmonary diseases such as emphysema and chronic bronchitis, and although it is the most preventable causes of death, it accounts for approximately 6 million deaths worldwide each year. Cigarette smoking during pregnancy and lactation remains one of the primary modifiable risk factors for undesirable fetal, obstetrical, and developmental outcomes. Consequently, the offspring of the smoking mother is exposed to nicotine via the blood and the milk of the mother. As a result, nicotine interacts with the developing offspring and therefore interferes with normal fetal lung development. Maternal smoking during gestation and lactation has been associated with both short and long term health risks ranging from intrauterine growth restriction to physiological abnormalities. Maternal smoking has also been strongly linked to an increased risk for pulmonary diseases and respiratory morbidity in the offspring of the smoking mother. The main objectives of this study were to determine the effects of maternal nicotine exposure during gestation and lactation on the alveolar wall composition during lung development in the offspring; if maternal nicotine exposure during gestation and lactation induces premature cellular senescence in the lungs of the offspring; to clarify the role of pulmonary fibroblasts in premature senescence; and to establish whether tomato juice supplementation will prevent premature aging in the lungs of rats that were exposed to nicotine via the placenta and mother's milk. From the data generated in this study it was evident that maternal nicotine exposure during gestation and lactation compromises the gas exchange function of the lungs of the F1 offspring. This was prevented by supplementing the mother's diet with tomato juice which is then received by the offspring via the placenta and mother's milk. This is conceivably achieved by maintaining the oxidant-anti-oxidant ratio of the mother and of the developing fetus and neonate, thereby averting premature senescence caused by nicotine exposure. Moreover, the present study also demonstrates that a decrease in fibroblast density is associated with emphysematous-like lesions in the lungs of the nicotine exposed F1 progeny. Since pulmonary fibroblasts are chief contributors to the extracellular matrix of the lungs, involved in alveolar multiplication and regeneration; premature aging or cessation of the metabolically active fibroblasts largely contributes to diminished lung structure and function.

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***Key Words:*** *cigarette smoking, pulmonary fibroblasts nicotine, maternal exposure, F1 offspring, lung development, tomato juice, premature aging,*

# DECLARATION

I declare that *The Effect of Maternal Nicotine Exposure on the Alveolar Wall Composition during the Phases of Lung Development* is my own work, that it has not been submitted before for any degree or examination at any other academic institution, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Jihaan Adonis May 2015

Signed.........................................

# DEDICATIONS

*This thesis is dedicated to my supervisor Professor Gert S. Maritz for the influence he has had on my academic path and for the opportunity and pleasure he has afforded me by learning from him through his mentorship and supervision. It is also dedicated to my parents, Mr. Gasant Adonis and Mrs. Gadija Adonis for their never ending encouragement and support that propelled me throughout my studies. Last but not least, to my son Hamaad Adonis, who has been both my greatest blessing and challenge.*

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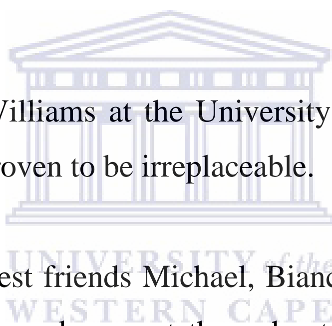


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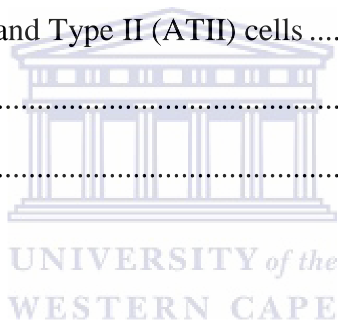

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# CHAPTER ONE
# *Literature Review*

## 1.1 Introduction

Smoking is known to be the most preventable cause of death (Cnattingius, 2004) and accounts for approximately 6 million deaths worldwide each year (W.H.O., 2011). Villalbi and colleagues (2011) have shown that tobacco persists to be the second major cause of death in the world and continues to be the cause of superfluous deaths globally. Cigarette smoking during pregnancy and lactation remains one of the primary modifiable risk factors for undesirable fetal, obstetrical, and developmental outcomes (Andres and Day, 2000a). Furthermore, epidemiological studies show that maternal smoking during pregnancy and lactation is associated with adverse postnatal health, including, hypertension, obesity and type 2 diabetes (Blake et al., 2000).

Tobacco smoke contains several potential cytotoxic agents, notably nicotine, which is a habit-forming substance and thereby promotes damage to various systems including the respiratory, nervous, reproductive and cardiovascular systems (Fowles and Bates, 2000). Numerous studies have shown that a number of diseases brought on and/or aggravated by tobacco smoke are induced by nicotine (Maritz et al., 2011a). Several laboratory studies have shown that nicotine alters the structural development of the lungs (Maritz et al., 2011a, Sekhon et al., 2001), as well as its metabolism as the lungs age (Bruin et al., 2008b). Consequently this leads to a gradual deterioration of organ function with a concomitant increase in susceptibility to disease (Maritz and Windvogel, 2003).

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## 1.2 Lung development

Lung development necessitates the incorporation of numerous regulatory factors which facilitate cell proliferation, differentiation, migration and death. These developmental platforms, such as signalling molecules and transcriptional factors are most likely to be re-recruited during lung repair or recovery subsequent to injury (Deutsch and Pinar, 2002). In order to understand the mechanisms of lung disease, it is imperative to have a general comprehension of normal lung development and function. The lung is designed to afford a large internal surface where both inspired air and

capillary blood come into intimate contact with each other for efficient gaseous exchange (Schittny and Burri, 2008).

### 1.2.1 Prenatal lung development

In humans, lung development is initiated at 3-4 weeks of gestation in comparison to that of rats which is 11.5 days post-conception (Warburton et al., 2005, Schittny and Burri, 2008). Lung development can be divided into two periods, namely, the prenatal (*in utero*) period and the postnatal period. In humans, the prenatal period consists of the embryonic, pseudoglandular, canalicular and terminal sac or saccular phases. The postnatal period is comprised of the lung expansion, rapid alveolarisation (alveolar) and equilibrated lung growth phases (Maritz et al., 2005). The timing of advancement from phase to phase may vary in other species and in some instances alveolarisation may occur postnatally (Hall et al., 2002).

#### *1.2.1.1 Embryonic*

The prenatal period is established by the embryonic stage from weeks 4-7 and day 13 of gestation in humans and the rat respectively (Pinkerton and Joad, 2000), where the respiratory diverticulum (lung bud) appears as an extension from the ventral wall of the foregut known as the laryngotracheal groove (Joshi and Kotecha, 2007, Sadler, 2009). Epithelial cells from the foregut endoderm invade the neighbouring mesenchyme to form the trachea (Joshi and Kotecha, 2007). The trachea then subdivides into the right and left bronchi and subsequently into the lobar and segmental bronchi. In humans the lobar and segmental bronchi emerge around the 5$^{th}$ week (Jeffery, 1998) and at this point, 18 major lobules are noticeable (Kotecha, 2000). Also, the two lungs are easily distinguishable by the 6$^{th}$ week (Hall et al., 2002). In humans pulmonary veins and arteries develops as a single avascular bud from the 6$^{th}$ aortic arch and persists to sprout by vasculogenesis around the airway buds from weeks 4-16 (Hislop and Pierce, 2000).

#### *1.2.1.2 Pseudoglandular*

This phase takes place from week 7-16 in humans and day 16 in the rat fetus (Pinkerton and Joad, 2000). This stage is so named as the lungs take on a distinct glandular appearance (Pinkerton and Joad, 2000). Airways are lined by columnar epithelium and separated by a poorly differentiated mesenchyme, and more distal structures are lined by cuboidal epithelium (Pinkerton and Joad,

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2000). The mesenchyme plays a key role in the development of the lung into a fully functional organ. Mesenchyme directs the growth and cytoarchitecture of a number of organs, including the lung in an organ specific manner (Windvogel, 2006b). In the lung the mesenchyme regulates growth and differentiation of the lung epithelium at the cellular-molecular level by means of various growth and differentiation factors that are hormonally regulated (Torday, 1992). During this phase cell proliferation is at its peak and large quantities of glycogen occur in respiratory epithelial cell types. This may also significantly modify gene expression and ultimately, lung development and growth (Pinkerton and Joad, 2000).

#### *1.2.1.3 Canalicular*

The canalicular stage occurs from weeks 16-24 in the human fetus and days 19-20 in the rat (Pinkerton and Joad, 2000). This stage is marked by the proliferation of the mesenchyme and the development of a rich blood supply within in the mesenchyme (El-Hashash et al., 2011), as well as the flattening of the respiratory epithelium that lines the airways (Thurlbeck, 1975). The rapid formation of capillaries can be observed which protrude into the epithelium and occasional areas of a thin blood-air barrier, similar to that of an adult, appear. Type I and II cell differentiation occurs and the production of surfactant in type II cells commences (Sadler, 2009).

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#### *1.2.1.4 Saccular*

The terminal or saccular stage of lung development in humans is marked from week 24 to near term and in the rat from day 21 (Pinkerton and Joad, 2000). The most peripheral airways form enlarged airspaces termed saccules which widen and lengthen by the addition of new generations. The impending gas regions increase significantly. As a result of cell differentiation, type I and II cells can clearly be distinguished (Haworth and Hislop, 2003). Lamellar bodies are characteristic of type II cells and are produced by the cells at about prenatal week twenty four in humans, and surfactant can be detected in the amniotic fluid four to five weeks later (Haworth and Hislop, 2003). A close correlation exists between the appearance of lamellar bodies in the type II cells and the presence of surfactant in the amniotic fluid. The loss of glycogen from the type II cells is associated with the formation of surfactant (Ridsdale and Post, 2004).

### 1.2.2 Postnatal lung development:

#### *1.2.2.1 Lung expansion*

In humans and rats this stage occurs just after birth (Burri, 1985) and the lung volume increases almost exclusively from an enlargement of the existing airspaces (Maritz and Dennis, 1998). Lung volume escalates proportionally to body weight during the first 10 days and thereafter is allied to lung volume (Burri et al., 1974).

#### *1.2.2.2 Rapid alveolarisation*

Depending on species this stage may be initiated before birth and continued postnatally. In altricial species such as rats (Burri, 1974, Burri et al., 1974), mice (Amy et al., 1977) and humans (Zeltner and Burri, 1987, Zeltner et al., 1987), alveolarisation is a relatively postnatal event. However, in precocial animals like in sheep (Alcorn et al., 1981) and guinea pigs (Sosenko and Frank, 1987) alveolar formation is initiated well before birth, and at term their lungs seem virtually developed. Burri et al. (1974) suggested that the establishment of secondary septa necessitates a double-layered capillary network in the pre-existing septum from which the novel septum is elevated and each section has an "exclusive" capillary bed. The preceding structural arrangement permits an upfolding of the capillary network of one septal side, whilst ensuring blood supply of the other side. Additionally, subsequent to maturation of the alveolar microvasculature from a double-layered to a single-layered capillary complex, the formation of novel alveoli was no longer identified in developmental studies achieved by morphological examination of lung sections. Hence, it was assumed that there is a cessation in growth of new alveolar septa or new alveoli following the completion of microvascular maturation. More recent findings using more modern technology and techniques such as synchrotron radiation-based X-ray tomographic microscopy (Schittny et al., 2008), and quantitative methods (Ochs et al., 2004, Hyde et al., 2007), showed that alveoli or the estimation of the free septal edge (Schittny et al., 2008) are formed until young adulthood. This can be seen in Rhesus monkeys, rats, mice and humans (Hyde et al., 2007, Mund et al., 2008, Schittny et al., 2008, Butler et al., 2012, Narayanan et al., 2012).

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Tschanz and colleagues (2014) recently demonstrated that alveolar formation of novel alveoli is biphasic throughout postnatal lung development. In rats, the first phase of alveolar formation (*days 4-10*) is known as "bulk alveolarisation", while the second phase (*days 21-60+*) is termed "continued alveolarisation". Bulk alveoli formation was observed between days 4 and 21 with a 17.4 times increase from 0.8 to 14.3 million alveoli, while between days 21 and 60, termed continued alveolarisation, a 1.3 times increase to 19.3 million alveoli was observed. At day 4 during the phase of bulk alveolarisation in rats the mean volume of alveoli decreases from ~593,000 $\mu m^3$ to ~141,000 $\mu m^3$ at postnatal day 21. However, it again increases to ~298,000 $\mu m^3$ at day 60 after birth (Tschanz et al., 2014). Additionally, it was concluded that "bulk alveolarisation" correlates with process of classical alveolarisation (alveolarisation prior to microvascular maturation completion) and that "continued alveolarisation" follows three suggested mechanisms of late alveolarisation (alveolarisation subsequent to microvascular maturation) (Burri, 2006, Schittny et al., 2008).

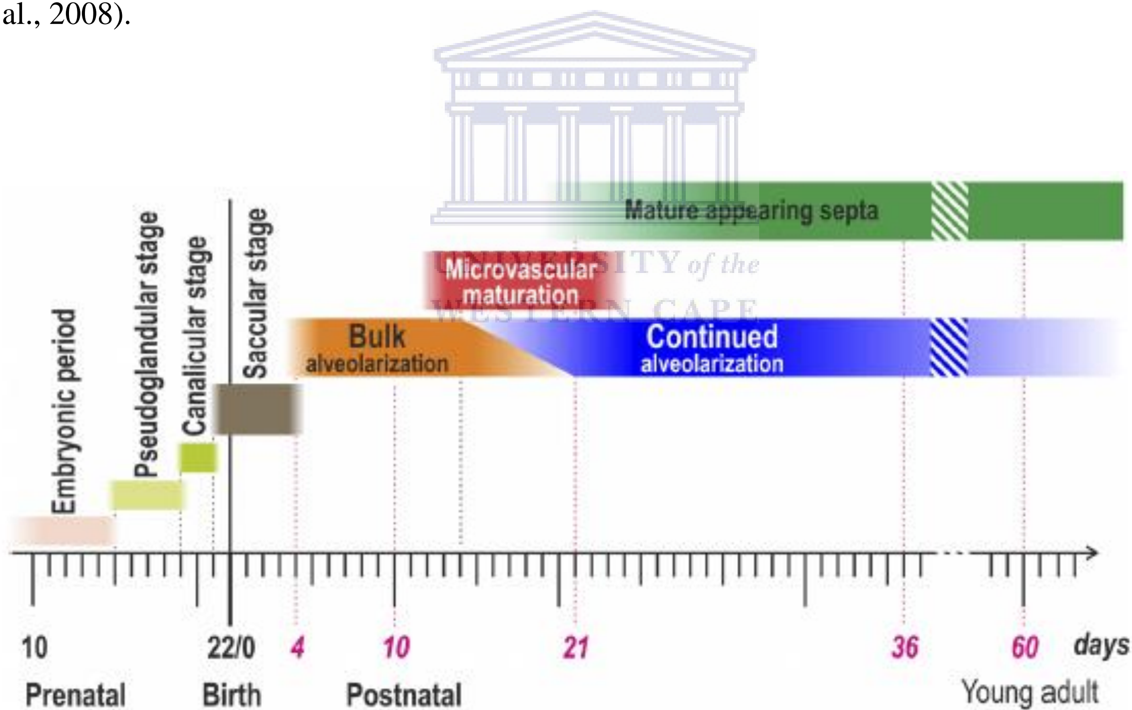


**Figure 1.2.2.2:** The Biphasic Formation of New Alveoli during Postnatal Lung Development. Adapted fromTschanz et al. (2014).

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The first proposed mechanism corresponds to classical ongoing alveolarisation whereby the development of novel alveolar septa occurs at sites where the alveolar microvasculature had not entirely matured, and thus the obligatory double-layered capillary network persists. This is about 5-10% of the capillary network (Roth-Kleiner et al., 2005). Secondly, it occurs at sites of matured single-layered capillary networks by a central and interim reduplication of the capillary bed that can establish conditions for septal upfolding. Aforesaid reduplication was undeniably found in vascular casts at sites where new interalveolar septa began to surface (Schittny et al., 2008).

The second mechanism can function ubiquitously in the lung parenchyma. Although considered as an augmented mechanism of late alveolarisation, a rat model done by Roth-Kleiner and colleagues (2005) and Schittny and co-workers (2008) respectively, observed that alveolarisation was experimentally dampened by several glucocorticoid treatments. Following treatment, undeveloped septa with dual capillary layers resurfaced and a considerable resurgence of alveolarisation was observed. Since alveolar walls possess an exclusive capillary bed, the third mode of alveolarisation occurs at the parenchymal border where alveoli lay adjacent to non-parenchymal structures namely bronchi, vessels, and pleural tissue. It has been suggested that an upfolding of novel septa may well occur throughout life at these sites (Burri, 2006). The former denotes the only type of alveolarisation that does not necessitate a double-layered capillary system since gas exchange only takes place on one side unlike that of the mature alveolar septa which require opposing sides of the capillary network. Furthermore, it is proposed that the three mechanisms of late alveolarisation eventuate during the course of postnatal lung development subsequent to most alveolar septal maturation.

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As reported by Tschanz and co-workers (2014), all modes fulfil the prerequisite of a “private” capillary bed per septal edge, where one side strives to fold up and form a new interalveolar septum. They concluded that continued alveolarisation, following the completion of microvascular maturation, may possibly serve as an explanation for the lungs potential to both structurally and functionally adapt to environmental fluctuations such as hypoxia and to recuperate subsequent to resection of lung tissue (Burri and Weibel, 1971, Ravikumar et al., 2009). Functional recovery of the lung was observed after pneumonectomy and other experimental/clinical studies for humans, mice, rats and dogs (Burri and Sehovic, 1979, Wandel et al., 1983, Nakajima et al., 1998, Takeda et al., 1999, Brown et al., 2001, Hsia, 2004, Fehrenbach et al., 2008, Butler et al., 2012). This suggests

that the lung is not only proficient in partially re-establishing the diffusion surface area by expansion of airspaces but also capable of forming new airspaces. Moreover, the biphasic pattern is more apparent for the growth in alveolar number than for the formation of new alveolar septa (Tschanz et al., 2014).

#### *1.2.2.3 Equilibrated lung growth*

This phase is also known as microvascular maturation. In the human it lasts from birth to the age of 3 years whereas in the rat it lasts from postnatal days 14 to 21 (Schittny and Burri, 2008). This stage is associated with final maturation of the lung by increasing the surface area through the formation of more alveoli as well as expansion of the existing alveoli (Schittny and Burri, 2008). At this stage the capillaries are intimately associated with the blood-air barrier which is vital for optimal gaseous exchange in the organism.

### 1.2.3 Factors affecting lung development

An intricate synergistic role exists between various factors that affect lung development. Earlier studies have documented that various environmental substances (e.g. endocrine disruptors), physiological stress (e.g. caloric restriction) and therapeutic treatments (e.g. diethylstilbestrol, DES) during embryogenesis and/or early postnatal lung development may encourage lung disease (Skinner, 2009). Other interrelated factors include developmental, genetic and mechanical factors. Examples of these factors include both fetal and maternal nutrition, endocrine factors, normal fetal lung fluid production, adequate intrathoracic and extrathoracic space, adequate amniotic fluid volume, positive transpulmonary pressure to conventional postnatal acclimatisation (Mesas-Burgos et al., 2009). The fetal lung is highly prone to changes in the environment during rapid cell proliferation. Certain windows of opportunity exist as a result of the vastly influential lung and may have adverse or beneficial effects. These effects are dependent on the timing, duration, severity and type of effect imparted on the fetal lung during development (Fowden et al., 2006).

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## 1.3 Alveolar wall cells

### 1.3.1 Alveolar Epithelial Type I and Type II Cells

Pulmonary alveolar walls are composed of Type I and Type II epithelial cells, endothelial cells and fibroblasts. Two cell types occupy the alveolar epithelium in normal adult lungs, namely the alveolar epithelial type I (ATI) cells, and the alveolar epithelial type II (ATII) cells. The ATI cells cover 95% of the internal surface area (ISA) of the lung. ATI cells are branched cells with compound apical surfaces that continue into adjacent alveoli (Weibel, 1984). Additionally, ATI cells are characterised by very large apical surfaces in contrast to most cells (i.e. ~5000μm$^2$ for human ATI cells), and are very thin cells (i.e. 0.2 μm in depth) (Weibel, 1984, Pinkerton et al., 1992). Furthermore, the gas exchange barriers of the lungs are occupied by the ATI cells along with endothelial cells joined by a fused basement membrane. ATI cells are also imperative in the regulation of alveolar fluid balance and surfactant secretion by ATII cells in response to stretch (Stevens et al., 2000, Boers et al., 1999, Kuhn et al., 1974). The ATII cells cover the remaining 2-5% of the lungs surface area (SA). ATII cells are cuboidal in shape found between ATI cells and enclose characteristic lamellar bodies and apical microvilli (Weibel, 1984). Moreover, the ATII cells possess many known functions which include the production, secretion and reuptake of pulmonary surfactant (Niden, 1967), regulation of alveolar fluid in normal lungs and during the resolution of pulmonary oedema (Rehan et al., 2007b), synthesis and secretion of immunomodulatory proteins essential for host defences e.g. surfactant proteins A and D (Roman, 2012, Virender et al.).

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### 1.3.2 Response of the epithelium to injury

Damaged ATI cells are replaced by ATII cells (Evans et al., 1975, Adamson and Bowden, 1974, Adamson and Bowden, 1975, Geiser, 2003, Uhal, 1997) (reviewed in (Uhal, 1997)). Studies done by Evans and colleagues in particular, provide the groundwork of existing knowledge regarding the response of the alveolar epithelium to injury. They studied ultrastructural changes to the alveolar epithelium following the exposure to the oxidant nitrogen dioxide ($NO_2$) for 48 h. In alveoli neighbouring to terminal bronchioles, the ATI cells were maximally damaged following 8-12 hr exposure (Evans et al., 1972). Subsequent to 48 h exposure, the denuded basement membranes were repopulated by cuboidal epithelial cells several of which were ATII cells (Evans et al., 1975). Proliferating cells were labelled with tritiated thymidine, which is integrated into newly synthesized DNA during the S phase of the cell cycle. Following 48 h exposure to $NO_2$, majority of the labelled

cells were ATII cells; however, after an additional 48 h recovery, the number of labelled ATII cells had diminished, though the number of ATI cells had increased. Additionally, over the 48 h recuperation period, a temporary upsurge was seen in label associated with alveolar epithelial cells (AEC) having both ATI and ATII cell characteristics (i.e. ATII-like cells but extended across the basement membrane or ATI-like cells possessing occasional microvilli and lamellar bodies) (Evans et al., 1975). These studies display three vital aspects of alveolar epithelial injury and repair: 1) the loss of AT1 cells can herald ATII cell proliferation, 2) alveolar epithelial repair involves the transformation of ATII cells (or a subset thereof) into novel ATI cells and 3) the transformation of ATII cells into ATI cells does so via intermediate cell types.

More recent studies have demonstrated that subpopulations of ATII cells may be present with dissimilar proliferative capabilities subsequent to injury (Reddy et al., 2004). However, the probability for these supposed ATII cell subpopulations to transdifferentiate into ATI cells is unknown. Injured ATI cells may also be reinstated by bone marrow and noncilliated bronchiolar epithelial progenitor cells (Daly et al., 1998, Kotton et al., 2001). For a long time it was speculated that ATI cells were terminally differentiated. However, studies intended to investigate the role of a mechanical load in fetal lung development propose that ATI cells have the potential to transdifferentiate into ATII cells (Flecknoe et al., 2002). It is unknown whether ATI cells are capable of replacing ATII cells in injured adult lungs.

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### 1.3.3 Fibroblasts

Fibroblasts are spindle shaped cells found in majority of tissues and organs of the body associated with extracellular matrix (ECM) molecules. Distinctive features include the expression of vimentin in the absence of desmin and α-smooth muscle actin (SMA) (McAnulty, 2007). Once activated, these cells display and an abundant endoplasmic reticulum (ER) and a prominent Golgi allied with the synthesis and secretion of ECM molecules including fibronectin, collagens, and proteoglycans, together with proteases capable of degrading the ECM. Both cytoskeletal proteins and cell surface integrins in association with the ECM facilitate cell motility and the initiation of contractile forces crucial in tissue homeostasis and wound healing (McAnulty, 2007).

Fibroblasts are embryologically of mesenchymal descent with a range of phenotypic entities varying from the non-contractile fibroblast to the contractile myofibroblast along with a number of

intermediate phenotypes having been described (Eyden, 2005) such as protomyofibroblast (DesmouliÃ¨re et al., 2003). One of the major functions of fibroblasts is the production and homeostatic maintenance of the ECM of the organ or tissue which they inhabit. They are metabolically highly active cells, capable of synthesizing and secreting majority of the ECM components, including collagens, proteoglycans, fibronectin, tenascin and laminin. Fibroblasts constantly synthesize ECM proteins and it has been predicted that each cell is able to synthesize approximately 3.5 million procollagen molecules/day (McAnulty et al., 1991). However, the quantity they secrete is regulated by lysosomal enzymes, for instance cathepsins B, D and L; amid 10% and 90% of all procollagen molecules being degraded intracellularly prior to secretion, depending on tissue and age. Control of this process seems to impart a mechanism for a hurried adaptation of the amount of collagen secreted subsequent to injury (McAnulty and Laurent, 1995). Additionally, fibroblasts produce matrix metalloproteinases (MMP) along with their inhibitors, that is, tissue inhibitors of metalloproteinase (TIMP), which regulate extracellular degradation of the ECM. Fibroblast ECM metabolism is modulated by complex mechanisms including cell-cell and cell-matrix interactions, along with a host of stimulatory and inhibitory mediators, which may be found in their resident environment (McAnulty and Laurent, 2002). Mediators involved include those which are; a) derived from other resident cells in the local environment, b) fibroblasts themselves, and c) mediators resulting from the circulation or infiltrating inflammatory cells.

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Apart from its function in producing the components of the ECM, fibroblasts are also in direct contact with alveolar epithelial cells and air-blood barrier endothelial cells. The fibroblasts appear to form an interconnected reticular network that extends from the distal alveoli to the more proximal conducting airways (Evans et al., 1999). This implies that the fibroblasts play an important role in maintaining lung structural and functional integrity. In addition, if these cells are compromised it will adversely affect lung structural integrity and function.

#### *1.3.3.1 Fibroblasts in tissue injury*

Subsequent to tissue injury both fibroblasts and myofibroblasts play a fundamental role in wound healing and tissue repair (reviewed by (DesmouliÃ¨re et al., 2003). The general processes following injury involves clot formation and platelet aggregation, discharging mediators to attract inflammatory cells to the site of the wound which will produce additional mediators implicated in the recruitment of fibroblastic cells stemming from a number of sources as explained above (McAnulty, 2007). In this early phase of injury, the present fibroblastic cells are exceedingly active, synthetically exchanging impermanent matrix with a more mature ECM including collagens and fibronectin under the regulation of inflammatory mediators, regenerating injured epithelial cells, and fibroblasts themselves. With the progression of granulation tissue deposition, the fibroblasts acquire the characteristics of myofibroblasts, along with the appearance of α-smooth muscle holding stress fibers (McAnulty, 2007). The manifestation of these myofibroblasts correlates with the contraction and closure of the wound through central adhesions between the ECM and myofibroblasts. In the concluding phases of resolution and remodelling, the assembly of MMPs and TIMPs by cells as well as fibroblasts becomes altered from a balance favouring ECM accretion to a matrix reducing environment while myofibroblasts are eliminated by apoptosis. Residual fibroblasts display a more dormant, noncontractile phenotype which may possibly have regressed from a myofibroblasts phenotype or stemmed from a source of cells uninvolved in the differentiation into myofibroblasts. Dysregulation of the injury-repair response may well advance into unsuccessful or over exuberant and pathological wound healing (McAnulty, 2007).

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Several diseases concomitant with diminished or excess accretion of ECM are possibly linked to dysregulation of the injury-repair response along with fibroblast function. The term "injury" is far reaching in this context and encompasses infectious, environmental, traumatic/mechanical, cancerous, drug induced and autoimmune insults. Therefore, associated pathologies involving fibroblasts, in their various phenotypic forms, play a crucial role in tissue maintenance, which in turn may affect almost all tissues and organs of the body (McAnulty, 2007). This is further supported by the suggestion that virtually half of all deaths are as a result of fibrosing conditions. Diseases associated with the imbalance ECM deposition or degradation, or contraction of tissues are likely to give rise to distorted tissue architecture, diminished function, and in most cases, especially with the involvement of vital organs, substantial morbidity and mortality. Dysregulation of a number of phases of the injury-repair response, along with an inadequate inflammatory

response, chronic or recurring injury, altered phenotypic forms, an imbalance of ECM metabolism and deposition or persistence of myofibroblasts, participate in anomalous tissue repair (McAnulty, 2007). Currently, no treatments exist which specifically target fibroblast-associated pathologies. However, present therapeutic strategies are predominantly revolving around the use of anti-inflammatory corticosteroids, immunosuppressants and treatment of specific tissue or disease symptoms. Furthermore, at most the aforementioned have restricted therapeutic effects (McAnulty, 2007).

#### *1.3.3.2 Role of pulmonary fibroblasts*

Lung fibroblasts are the primary cell type responsible for synthesis and secretion of the chief alveolar extracellular matrix components, collagen, proteoglycan, and elastin, as well as maintenance of the alveolar ECM, which is the crucial foundation over which other cells differentiate and proliferate. Elastin in particular is a fundamental element of the alveolar extracellular matrix since it provides the lung with its key mechanical characteristic, elasticity (Plantier et al., 2007). Elasticity is required for ventilation and elastin synthesis is a vital feat for both alveolar growth and regeneration. Apart from its invaluable role as ECM contributor, in both fetal (Warburton and Bellusci, 2004) and adult lungs, alveolar fibroblasts partake in compound cellular interactions that result in alveolar growth and multiplication (Plantier et al., 2007).

Fibroblasts act together with both epithelial and endothelial cells via the discharge of soluble factors that act in a paracrine manner. Fibroblasts in particular, are the chief, if not the exclusive source of keratinocyte growth factor (KGF) in the distal lung and contributes considerably to the upsurge of lung hepatocyte growth factor (HGF) levels in response to alveolar damage (Stern et al., 2000, Marchand-Adam et al., 2005, Cohen et al., 2006). It has been reported by Sirianni et al. (2003) that the role of fibroblasts in the control of cell-cell interactions in alveoli may not be restricted to the secretion of paracrine signal molecules as cytoplasmic expansions deriving from those cells arrive at the alveolar epithelial and endothelial cells by means of their respective basement membranes. Furthermore, fibroblasts play a key role in alveolar multiplication and regeneration and is believed that the fulfilment of such phenomenon necessitates metabolically active and proliferating fibroblasts (Plantier et al., 2007).

It is quite clear that the characteristics of all the cells are important for lung development and for the maintenance of lung structural integrity and also lung function. Any interference with these cells by, e.g., smoking or nicotine or any drug, may compromise lung development and lung structure and function. Development of the lung in utero and also after birth is a controlled process. Any interference with normal development during especially the phases of rapid cell proliferation in utero and early neonatal lung development may induce programming of the lungs to become more prone to diseases later in life.

## 1.4 Pulmonary innate immunity

The innate immune system is a non-specific defence mechanism which plays a central role in lung antimicrobial defences from birth. Innate immunity in the lung is comprised of lung leukocytes along with epithelial cells that line the alveolar surface and the conducting airways (Martin and Frevert, 2005). The significance of innate immunity is well demonstrated by the fact that pulmonary internal surface area which is about 70 $m^2$, is subjected to the inhaled atmospheric air. Given that many unknown substances occur in the inhaled air, from the first breath at birth, this necessitates an effective protection mechanism to avert infection and lung parenchymal damage (Derscheid and Ackermann, 2013). Additionally, the innate immune system also has influential interactions with other systems such as apoptosis and signalling pathways prompted by mechanical stretch (Martin and Frevert, 2005). Moreover, innate immunity also drives adaptive immunity which includes both cell-mediated and humoral immunity mechanisms. However, unlike that of the adaptive immune system, the innate immune system is not highly specific to a specific pathogen. Therefore, the innate immune system is of particular importance in infants, especially preterm neonates, immunosuppressed individuals, and the elderly due to the fact that the adaptive immune system function is defective in each of these groups (Derscheid and Ackermann, 2013).

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### 1.4.1 Alveolar Macrophages (AM)

Alveolar macrophages (AM) occur freely in the alveoli and constitute approximately 95% of airspace leukocytes, while only 1 to 4% is lymphocytes and around 1% is neutrophils. This is indicative that the alveolar macrophage is the most crucial phagocytic cell of pulmonary innate immunity. Alveolar macrophages play a crucial role in the protection of the lung. It does so by directly binding, phagocytose and kill pathogens. Alveolar macrophages derive from fetal monocytes that seed the lungs during late embryogenesis (Ginhoux, 2014). The first signs of AM

differentiation appeared around the saccular stage of lung development. Mature AM were undetectable before birth and only fully colonized the alveolar space by 3 d after birth. Development of AM is dependent on granulocyte-MF colony-stimulating factor (GM-CSF) and M-CSF for their development. It has been found that around the perinatal period, high expression of GM-CSF mRNA in epithelial cells and protein in BAL fluid is high, but decreased rapidly after birth (Guilliams et al., 2013). From this it appears that the epithelial cells plays and important role in creating an environment for the development and differentiation of the AMs.

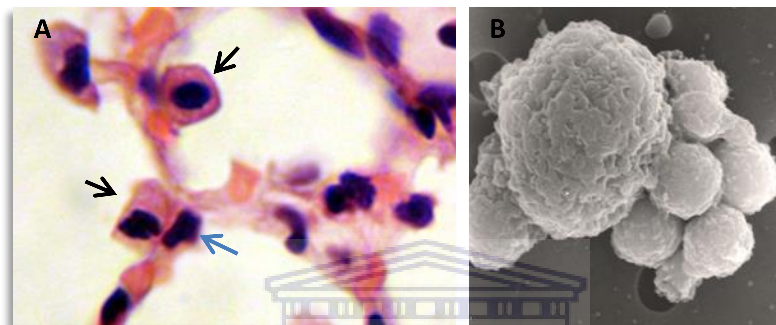


**Figure 1.4.1:** A) Alveolar macrophages occurring freely in the alveoli (Black arrow) and type II alveolar cell (Blue arrow). B) Scanning electron micrograph of an alveolar macrophage isolated from a bronchioalveolar lavage.

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Although there are studies that demonstrate that the phagocytic capacity of the AM of neonates is not as well developed as in the adult lung, other studies suggest that there is no difference in the capacity of the AM between neonates and adult lung. In addition to phagocytosis AM also secrete a large number of mediators, some of which act directly with pathogens while others, such as chemokines exert their effects indirectly by recruiting other components of the immune system. This latter process is relatively inefficient in the neonate (Martin et al., 1995).

In a study by Wongtrakool et al. (2012) it was demonstrated that *in utero* nicotine exposure induces a TH2 milieu in the neonatal lung resulting in a skewed distribution of the AM towards M2 activation, thereby compromising AM function. As a consequence, phagocytosis becomes impaired and thus a decline in its ability to protect the lung against foreign inhaled substances rendering it more susceptible to disease. Whether this effect of nicotine is permanent is not known. It is known though that cigarette smoke also impairs clearance of bacteria from the lungs leading to inflammation and morbidity related to epithelial damage (Mehta et al., 2008).

### 1.4.2 The role of alveolar Type I and Type II cells in innate immunity

Although the alveolar macrophages and dendritic cells play a key role in pulmonary innate immunity, alveolar epithelial cells are also quite crucial. The alveolar type I and type II cells are morphologically very different and contribute significantly to protection of the lung. Termed "defenders of the alveolus", alveolar type II (ATII) cells account for 3-5% of the internal surface area (ISA) of the lung. ATTII cells produce a vast number of cytokines and chemokines including interleukin-6 (IL-6), interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inhibitory protein-1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), and growth related oncogene-α (GRO-α) in response numerous forms of lung injury induced by inhaled viruses, bacteria or mechanical ventilation (Wong et al., 2012). Furthermore, the ATII cells produce surfactant which is not only important in alveolar stability, but also augments chemotaxis, and the engulfment of bacteria by the alveolar macrophages via phagocytosis (Tenner et al., 1989, Wright, 2004).

Alveolar type I (ATI) cells covers approximately 95% of the ISA of the lung (Stone et al., 1992) and not only acts as a physical barrier to counteract inhaled particular, but is also involved in the active cellular response of the lungs to these inhaled particles. Contrary to earlier reports, it has been suggested that ATI cells actively participate in innate immunity. These cells express toll-like receptors such as Toll-like receptor 4 (TLR-4) and RAGE, however, they also produce pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β in response to lipopolysaccharides (LPS). Studies suggest that the roles of ATI and ATII cells in inflammation is unalike and that these two cell types that line the enormous ISA of the lungs work simultaneously to safeguard the lungs against respiratory diseases. This is further supported by the observation that several of the inflammatory mediators are highly expressed in the ATI cells than in the ATII cells (Wong et al., 2012).

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## 1.5 Cigarette smoking

Cigarette smoke contains more than 4700 chemical compounds which includes oxidants and free radicals (Rahman and MacNee, 1996). Approximately 60 of these were identified as both human and/or animal carcinogens (Fowles and Bates, 2000). The most highly recognized component of cigarette smoke is nicotine, a tertiary amine (Buccafusco, 2004), which is responsible for the habitual use of tobacco. It is one of the leading causes of disease and premature death in both

developed and developing countries (Moore et al., 2009). Cigarette smoke contains free radicals in both the gas and the tar phases and contains approximately $10^{15}$ radicals per puff (Rahman and MacNee, 1996). According to Rahman and MacNee (1996) oxidants found in cigarette smoke can cause direct damage to the lung matrix itself, particularly the collagen and elastin. It can also interfere with elastin repair and synthesis. This interference contributes to the degradation of elastin thus leading to the possible development of emphysema (Rahman and MacNee, 1996).

### 1.5.1 Maternal exposure to tobacco smoke

Environmental tobacco smoke (ETS) remains an important worldwide public issue and has been grouped by the Environmental Protection Agency (EPA) as a human lung class A carcinogen, along with asbestos and radon. Tobacco smoking during pregnancy remains widespread particularly in indigenous populations, and most likely promotes respiratory illnesses in the exposed offspring. It is now accepted that constituents of tobacco smoke, notably nicotine, can have a multitude of deleterious effects on the organs of the fetus and neonate , potentially with life-long consequences (Maritz and Harding, 2011a).

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While an increasing body of evidence exists which strongly link maternal tobacco smoking to fetal morbidity and mortality and obstetric disease, many pregnant females continue to smoke (Jimenez-Ruiz et al., 2006).Maternal smoking during gestation and lactation has been associated with both short and long term health risks ranging from intrauterine growth restriction to physiological abnormalities (Brion et al., 2010). Low birth weight (LBW), sudden infant death syndrome (SIDS), perinatal complications, altered neurodevelopment, obstructive lung diseases, cancers and childhood infections (Hofhuis et al., 2003b) as well as an increased prevalence of lifestyle cardiovascular risk factors amongst offspring of smoking parents (Horta et al., 2011). Studies show that maternal smoking increases the comparative risk of admission to a neonatal intensive care unit (NICU) by virtually 20%. As a result of maternal smoking, it further increased the length of stay whereas for non-NICU infants it seemed to decrease. Moreover, among mothers who smoke, smoking adds over $700 (± R 8054.08) in neonatal costs. These costs are decidedly avoidable given that the hostile effects of maternal nicotine exposure occur in the short-term and can be prevented by even the brief cessation of maternal smoking. These expense estimates can be utilised by local and state public health officials, managed care plans and others to assess alternative smoking cessation programs (Adams et al., 2002).

Maternal smoking has also been strongly linked to an increased risk for pulmonary diseases and respiratory morbidity in the offspring of the smoking mother (Horta et al., 2007, Kramer, 1987). In addition, epidemiological studies have shown a close relation between abnormal pulmonary function in infants and children and maternal exposure to cigarette smoke (Brion et al., 2008, Brion et al., 2010, Jaddoe et al., 2008).As a result of this, the developing fetus, or lactating infant whose mother smoked during pregnancy and/or lactation, are more likely to have decreased lung function and subsequently or additionally increased susceptibility to respiratory diseases later in life (Ruiz, 2006). Even though there is a substantial body of supporting evidence that states smoking during pregnancy is detrimental (Schwartz et al., 1972), an estimation of about 15%-20% of woman smoke throughout gestation (Andres and Day, 2000a, Bergmann et al., 2003). Previous studies have approximated that 5%-10% of fetal and neonatal deaths are as a result of maternal tobacco or nicotine exposure (Proskocil et al., 2005).

### 1.5.2 Nicotine

Nicotine, is a natural ingredient operating as a botanical insecticide in tobacco leaves, and is the principle alkaloid found in tobacco smoke. Nicotine underlies tobacco addiction, contributes to tobacco use patterns, and is used as a pharmacological aid for the cessation of smoking, otherwise known as nicotine replacement therapy (NRT) (Benowitz et al., 2009). It's about 1.5% in weight of commercial cigarette tobacco and comprises approximately 95% of the total alkaloid content (Benowitz et al., 2009).

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Nicotine is the primary psychoactive component of tobacco smoke and is responsible for the addictive nature of, as well as the soaring incidence of relapse of those who attempt to quit. The most extensively used biomarker of nicotine intake is cotinine, which may be measured in blood, urine, saliva, hair or nails (Benowitz et al., 2009). The effects of nicotine are exerted on the central nervous system (CNS) and other viscera such as the lungs by binding to nicotinic acetylcholine receptors (nAChR) where it mimics the biological action of the neurotransmitter acetylcholine (Lavezzi et al., 2005). These receptors are members of the ligand-gated ion channels located in the brain. They are also said to exist on neurons and non-neuronal tissue such as muscles and immune cells (Gallowitsch-Puerta and Tracey, 2005). Both cancerous and normal lung cells have an affinity towards nAChR (Pontieri et al., 1996, Maus et al., 1998). Literature suggests that the nicotine and

nAChR interface on the surface of rodent bronchiole epithelium, promotes cell proliferation (Cattaneo et al., 1997).

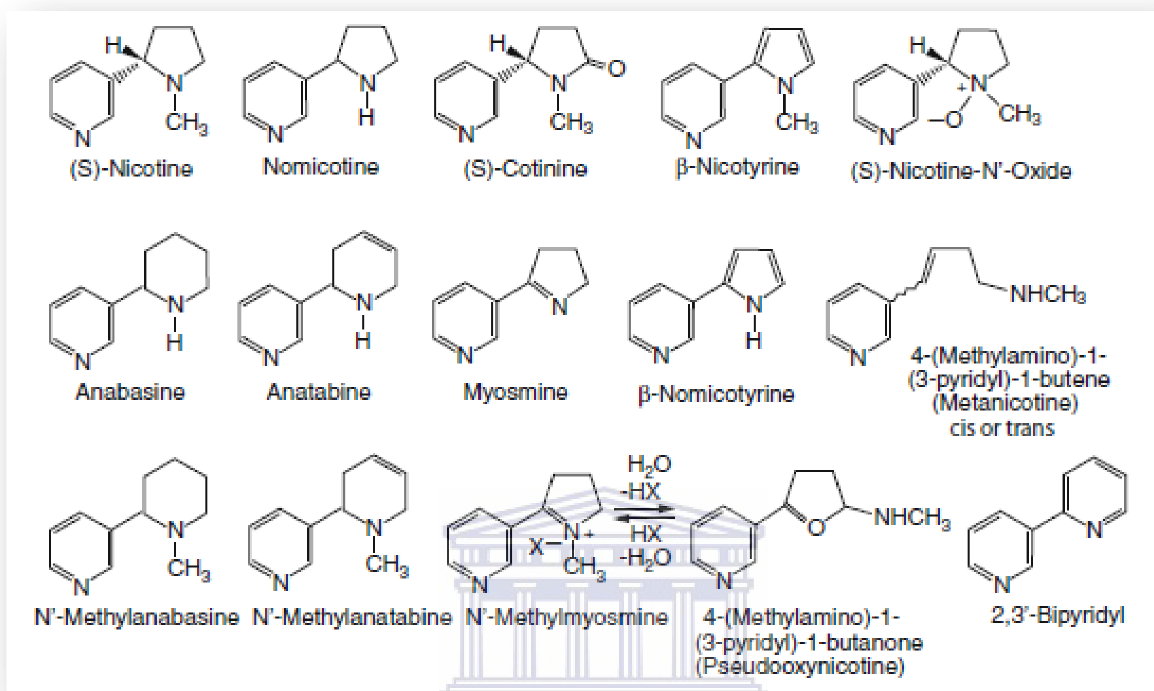


**Figure 1.5.2:** Structure of nicotine and associated tobacco alkaloids (Benowitz and Jacob, 1998).

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### 1.5.3 Nicotine metabolism

Nicotine is extensively metabolized into a number of metabolites by the liver. Six chief metabolites of nicotine have been identified; however, quantitatively the most essential metabolite of nicotine in mammals is the lactam derivative cotinine. In humans, approximately 70-80% of nicotine is transformed into cotinine (Benowitz et al., 2009). It is understood that an increased blood flow to the liver as well as prompt breakdown of nicotine and cotinine occurs in the mother. Fetal liver metabolism of nicotine is lengthy since the enzymatic protection mechanisms of the fetus are not fully matured. It is for this reason that a longer half-life of nicotine is thus to be expected (Frank and Sosenko, 1987, Walther et al., 1991). Thus, this accounts for more hurried metabolism of nicotine and its metabolite cotinine during pregnancy (Dempsey and Benowitz, 2001). This observation is therefore validated by the elevated concentrations of nicotine in fetal tissue as opposed to maternal blood (Luck and Nau, 1987, Lambers and Clark, 1996). The growing fetal lungs as well as other organs are as a consequence exposed to high concentrations of nicotine for a

longer period of time and thus its potential destructive effects on cell structure and integrity (Kleinsasser et al., 2005).

It should also be noted that proliferating cells at this stage of fetal development are highly susceptible to the manipulation by foreign materials such as nicotine (Rehan et al., 2007a). It is therefore anticipated that exposure to nicotine during gestation and early postnatal life via the mother's milk may also interfere with the development and growth of the fetus and neonate. Earlier studies have shown that the nicotine content of milk of the mother is 2 to 3 times higher than in the mother's plasma (Luck and Nau, 1984a, Dahlstrom et al., 1990). Throughout gestation and lactation, nicotine can have a direct effect on cells by decreasing the supply of nutrients to the offspring (Guo et al., 2005b).

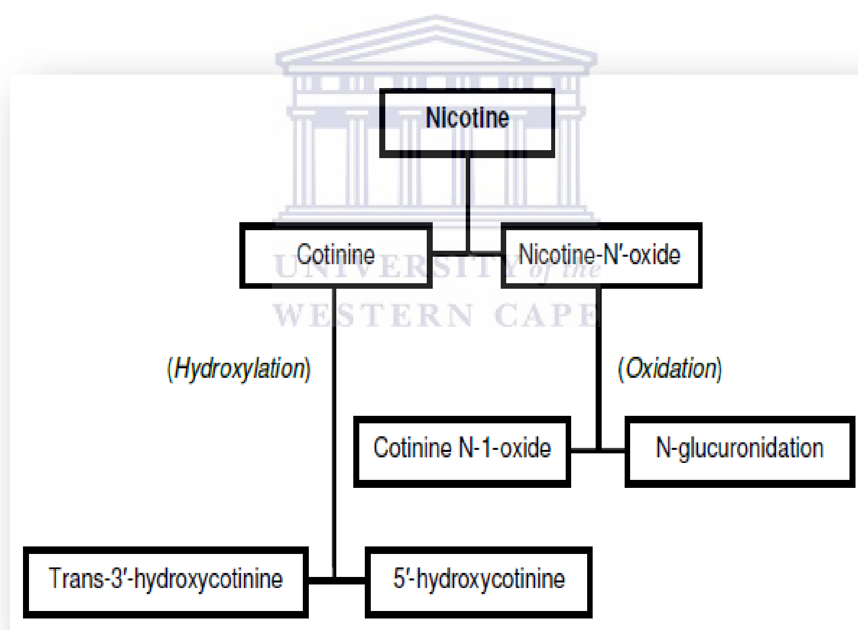

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**Figure 1.5.3:** The main metabolites of nicotine are cotinine and nicotine-N'-oxide. Cotinine is metabolized by hydroxylation to trans-3'-hydroxycotinine, N-oxidation to cotinine N-1-oxide, and N-glucuronidation (Pogocki et al., 2007).

## 1.6 Emphysema

Known as an obstructive lung disease and more specifically falls under the umbrella term of chronic obstructive pulmonary lung diseases (COPD). Emphysema is characterized by the distal airspaces of the terminal bronchioles that are abnormally and permanently enlarged. This enlargement is caused by alveolar wall destruction (Snider et al., 1985) and results in restricted airflow and blood oxygenation (Shapiro, 1995). Destruction of the alveolar walls results in enlarged alveoli and a decrease in the surface area available for gas exchange. Emphysema differs from restrictive lung diseases such as asthma in that it is an obstructive lung disease; thus individuals who suffer from it experience difficulty expelling air from the lungs thus leading to an accumulation of air in the alveoli due to damaged elastic tissue and thus an increase in compliance. Although the pathogenesis of emphysema is yet to be elucidated, numerous studies have proposed that genetic factors may contribute to determining the individual susceptibility to developing emphysema (Ito et al., 2005). The development of emphysema has been associated with smoking and atmospheric pollution by several scholars (Snider et al., 1985). Factually, emphysema has been coupled to extreme lung inflammation caused by chronic inhalation of cigarette smoke and the subsequent protease/anti-protease imbalance (Shapiro, 1995). Several studies have proposed that connective tissue, specifically elastin, is the primary target of destruction involved in the development of emphysema. As a result of elastin destruction, the changes imparted are said to be permanent (Mercer and Crapo, 1992).

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Two major types of emphysema have been characterized by pathologists which are morphologically similar (Friedman, 2008). The first of these is centrilobar emphysema which is the most commonly observed emphysema. This form of emphysema is largely associated with chronic exposure to cigarette smoke (Pryor et al., 1983, McCusker, 1992). As the name implies, "centrilobar" emphysema is distinguished by the collapse of alveolar walls in the central part of the acini, originally sparing the peripheral portions of the acinus and lobule. Usually this form of emphysema manifests in the upper areas of the lung. Conversely, panlobar emphysema is distinguished by the breakdown encompassing all parts of the lobule extending to the periphery and is generally more acute in the lower lung areas (Friedman, 2008). Furthermore, it is recognized by the enlargement in airspace throughout the acinus and is frequently due to a deficiency in $\alpha_1$-protease production (McCusker, 1992).

Moreover, evidence is accumulating which suggests that alveolar cell apoptosis is implicated in the pathogenesis of emphysema (Rangasamy et al., 2004). Given that emphysema is considered as the progressive diminishing in alveolar gaseous exchange area, which is highly likely to result in the loss of alveolar capillaries, proposals have been made that a possible link between alveolar cell apoptosis and a disturbance in cellular and molecular signalling involved in alveolar structural maintenance and repair exists. This potential link has extensive implications given that the pathogenesis of alveolar destruction induced by cigarette smoke may have mutual features with alveolar expansion due to aging (Tuder et al., 2006).

### 1.6.1 Phenotypic variations in fibroblasts

Fibroblasts play a key role in pulmonary tissue repair and alterations in fibroblast phenotype may well be implicated in the pathogenesis of emphysema (Müller et al., 2006). It has been reported that lung fibroblasts from patients with emphysema show a diminished proliferation rate (Holz et al., 2004, Nobukuni et al., 2002), altered growth factor response (Noordhoek et al., 2003) and reduced number in population doublings in long-term culture (Holz et al., 2004). Nakamura et al. (1995) observed that cigarette smoking impeded both fibroblast proliferation and recruitment. This impact of cigarette smoke can possibly be attributed to cigarette smoke induced premature aging of pulmonary fibroblasts (Nyunoya et al., 2006). Along with clinical observations, these findings offer validation to the hypothesis that premature aging of fibroblasts is implicated in the pathogenesis of emphysema. This is supported by the fact that increased age is also associated with emphysema (Raherison and Girodet, 2009). Bird and colleagues (2003) indeed reported that senescent cells not only display changes in morphology and metabolic profile, but also expend their capability to divide and respond to mitogenic stimuli. This phenotype is prompted by oxidative stress (Balin et al., 2002), in combination with epigenetic manipulations in gene expression. Consequently, since fibroblasts contribute to a portion of lung integrity, a senescent phenotype could alter both structural maintenance and tissue microbalance (Müller et al., 2006).

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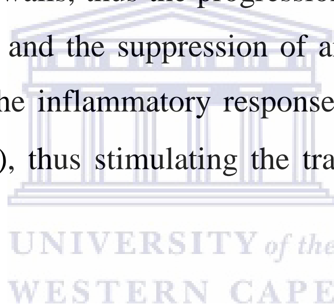
### 1.6.2 The proteinase-antiproteinase theory

Numerous studies have indicated that protease pathogenesis induces several lung diseases, as well as asthma and COPD (Demedts et al., 2006), also known as diseases characterized by impaired airflow (Eriksson, 1999). Typically, an imbalance between proteolytic and anti-proteolytic molecules exists in the lungs of patients who suffer from COPD (Demedts et al., 2006). This results in elevated proteolytic activity which triggers the damage of healthy lung parenchyma, prompting the development of emphysema. The elevated proteolytic activity may be due to inflammation, characterized by the discharge of proteolytic enzymes by inflammatory mediator cells such as neutrophils and macrophages, or could otherwise be a result of genetic influences such as an alpha-1 antitrypsin deficiency (Demedts et al., 2006).

A disturbance in the proteases and anti-proteases balance, by for example oxidants, consequently causes the degradation of alveolar walls, thus the progression into emphysema. Antioxidants does so by the stimulation of proteases and the suppression of anti-proteinases. Furthermore, oxidants play a crucial role by mediating the inflammatory response by activating the transcription factor nuclear factor-kappa beta (NF-κB), thus stimulating the transcription of pro-inflammatory genes (Demedts et al., 2006).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### 1.6.3 Oxidant-antioxidant status

By definition, oxidative stress is the imbalance between the production of reactive oxygen species (ROS) and the antioxidant defences in favour of the oxidants (Betteridge, 2000). Under normal physiological conditions, antioxidants inactivate the ROS intermediates by scavenging free radicals. Oxidative stress cannot only directly cause tissue damage but also influence molecular mechanisms that control lung inflammation (Guo and Ward, 2007). Lung epithelia is frequently exposed to oxidants either via those generated by normal cellular processes such as those produced by airspace phagocytes in lung inflammation, or by direct inhalation of ambient air which contain oxidants such as ozone, nitrogen dioxide (Guo and Ward, 2007), cigarette smoke and diesel exhausts (Rahman et al., 2006). A significant amount of oxygen passes through the lungs thereby rendering it highly susceptible to oxidative stress. It’s for this reason that a sufficient, if not a greater amount of antioxidants are required for protection against these exposures (Arab et al., 2002).

The primary oxidants present in the lung are reactive oxygen species (ROS) which comprise of radicals such as superoxide ($O_2^-$), hydroxyl (OH) and peroxyl ($RO_2^-$); non-radicals which include hydrogen peroxide ($H_2O_2$) and ozone ($O_3$). Oxidants that form part of the reactive nitrogen species (RNS45), such as nitric oxide (NO) and nitric dioxide ($NO_2$), as well as the non-radicals such as peroxynitrite ($ONOO^-$) (Evans and Halliwell, 2001). These oxidative and nitrosative species which exert both an indirect and direct effects on tissue, are chief contributors to inflammatory lung injury (Lang et al., 2002). Other molecules such as lipid and protein radicals can also contribute to oxidative stress (Musellim et al., 2006). The formation of these oxidants are intensified by the exposure to external physical and chemical mediators such as nitrogen oxides, ozone, ionizing and ultraviolet radiation, mineral dusts and tobacco smoke (De Paepe et al., 1999).

The major oxidant sources in the lung include eosinophils, alveolar epithelial cells, neutrophils, endothelial cells and bronchial epithelial cells. Oxidative stress can bring about the peroxidation of membrane lipids, rises in intracellular calcium ions, cytoskeleton disruption, deoxyribonucleic acid (DNA) damage, and the reduction of nicotinamide nucleotides (Musellim et al., 2006). As a result, this leads to impaired cellular function, induction of apoptosis and the stimulation of dysfunctional matrix remodelling (Foronjy and D'Armiento, 2006). It was suggested by Harman (2003) in the free radical theory of ageing (FRTA) that ageing or cellular senescence is a consequence of free radical reactions (FRR's). This study supported the findings of Parrinello (2003) who stated that an increase in ROS and subsequently oxidative stress participates in the progression into senescence.

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Kondoh et al (2005) also illustrated a link between senescence and the glycolytic pathway in that glycolysis slows down as the cell becomes older. It is interesting to note that maternal nicotine exposure during gestation and lactation slows down glycolysis (Maritz and Harding, 2011b). It was indeed suggested that nicotine induces premature lung aging (Maritz and Harding, 2011b, Maritz and Mutemwa, 2012).

#### *1.6.3.1 Tobacco smoke as source of oxidants*

Tobacco smoke use or exposure prompts both the airway and parenchymal inflammatory cells to generate proteinases, especially elastase, from neutrophils and several metalloproteinases from the alveolar macrophages. As a consequence, the antiproteinase defence in the epithelial lining fluid

becomes overwhelmed. Majority of lung tissue studies on the pathogenesis of emphysema have been centered on the mechanisms involved in chronic injury induced by cigarette smoke to the pulmonary parenchyma; notably, perpetual inflammation, oxidative stress, protease-antiprotease imbalance, and excessive apoptosis of the alveolar cells (MacNee, 2000, Barnes et al., 2003, Tuder et al., 2003).

Antioxidant defences are found both within the fluid epithelial lining of the lung and the lung cells. They are the first line of defence (Rahman et al., 2004) and are classed into enzymatic and non-enzymatic antioxidants. The enzymatic antioxidants of the lung consist of glutamate, cysteine ligase, glutathione peroxidise, glucose-6-phosphate dehydrogenase, as well as catalase, superoxide dismutase, heme oxygenase-1, peroxiredoxins, glutaredoxins, thioredoxins (Musellim et al., 2006, Chu et al., 2005). Non-enzymatic antioxidants include glutathione, α-tocopherol, uric acid, lipoic acid and bilirubin, vitamins C and E, and β-carotene (Rahman et al., 2004). Moreover, fluid linings in the airways and extracellular spaces of healthy lungs are preserved by millimolar concentrations of reduced glutathione (GSH), vitamin C and urates to guard the normal physiological function of the lungs. Therefore, in healthy lungs the oxidant-antioxidant levels are balaced in favour of a reduced state. A disruption in the equilibrium of the oxidant-antioxidant status in favour of the oxidants leads to oxidative stress and is one of the initial incidents that results in inflammatory counteraction in the lung (Musellim et al., 2006).

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The effect of the oxidants in cigarette smoke is opposed by the antioxidants that occur in the epithelial layer of the alveolar walls and in the fluid layer covering these cells (Thorley and Tetley, 2007). This normal protection ensures that cell aging is normal, but when it becomes depleted such as during smoking, this protection is inadequate resulting in type one cell death, increased type 2 cell proliferation and premature aging of the lungs (Maritz and Harding, 2011b). This can induce emphysema over time. It is therefore, important to aim at restoring the anti-oxidant capacity of the lungs and thus its ability to protect itself against oxidants in the inhaled air as well as against those entering the lungs in the blood. This can partly be achieved through the intake of nutrients that are rich in antioxidants such as vitamins A, E and C.

Fruits and vegetables are an excellent source of antioxidants. Substantial interest has been fixed on the β-carotene, vitamin C, and vitamin E content of fruits. Although, fruits also contain many other

constituents that hold antioxidant activities (Wang et al., 1996). Low molecular weight polyphenolic compounds known as flavonoids are extensively distributed in fruits and vegetables and a portion of the antioxidant capacity of fruits and vegetables are ascribed to these compounds (Hertog et al., 1992, Ortuno et al., 1995). Several flavonoids such as hesperidin, luteolin, kaempherol, quercitin, eridictyol, myricetin, lycopene and catechin have been isolated from commonly consumed fruits and vegetables such as oranges, lemons, apples, tomatoes, celery, broccoli and parsley to name a few. Furthermore, it has been shown to confer antioxidant (Bors and Saran, 1987, Bors et al., 1990, Hanasaki et al., 1994, Garg et al., 2001) antihemorrhagic, anti-inflammatory, anticancer, and antiallergic properties (Das, 1994).

### 1.6.4. Carotenoids

Carotenoids are naturally occurring dietary constituents that are contained in most fruits including oranges or yellow and green vegetables. Carotenoids are said to exert antioxidant activities and prevent free-radical induced cellular damage (Bendich, 1993). Evidence has demonstrated that carotenoids are a forthcoming approach in protecting lung function and integrity. According to Arab et al. (2002), carotenoids such as lycopene together with other antioxidant vitamins, including vitamin C and vitamin E that are present in both the lung epithelial and fluid lining of the lung, may provide additional level of defence against oxidative and ozone induced damage. However, lycopene is an antioxidant abundantly contained in tomato juice (Di Mascio et al., 1989). Kasagi et al. (2006) applied lycopene to evaluate the hypothesis that oxidant-antioxidant imbalance could be of importance instead of inflammatory cell influx with the burden of proteinases.

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#### *1.6.4.1. Tomato Juice and Lycopene*

Lycopene is a bioactive carotenoid found in many fruits in vegetables and is the primary carotenoid in fresh tomatoes and tomato products (Kaplan et al., 1990, Takeoka et al., 2001). It has been shown that lycopene confers many beneficial health effects (Giovannucci, 1999) and is said to be the most effective biological carotenoid in quenching singlet oxygen (Di Mascio et al., 1989). Since lycopene possess antioxidant properties, it has encouraged interest in the tomato as a food with possible anticancer characteristics (Giovannucci, 1999). Although β-carotene had been efficient against some chemically stimulated cancers, it was not competent in eliminating tumours in the respiratory tract (Obermueller-Jevic et al., 2002). Both β-carotene and lycopene is present in lung

tissue and thus adds to the possibility that carotenoids are suitable defence mechanisms against oxidative stress (Takeoka et al., 2001).

Research done by Agarwal et al. (2001) showed that the consumption of tomato products conferred protection against oxidative damage to serum lipids, low density lipoproteins (LDL's), proteins, lipids, and lymphocyte DNA. Also noted from their study was that lycopene supplementation causes a decrease in DNA damage, which as suggested, might play a considerable role in lowering the development of cancer by lessening the oxidation of DNA and proteins (Arab et al., 2002). Furthermore, preceding literature also discloses that an increase in oxidized proteins have a vital role in the development of chronic diseases as well as the process of aging. Several proteins function as metabolic enzymes and as a result, their oxidative damage may therefore cause a loss in its particular function (Stadtman, 1992, Hu, 1994).

As demonstrated by Liu et al. (2006), purified lycopene was shown to act concomitantly with other bioflavonoids like glabridin, the phenolic compounds carnosic acid and rosmaniric acid along with garlic in impeding *in vitro* LDL oxidation. Literature on the sole antioxidative effects of lycopene in a biological organism is limited (Mein et al., 2008). However, studies report that considerable inhibition of N-methyl-N-nitrosourea-testosterone induced carcinogenesis in male Wistar-unilever rats following tomato powder intake (13 mg/lycopene/kg diet), while no differences were observed subsequent to lycopene supplementation *per se* (161 mg/lycopene/kg diet) (Basu and Imrhan, 2007). This implies that lycopene together with other antioxidants, present in tomatoes, may be more effective in deterring lipid peroxidation as opposed to lycopene in isolation. This suggests that these nutrients in fruit and vegetables are working together for the best effect. In isolation it becomes ineffective.

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## 1.7 Cellular senescence

Biological aging comprises of an array of molecular, cellular and structural modifications founded on several mechanisms. Although conventionally associated with chronological age, biological aging can occur earlier in life, with partial dependence on the individual's chronological age (premature aging) (Balcombe and Sinclair, 2001). Cellular senescence has been defined as the state of constant arrest in the G1/G0 phase of the cell cycle. During this phase primary somatic cells possess a complete irreversible loss of their replicative capacity (Hayflick, 1965).

According to Hayflick and Moorhead (1961, 1965), *in vitro* studies have shown that the proliferation of normal human lung and skin fibroblasts are curbed to a certain number of divisions and that the replicative capacity of the cells deteriorate with age. The maximum number of divisions for cells is known as the "Hayflicks limit" and ranges between 50 to 70 divisions in tissue culture. The instant the Hayflicks limit is achieved, the cells will cease to divide and enters a type of cell arrest known as replicative senescence (Serrano and Blasco, 2001). As a result, the cells will undergo a series of functional, biochemical and morphological changes which are indicative of cellular aging (Dimri et al., 1995), including the loss of proliferative ability in otherwise viable cells (Karrasch et al., 2008). The incidence of acute, self-limiting lung diseases, prior tobacco consumption and exposure to environmental pollutants in elderly people makes it challenging to solely distinguish pulmonary alterations that could be ascribed to 'normal aging' (Dyer and Stockley, 1999). Throughout a lifetime the human lung is subjected to a multitude of factors capable of altering lung structure and function by enhancing the aging of the lung. It is for this reason that it is highly likely that all these factors may enhance the rate at which age associated deterioration of lung structure and function occur (Dyer and Stockley, 1999).

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The notion of the 'Hayflick limit' suggests that normal somatic cells possess a cell division counting mechanism or 'clock'(Hayflick and Moorhead, 1961). According to the telomere hypothesis of senescence, the tallying process is progressive with telomere shortening which occurs during cell division (Olovnikov, 1973). Telomeres form protective caps at the terminus of the chromosome which prevents it from being identified as double-strand (ds) breaks as well as preventing the DNA ends from degradation or recombination (Chen et al., 2001, Lange et al., 2005). Given that the chromosome does not possess the ability to duplicate end of linear molecules, telomeres shorten with each cell division (Blasco, 2005). Eventually, telomeres reach a

considerably short length and appear as ds DNA breaks. These ds DNA breaks stimulates the expression of the tumour suppressor protein p53 resulting in telomere-initiated senescence or apoptosis (de Lange, 2005, Zglinicki and Martin-Ruiz, 2005). The telomere is lengthened by telomerase, a ribonucleoprotein with DNA polymerase activity (Greider and Blackburn, 1985). By using a generation of telomere-deficient mice, Blasco et al. (1997) demonstrated that telomerase is the most vital cellular activity which accounts for the maintenance of telomere length. Thus, rapid cell division, such as those induced by cellular damage, may result in an increase in the number of senescent cells in, e.g., the lungs. This may make the lung more susceptible to disease related to aging.

Senescent cells assume distinct, flat and distended cell morphology; this appearance can be used to differentiate between senescent cells and non-senescent cells (Rattan, 1995). Senescent cells also display a senescence associated β-galactosidase activity (Dimri et al., 1995) which may be suggestive of an increased lysosomal-mass (Lee et al., 2006).

### 1.7.1 The relationship between senescence and apoptosis

Apoptosis is a distinct form of programmed cell death typified as the loss of cell function and prompt morphological changes, culminating in cell death without inflammation (Del Riccio et al., 2004). Apoptosis and senescence takes place during normal development and growth of cells and organisms. Hengartner (1995) proposed that senescence is a crucial cellular mechanism in the maintenance and preservation of physiological equilibrium within a system.

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Senescent cells are found to be arrested in the G1/G0 phase of the cell cycle. As a result these can no longer respond to growth factors and become irreversibly resistant to apoptosis (Kong et al., 2011). It is still unclear what determines if a cell undergoes apoptosis or senescence. Although cells are generally capable of both, these processes appear to be absolute (Campisi and D'Adda Di Fagagna, 2007). Cell types seem to be a factor as damaged fibroblast and epithelial cells tend to undergo senescence whereas damaged lymphocytes tend to undergo apoptosis. It has been reported that attempts to hinder senescence by telomerase overexpression, does not impede cellular senescence but rather protects cells from undergoing apoptosis (Gorbunova et al., 2002, Massard et al., 2006). Similarly, manipulations of the expression levels of Bcl-2 or caspase inhibition may result in cells that typically undergo apoptosis to enter senescence (Rebbaa et al., 2003, Nelyudova

et al., 2007). Furthermore, these studies strongly suggest a complex cross regulation between apoptosis and senescence (Seluanov et al., 2001).

### 1.7.2 The role of senescence in cell proliferation

Cell proliferation is defined as the increase in cell number as a result of cell growth or cell division. The balance between cell proliferation and apoptosis is vital normal development and tissue-size homeostasis in the adult (Guo and Hay, 1999). It was found by Nyunoya and colleagues (2006) that exposure to cigarette smoke ceased the proliferation of lung fibroblasts and upregulated two pathways allied to cell senescence, p53 and p16-retinoblastoma protein (pRB) pathways. They also observed that while a single exposure to cigarette smoke inhibited normal fibroblast proliferation, multiple or incessant exposure to cigarette smoke shift cells into an irreversible state of senescence. This inability to repair lung injury may be a fundamental feature of emphysema (Nyunoya et al., 2006).

### 1.7.3 The relationship between Apoptosis and proliferation and their role in Emphysema

Numerous threads of evidence relate apoptosis to proliferation. Cellular proliferation, differentiation, and death are essential processes in multicellular organisms, and play a role during developmental phases as well as aging to maintain cell populations in tissues, thus contributing to homeostasis. Originally, uninhibited proliferation can be coupled with an excessive level of apoptosis. Several latent oncogenes such as *c-Myc,* seem to prompt apoptosis, which implies that crosstalk between the cell proliferation and apoptosis pathways is present (Evan et al., 1992). Previous studies have shown that emphysematous lungs are linked with amplified levels of apoptosis in both alveolar epithelial and endothelial cells (Yokohori et al., 2004). Consequently, the integrity of the alveolar wall structure in the lungs become diminished and due to the loss of cells by apoptosis. To counterbalance the alveolar loss, cellular proliferation will therefore escalate (Tsuji et al., 2006). Supporting studies have observed that patients with emphysematous lungs had increased levels of proliferation in comparison to those with normal lungs (Imai et al., 2005). The latter stands in agreement that a direct correlation between apoptosis and cellular proliferation in the pathogenesis of emphysema subsists.

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Hence, based on the cited literature, the aims of this study were to determine:

- Whether maternal nicotine exposure during pregnancy, and until weaning was completed at day 21, affects the phases of fetal- and postnatal lung development
- Whether maternal nicotine exposure induces premature aging in the lungs of the offspring
- What the role of fibroblasts are in premature aging in the lungs of the offspring
- Whether tomato juice supplementation would avert premature aging in the lungs of the offspring

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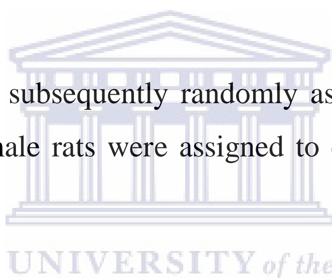
# CHAPTER TWO
## *Methods and Materials*

### 2.1 Animal preparation and ethical clearance

White virgin female rats (Wistar descendants) were used in the present study. Animals were sheltered in the animal rooms at the Department of Medical Biosciences of the University of the Western Cape. Ethical clearance for this study has already been approved by the Ethical Committee of the University of the Western Cape in accordance to the guidelines of the Medical Research Council for Animal Care. Animals were fed a stock diet (Epol rat cubes and water as required) throughout the study. Room temperature was kept at 22˚±1˚C and a day-night cycle of 12 hours (06:00 – 18:00) was maintained.

### 2.2 Treatment of animals

Animals were mated overnight and subsequently randomly assigned to a control and three different experimental groups. At least 6 female rats were assigned to each group. The pregnant experimental animals were divided into 4 groups:



- Group 1 received 1 mg of nicotine subcutaneously/kg body weight/day.
- Group 2 received tomato juice only.
- Group 3 received both nicotine (1 mg/kg body weight/day) and tomato juice.
- Group 4 was the control, receiving saline injections subcutaneously where the volume of saline was determined by the initial body weight of the mother.

The treatment of all four groups began as soon as the animals were mated and was terminated at weaning when the offspring that were born to these mothers, were 21 days old. It is important to note that the offspring were not treated with nicotine, saline or tomato juice. Only the mothers received nicotine, saline, or tomato juice. Since nicotine crosses the placenta and occurs in the milk of smoking mothers (Luck and Nau, 1984b), the offspring would therefore be exposed to nicotine only via the placenta and the mother's milk. Nicotine doses were not changed as the body weights of the animals increased during pregnancy; it remained standard according to the initial body weight of the female rats before mating. Nicotine doses were administered via subcutaneous injections with a 1ml tuberculin syringe.

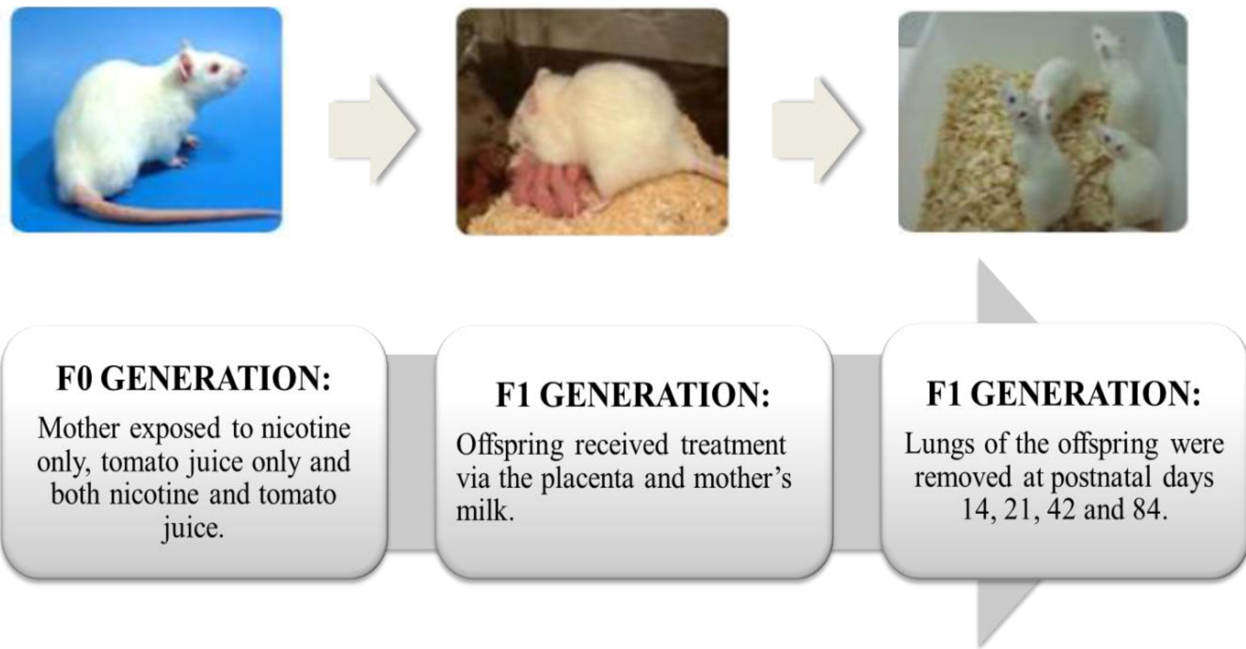



**Figure 2.2:** Demonstration of the rat model representing the animals exposed to nicotine, tomato juice and a combination of nicotine and tomato juice *in utero*. The F0 generation was mated to produce the F1 generation. The F1 offspring were exposed via the mother's placenta and breast milk.

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All Gold Tomato juice containing 5.3 mg lycopene/100 ml was diluted 50/50 with distilled water on a daily basis due to its content being too viscous to move though the drinking bottles. It was freely available for the animals to drink from 200 ml glass water bottles. The water and tomato juice intake was measured daily at a set time, and the average intake was recorded per week. The lycopene intake was calculated based on the tomato juice intake by the animals and recorded as the average weekly intake (mg/100 g body weight/week). Apart from lycopene (5.3 mg/100 ml tomato juice), the tomato juice also contains protein (0.8 g/100 ml), carbohydrates (3.4 g/100 ml), fibre (0.55 g/100 ml), and sodium (200 mg/100 ml). The pregnant rats within each of the experimental groups were weighed on a weekly basis to monitor any changes in their body weights and to determine if any abnormalities occur in body weight due to their different treatments. After birth the number of rat pups per litter was kept between 8 and 10 pups to ensure that the nutrient supply from the mother was adequate to support the normal growth and development of the offspring.

## 2.3 Lung extraction procedure

The lungs were extracted from weaned pups at four different age groups namely postnatal days 14, 21, 42 and 84. Four litters of pups were taken from each of the 4 age groups, and the lungs of at least 3 pups from each of the litters were used. The rat lungs were fixed in 10% buffered formalin solution after the removal from the thorax. The following reagents were used to make up 10% buffered formalin solution:

- Formaldehyde 100 ml
- Distilled water 900 ml
- Sodium dihydrogen phosphate, monohydrate ($NaH_2PO_4.H_2O$) 4 g
- Disodium hydrogen phosphate, anhydrous ($Na_2HPO_4$) 6 g

Before the rats were sacrificed, they were weighed and the weight was recorded. The chest circumference and the crown-rump length were subsequently measured and recorded. To remove lung tissue, the offspring were anaesthetized with an intraperitoneal injection of Sodium Pentobarbitone (90 mg/kg/BW). Once the rats were completely unconscious, the thoracic cavity was carefully opened. Special care was taken to prevent damage to the lungs. Thereafter, the trachea was surgically cannulated and the diaphragm punctured. Lungs that were damaged during the dissection were discarded. The fixative (10% buffered formalin, pH 7.2) was allowed to flow into the lungs via the trachea while a transpulmonary pressure gradient of 25 cm fixative was maintained. After 30 minutes the lungs were ligated at the hilum of the lungs and removed via excision *en bloc* for morphologic and morphometric studies. The lungs were each separately labelled and stored in buffered formalin (pH 7.2). Buffered formalin solution was made up of 4 g Sodium Phosphate (Anhydrous) and 6 g Sodium Phosphate (dehydrogenous) added to 900 ml distilled water. Once the chemicals were dissolved in the water, 100 ml Formaldehyde solution was added to make up a 1000 ml buffered formalin solution.

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## 2.4 Determination of lung

Lung volumes were measured by the fluid displacement method as described by Scherle (1970), whereby a 100 ml beaker containing buffered formalin at a pH of 7.2 was weighed on a scale, and then zeroed. With the support of a surgical forceps, the extracted lung was then immersed in the beaker at a level of buoyancy, and the final lung volume was determined. Lung volumes were determined before and after the 24-hour fixation period to detect shrinkage.

## 2.5 Lung tissue processing

An automatic tissue processor was used. Care was taken as not to damage any of the lung tissue. For each lung, the left inferior lobe was gently cut off with a scalpel and perfectly placed in a properly labelled cassette. When all the cassettes containing the lung tissue were ready, they were placed into the tissue processing rack of the automatic tissue processor. The processor was programmed so that the lungs to be processed underwent an 18 hour cycle as follows:

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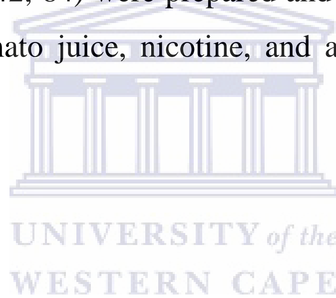
**Table 2.5.1:** Illustration of the tissue processing procedure

| AGENT | TIME |
|---|---|
| ***Dehydration Process*** | |
| **1. 70% Ethanol** | 2 hours |
| **2. 80% Ethanol** | 2 hours |
| **3. 90% Ethanol** | 2 hours |
| **4. Absolute Ethanol I** | 2 hours |
| **5. Absolute Ethanol II** | 2 hours |
| | 2 hours |
| ***Deparaffination Process*** | |
| **6. Xylene bath I** | 2 hours |
| **7. Xylene Bath II** | 2 hours |
| ***Tissue Embedding Preparation*** | |
| **8. Wax bath I** | 2 hours |
| **9. Wax bath II** | 2 hours |

## 2.6 Tissue embedding and microscopy

Tissues were manually removed from cassettes in order to begin the tissue embedding procedure. Lung tissue was carefully aligned and fixed into a mould block using warm wax to fill the mould. The mould hardened on an ice plate utilizing a tissue embedding machine. Tissue blocks were trimmed at right angles to enable accurate morphometric measurements. The paraffin tissue blocks were then cut with a microtome into sections of 5 µm for the tinctorial stains and 3 µm for immunohistochemistry staining for further microscopy. Every third section was used for morphology and morphometric evaluation. This prevented double counting of tissue constituents. Once the sections were cut, they were floated on a warm water bath, and was then shifted onto a glass microscopic slide for further staining processes. Once slides were dried, it was placed in an oven at 60°C for 30 minutes to allow fixation of lung tissue to the slide. All slides were initially examined to eliminate sections with evidence of inadequate preparation. A total of 32 slides for each postnatal group (day 14, 21, 42, 84) were prepared and equally divided amongst each of their experimental groups (control, tomato juice, nicotine, and a combination of nicotine and tomato juice).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## 2.7 Tinctorial stains

### 2.7.1 Hematoxylin and Eosin (H&E)

***Principle***

This stain is a gold standard and is practiced in every histology laboratory. It will be used for morphometric analysis as well as morphology of the alveoli, alveolar wall cells as well as surrounding structures.

**Table 2.7.1:** Hematoxylin &Eosin Staining Protocol

| CHEMICAL SOLUTION | TIME |
|---|---|
| ***Deparaffination and Rehydration*** | |
| **1. Xylene Bath I** | 5 mins |
| **2. Xylene Bath II** | 5 mins |
| **3. Absolute Ethanol I** | 5 mins |
| **4. Absolute Ethanol II** | 5 mins |
| **5. 90% Ethanol** | 5 mins |
| **6. 80% Ethanol** | 5 mins |
| **7. 70% Ethanol** | 5 mins |
| ***Haematoxylin Staining*** | |
| **8. Haematoxylin** | 15 mins |
| **9. Rinse in tap water** | |
| **10. Scott's tap water** | 2 mins |
| **11. 1% Acid Alcohol- destaining agent** | 2 mins |
| ***Eosin Staining and Dehydration*** | |
| **12. Eosin- counter stain** | 3 mins |
| **13. Rinse in tap water** | 2 mins |
| **14. 80% Ethanol** | 2 mins |
| **15. 90% Ethanol** | 2 mins |
| **16. Absolute Ethanol** | 2 mins |
| **17. Xylene Bath I** | 2 mins |
| **18. Xylene Bath II** | |
| **19. Mount for observation using DPX and slide coverslips** | |

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### 2.7.2 Masson's Trichrome (Connective tissue)

***Principle***

The Masson's Trichrome Stain employs three dyes, selectively staining erythrocytes, fibrin, collagen fibers and muscle tissue. The universal rule in trichrome staining is that tissues that are less porous are coloured by the smallest dye molecule; with the penetration of a larger dye molecule, it will always do so at the expense of a smaller dye molecule.

**Table 2.7.2:** Masson's Trichrome Staining Protocol

| CHEMICAL SOLUTION | TIME |
|---|---|
| ***Deparaffination and Rehydration*** | |
| **1. Xylene Bath I** | 3 mins |
| **2. Xylene Bath II** | 3 mins |
| **3. Absolute Ethanol I** | 3 mins |
| **4. Absolute Ethanol II** | 3 mins |
| **5. 90% Ethanol** | 3 mins |
| **6. 80% Ethanol** | 3 mins |
| **7. 70% Ethanol** | 3 mins |
| **8. Wash in tap water** | 3 mins |
| **9. Quick dip in d$H_2O$** | 3 mins |
| ***Hematoxylin Staining*** | |
| **10. Weigerts Hematoxylin** | 5 mins |
| **11. Tap water** | 3 mins |
| **12. Rinse in d$H_2O$** | |
| ***Masson's Fuchsin Ponceau-Orange G (MPFOG)*** | |
| **13. Counterstain with MFPOG** | 30 mins |
| **14. Rinse in Acetic acid water** | 1 min |
| ***Phosphotungstic Acid*** | |
| **15. Mordant in Phosphotungstic acid solution** | 5 mins |
| **16. Rinse in acetic acid water** | 1 min |
| ***Light Green Staining*** | |
| **17. Light Green solution** | 20 mins |
| **18. Treat with acetic acid water** | 5 mins |
| ***Dehydration*** | |
| **19. 80% Ethanol** | 2 mins |
| **20. 90% Ethanol** | 2mins |
| **21. Absolute Ethanol** | 2 mins |
| **22. Xylene Bath I** | 2 mins |
| **23. Xylene Bath II** | 2 mins |
| **24. Mount for observation using DPX and coverslips** | 2 mins |

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## 2.8 Immunohistochemistry (IHC)

All immunohistochemistry was done using the Bond™ Polymer Refine Detection (Catalogue No. DS9800) from Leica Biosystems Ltd. This detection system is for *in vitro* diagnostic use. Bond Polymer Refine Detection is a biotin-free, polymeric horseradish peroxidase (HRP)-linker antibody conjugate system for the detection of tissue-bound mouse and rabbit IgG and some mouse IgM primary antibodies. It is intended for staining sections of formalin-fixed, paraffin-embedded tissue on the Bond™ automated system. Immunohistochemical techniques can be used to demonstrate the presence of antigens in tissue and cells. Bond Polymer Refine Detection utilizes a novel controlled polymerization technology to prepare polymeric HRP-linker antibody conjugates. The detection system avoids the use of streptavidin and biotin, and therefore eliminates non-specific staining as a result of endogenous biotin.

**Bond Polymer Refine Detection works as follows:**

The specimen is incubated with hydrogen peroxide to quench endogenous peroxidase activity.

1. A user-supplied specific primary antibody is applied.
2. Post Primary IgG linker reagent localizes mouse antibodies.
3. Poly-HRP IgG reagent localizes rabbit antibodies.
4. The substrate chromogen, 3,3'-Diaminobenzidine tetrahydrochloride (DAB), visualizes the complex via a brown precipitate.
5. Hematoxylin (blue) counterstaining allows the visualization of cell nuclei.

### 2.8.1 Protocol: Leica Bond Max staining protocol

A standard protocol was used for all the antibodies mentioned in the present study. Table 2.8.1 illustrates the method for IHC applied staining.

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**Table 2.8.1:** Illustrates the IHC staining protocol as provided by Leica Biosystems Ltd

| Step | Type | Incubation Time | Temperature | Dispense Type |
|---|---|---|---|---|
| **1** | **Peroxide Block** | **5 min** | **Ambient** | **selected vol.** |
| **2** | Bond Wash Solution | 0 min | Ambient | selected vol. |
| **3** | Bond Wash Solution | 0 min | Ambient | open |
| **4** | Bond Wash Solution | 0 min | Ambient | selected vol. |
| **5** | **Primary Antibody** | **15 min** | **Ambient** | **selected vol.** |
| **6** | Bond Wash Solution | 0 min | Ambient | selected vol. |
| **7** | Bond Wash Solution | 0 min | Ambient | selected vol. |
| **8** | Bond Wash Solution | 0 min | Ambient | selected vol. |
| **9** | **Post Primary** | **8 min** | **Ambient** | **selected vol.** |
| **10** | **Bond Wash Solution** | **2 min** | **Ambient** | **selected vol.** |
| **11** | **Bond Wash Solution** | **2 min** | **Ambient** | **selected vol.** |
| **12** | **Bond Wash Solution** | **2 min** | **Ambient** | **selected vol.** |
| **13** | **Polymer** | **8 min** | **Ambient** | **selected vol.** |
| **14** | **Bond Wash Solution** | **2 min** | **Ambient** | **selected vol.** |
| **15** | **Bond Wash Solution** | **2 min** | **Ambient** | **selected vol.** |
| **16** | Deionized Water | 0 min | Ambient | selected vol. |
| **17** | Deionized Water | 0 min | Ambient | selected vol. |
| **18** | **Mixed DAB Refine** | **10 min** | **Ambient** | **selected vol.** |
| **19** | Deionized Water | 0 min | Ambient | selected vol. |
| **20** | Deionized Water | 0 min | Ambient | selected vol. |
| **21** | Deionized Water | 0 min | Ambient | selected vol. |
| **22** | **Hematoxylin** | **5min** | **Ambient** | **selected vol.** |
| **23** | Deionized Water | 0 min | Ambient | selected vol. |
| **24** | Deionized Water | 0 min | Ambient | selected vol. |
| **25** | Deionized Water | 0 min | Ambient | selected vol. |

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### 2.8.2 Staining for fibroblasts and Type II alveolar epithelial cells (Vimentin)

***Principle***

Vimentins are class-III intermediate filaments found in numerous non-epithelial cells, especially mesenchymal cells. Vimentin is attached to the nucleus, endoplasmic reticulum, and mitochondria, either laterally or terminally. Vimentin is highly expressed in fibroblasts, some T-and B-lymphocytes, in many hormone-dependent mammary carcinoma cell lines, and little or no expression in Burkitt's lymphoma cell lines. Vimentin is localized in the cytoplasm and found in connective tissue and the cytoskeleton. Since they are expressed in a highly developmentally-regulated fashion; vimentin is the major cytoskeletal component of mesenchymal cells. Because of this, vimentin is often used as a marker of mesenchymally-derived cells or cells undergoing an epithelial-to-mesenchymal transition (EMT) during both normal development and metastatic progression. Furthermore, studies done by Fujino and co-workers (2011) showed that AT11 cells that expressed surfactant protein A (SP-A) and surfactant protein D (SP-D), were also positive for vimentin. Therefore, in the current study I was able to stain for pulmonary fibroblasts and concomitantly stain for Type II alveolar cells.

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***Method***

- Tissue sections of 3 μm in thickness were cut and placed on slides.
- The tissue was dried in a 60°C oven for 1-2 hours.
- Slides was deparaffinised in 2 washes of Xylene for 5 minutes each and rehydrated in a series of graded alcohol.
- Using the Leica Bond Max staining protocol F (Table xxx), the primary antibody; Anti-Vimentin antibody [EPR3776]-Cytoskeleton Marker (Catalogue No. ab92547) was used to stain fibroblast activity at a 1/500 dilution as suggested by Abcam® for IHC-P.
- Mount with DPX and slide coverslips.

### 2.8.3 Staining for senescence (β-galactosidase)

***Principle***

The purpose for this stain is to detect senescent cells in the alveolar walls of the lungs. Senescence-associated beta-galactosidase (SA-β-gal) is a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides only in senescent cells. It is localized in the cytoplasm and coded by a gene (*lacZ*) in the *lac operon* of *Escherichia coli* (*E. coli*). This metalloenzyme has three enzymatic mechanisms. First, by cleaving the disaccharide lactose into glucose and galactose, this can then enter glycolysis. Second, it can catalyze the transgalactosylation of lactose to allolactose, and, third, the allolactose can be cleaved into the monosaccharides. It is the allolactose that binds to the *lacZ* repressor and generates the positive feedback loop that regulates the amount of β-galactosidase in the cell. This will indicate cellular senescence.

***Method***

- Tissue sections of 3 µm in thickness were cut and placed on slides.
- The tissue was dried in a 60°C oven for 1-2 hours.
- Slides was deparaffinised in 2 washes of Xylene for 5 minutes each and rehydrated in a series of graded alcohol.
- Using the Leica Bond Max staining protocol F (Table xxx), the primary antibody; Anti-beta Galactosidase antibody [DC1 4C7] (Catalogue No. ab116) was used to stain senescent cells at an assay dependent concentration as set out by Abcam® for IHC-P. In the present study a 1/250 dilution was used.
- Mount with DPX and slide coverslips.

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### 2.8.4 Staining for proliferation (PCNA)

***Principle***

PCNA (Proliferating Cell Nuclear Antigen) was previously known as cyclin. PCNA is a 36 kDa nonhistone protein found in the nucleus that plays a role in the initiation of cell proliferation by mediating DNA polymerase. PCNA levels are elevated in the S, G2, and M phases of cell mitosis in normal and malignant tissues. PCNA expression has a broad correlation with mitotic activity and can be used as a marker for cell proliferation. PCNA has proven useful for proliferative studies of normal and neoplastic tissues both *in vivo* and *in vitro*.

***Method***

- Tissue sections of 3 µm in thickness were cut and placed on slides.
- The tissue was dried in a 60°C oven for 1-2 hours.
- Slides was deparaffinised in 2 washes of Xylene for 5 minutes each and rehydrated in a series of graded alcohol.
- The Anti-PCNA antibody [PC10] (Catalogue No. ab29) was used to stain senescent cells at a dilution of 1/10000, heat mediated antigen retrieval with citrate buffer pH 6 before commencing with the IHC staining protocol F (Table 2.8.1).
- Mount with DPX and slide coverslips.

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### 2.8.5 Staining for apoptosis (AIF)

***Principle***

Apoptosis inducing factor (AIF) is a positive intrinsic regulator of apoptosis (caspase-dependent pathway) by causing both chromatin condensation and DNA fragmentation. Probable oxidoreductase that has a dual role in controlling cellular life and death; during apoptosis, it is translocated from the mitochondria to the nucleus to function as a proapoptotic factor in a caspase-independent pathway, while in normal mitochondria, it functions as an antiapoptotic factor via its oxidoreductase activity.

***Method***

- Tissue sections of 3 µm in thickness were cut and placed on slides.
- The tissue was dried in a 60°C oven for 1-2 hours.
- Slides was deparaffinised in 2 washes of Xylene for 5 minutes each and rehydrated in a series of graded alcohol.
- Using the Leica Bond Max staining protocol F (Table xxx), the primary antibody; Anti-AIF antibody [E20] - Mitochondrial Marker (Catalogue No. ab32516) was used to stain apoptotic cells at an assay dependent concentration as set out by Abcam® for IHC-P. In the present study a 1/500 dilution was used.
- Mount with DPX and slide coverslips.

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## 2.9 Morphology and Morphometry

The following parameters were used to determine the influence of maternal exposure to nicotine, tomato juice only or both nicotine and tomato juice on lung development in the F1 offspring. For morphometry, samples were taken from the left and the right lobes of the lung. Large airways and blood vessels were avoided when determining the measurements. The mean alveolar intercept (Lm) was determined as described by Weibel and Knight (Weibel, 1963). The alveolar ($V_a$) and parenchymal (Uksusova and Nizovtsev, 1982) density were determined as described by Bolender and colleagues. At least 25 randomly selected, non-overlapping fields from each section were analysed (Bolender et al., 1993). Seventy five fields from each animal were counted. Alveolar wall thickness was determined as described by Bolender et al (1993).

The morphometric techniques used in this study included:

- Volume Density ($V_t$ and $V_a$)
- Mean Linear intercept (Lm)
- Alveolar wall thickness (Tsept)

### 2.9.1 Volume Density ($V_t$ and $V_a$)

***Principle***

Alveolar volume is indicative of the size of the alveolus and therefore the volume of air that occupies it. It is expected that in alveoli with larger volumes, the total surface area for gaseous exchange in the lung would be reduced. This would also mean that there would be a possibility of a reduced alveolar surface area for gaseous exchange and fewer alveoli. According to Blanco et al (1991), the average size of an individual alveolus increases with age.

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***Method***

The alveolar air volume density (Va) and alveolar tissue volume density ($V_t$) (Uksusova and Nizovtsev, 1982) were determined as illustrated by Bolender et al. (1993). A 122-point eyepiece graticule was used at 100 x magnifications for the point counting technique which helped in determining the alveolar air volume density $V_a$ and $V_t$.

***Determination of $V_t$***

A 10x eyepiece and a 10x objective were used to obtain a total magnification of 100x. Two blocks were taken from the upper lung lobe, 1 from the middle lobe and two from the lower lobe. Non-parenchymal tissue included bronchus and blood vessels which had a diameter of >1.1 mm. The alveoli that were found within the graticule and those that touched the lower and right borders of the graticule were included. The alveoli were excluded from the count including those that were outside the square on the upper and the left side of the graticule. Furthermore, the fields surround by non-parenchymal tissue was excluded from the counts. At least 5 randomly selected non-overlapping fields were analysed for each slide.

The alveoli that contributed to the count included:

- Those that were found within the graticule, and
- Those that touched the right lower borders of the graticule.

The alveoli that did not contribute in the count included:

- Those found outside the square on the upper left side of the graticule.
- Areas that contained non-parenchymatous tissue were excluded from the counts. At least 5 randomly selected non-overlapping fields per slide were analysed.

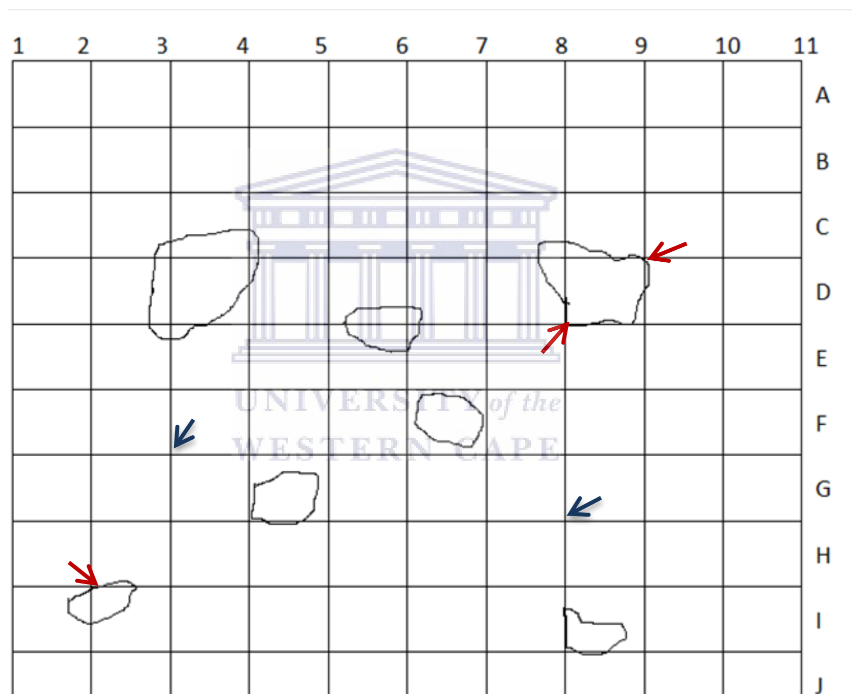


**Figure 2.9.1:** Illustration of the eyepiece grid used for point counting. The grid contains 122 points (Where lines cross, blue arrow). Points (blue arrows) that are over lung tissue (red arrows) were counted and the tissue density ($V_t$) calculated. In this example are 3 "hits" (red arrows) out of 122 points. Thus: 3/122x100 = 2.46%

## 2.9.2 The Mean Linear Intercept (Lm)

***Principle***

The Mean linear intercept (Lm), is the average distance found between alveolar walls and indicates the diameter of the alveolus (Dunnill, 1962). During normal lung maturation, the Lm increases with decreasing air-tissue interface. In microscopic emphysema however, the Lm has been observed to increase, indicating alveolar wall destruction and an increase in alveolar volume. Increase in Lm is indicative of a decrease in surface area available for gaseous exchange.

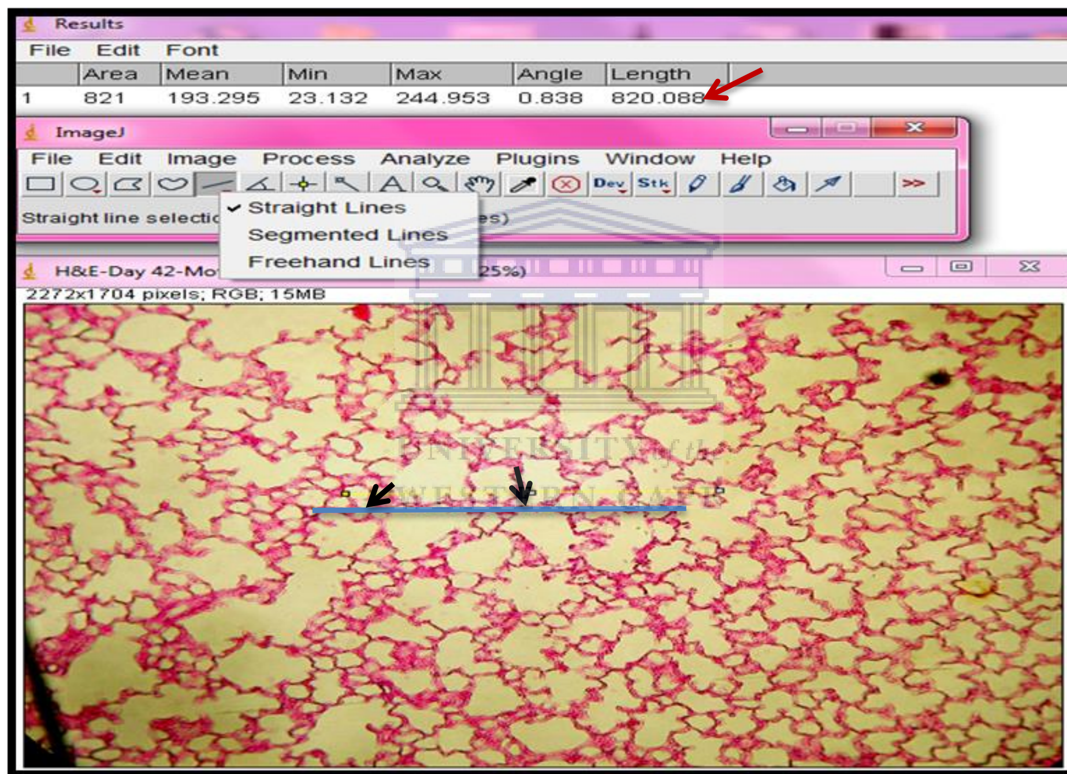


**Figure 2.9.2:** Illustration of a field (N) with a line drawn with the aid of Image J. The length of the line (red arrow) is determined by Image J and the number of intercepts (black arrows example of intercepts) counted for determination of Lm.

***Determination of Lm:***

Equation: Lm = N x L/m

Where: N = number of fields counted

L = length of cross line (see red arrow)

m = sum of all intercepts

***Example of the calculation:***

| Field | Number (#) of intercepts | Length of cross line |
|---|---|---|
| **1** | 20 | 820.088 |
| **2** | 26 | 622.048 |
| **3** | 35 | 868.021 |
| **4** | 29 | 760.011 |
| **5** | 32 | 994.002 |

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Total # of intercepts: 142

Sum of all intercepts: 4064.17

Thus: Average = 4064.17/5

= 8.12.83

Where:

812.84 µm = average length of the cross line

142 = total # of intercepts

Lm = N x L/m

= 5 x 812.83/142

= 28.62 µm

An intercept is where the cross line passes through the alveolar wall. Crossing an alveolar wall = 2 intercepts. The line just touches an alveolar wall = 1 intercept. The number of alveolar intercepts (m) was determined using an eyepiece micrometer at 100x magnification. For each slide 5 fields were used to determine the mean linear intercept.

The following alveolar walls contributed to the intercept count:

- Those that touched without crossing the left side of the vertical line,
- Those that touched without crossing the upper end of the horizontal line, and
- Those that intercepted the cross hairs.

Cut blood vessels were each counted as half an intercept.

Structures and Alveolar walls that did not contribute to the mean linear intercept included:

- Those that touched but not cross the right border of the vertical arm, and
- Those that touched but not cross the lower border of the horizontal arm.

### 2.9.3 Interalveolar Septal Thickness (Tsept)

***Principle***

The interalveolar septal thickness (Tsept) is the distance between alveoli that are adjacent to each other or the thickness of the wall of the alveoli between adjacent alveoli. Tsept is determined by utilizing the point counting and linear intercept method as described by Weibel (1963). The Weibel no. 1 graticule at 100x magnification will be used to determine the number of points on the alveolar septum and number of alveolar intercepts. For each slide, 5 non-overlapping fields will be were randomly selected and analysed for each slide.

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***Determination of Tsept***

Equation: **T*sept* = z x Pse/2x Ise**,

Where: **z** = lengths of lines on graticule (um)

**Pse** = points on alveolar walls

**Ise** = number of intercepts of alveolar walls

### 2.9.4 Lung Compliance ($C_{St}$)

An estimate of the lung compliance is a measure of the ability of the lung to stretch when a volume of air is inhaled, and its ability to recoil when it is exhaled. Lung compliance was determined by determining the volume of the lungs of the F1 control and experimental groups at different ages at a constant transpulmonary pressure of 25 cm $H_2O$.

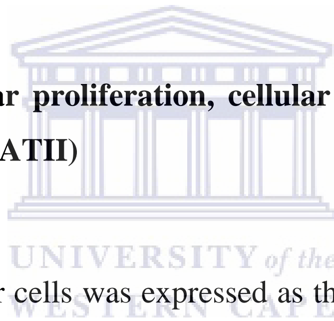
***Determination of lung compliance***

Equation: Lung Compliance = Lv/p ÷ BW

Where: Lv = Lung Volume of Animal

P = 25 cm $H_2O$ (pressure constant)

BW = Body Weight of Animal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### 2.9.5 Quantification of: Cellular proliferation, cellular senescence, pulmonary fibroblasts, and type II alveolar cells (ATII)

***Method***

The number of senescent and other cells was expressed as the number of senescent (or other cells) cells /100 µm of alveolar wall. The total number of a specific cell type per length of alveolar wall was determined by using the Image J software application. Pictures were taken at 400x magnification and used for determining cell numbers. For each slide, 5 randomly selected non-overlapping fields were used and a freehand line was drawn along the alveolar wall, where the line started at, for example a senescent cell and ended at a senescent cell. The number of a specific cell type occurring between the start and the end points of the line was counted and the number of cells was expressed as number of the specific cell type /100 µm of the alveolar wall. Slides of lung tissue of between 3 and 5 animals from at least 3 litters were used.

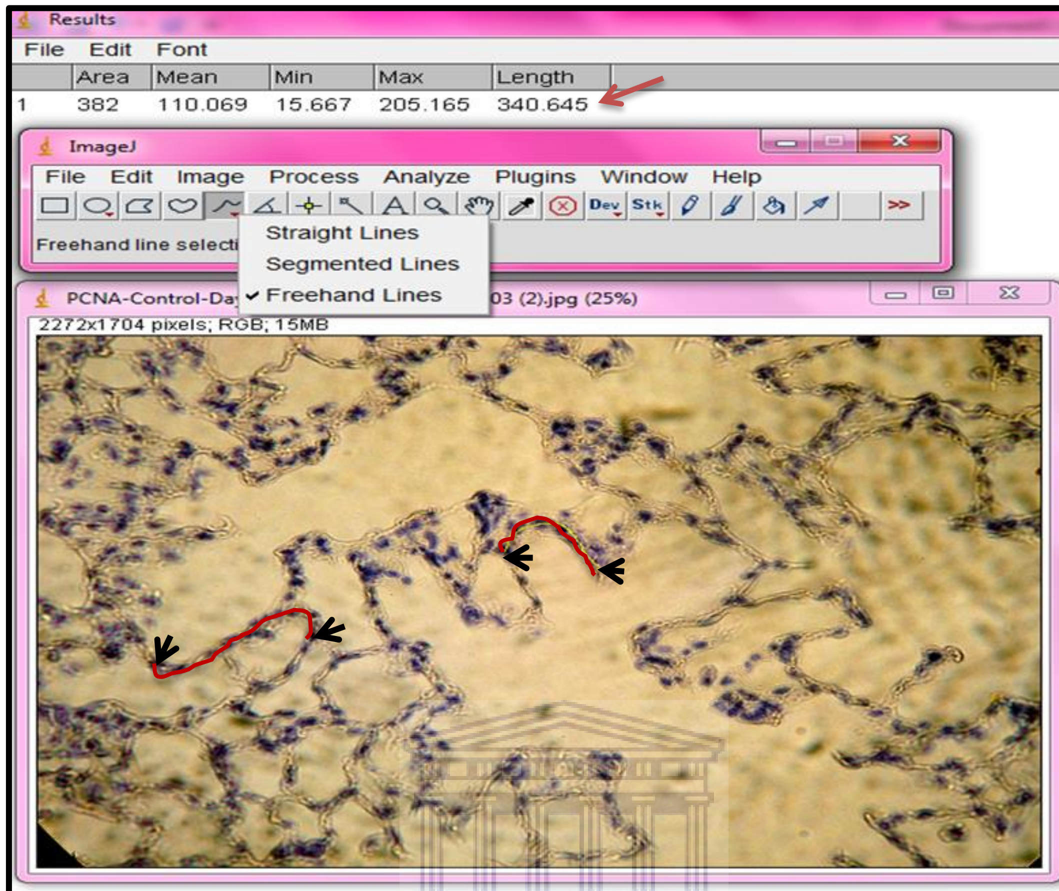


**Figure 2.9.5:** Illustration of the quantification of cells per length of alveolar wall using Image The red lines indicate the alveolar wall where cells (e.g., fibroblasts) were counted The arrows indicate the starting point and end points which are, e.g. fibroblasts. All fibroblasts between those cells were counted. Both the starting and final cells were included in the count.

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***Specific cell types were calculated as follows;***

**E.g.** Fibroblasts/100 µm =N*100/L

Where: N = number of fibroblasts counted

L= length of area containing fibroblasts (see red line)

Fibroblast number = 6*100/340.645

=1.76 cells/100 µm

This method was used to quantify and calculate the cellular proliferation, cellular senescence, type II alveolar cells, and fibroblasts respectively.

### 2.9.6 Determination of Alveolar Macrophage (AM) number

Alveolar macrophages (AMs) are the most abundant phagocytes and major effectors of innate immunity in the alveolar space of the lung. Impaired AM function in smokers is associated with the increased risk of pulmonary infections and is implicated in the pathogenesis of chronic obstructive pulmonary disease. Quantification of the AMs was done to evaluate if a decrease in the number of AMs would affect pulmonary innate immunity.

***Method***

The Hematoxylin and Eosin (H&E) slides were used to quantify the AM number. Alveolar macrophage (AM) numbers were determined by using an eyepiece grid (format reticule) of a specific surface area (80 $\mu m^2$). All AM (Arrows) inside the specified area (Red angles) were counted (see Figure 2.9.6.1). Only cells that are free and where nuclei were clearly seen were counted and expressed as number of AM/80 $\mu M^2$. Samples from lungs of at least 5 different animals were used.

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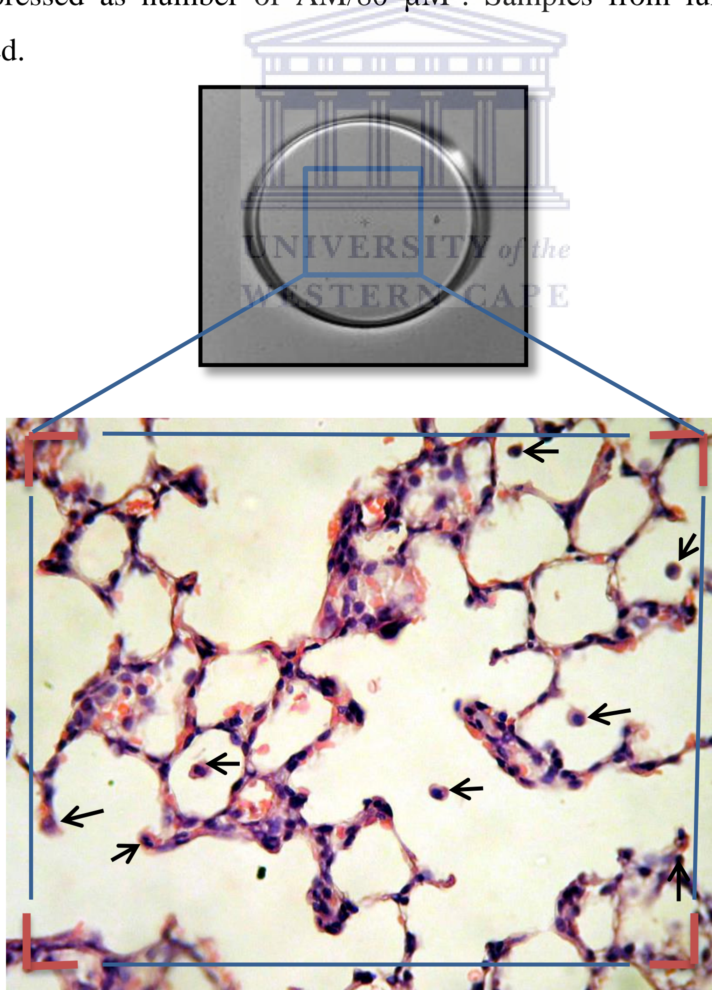

**Figure 2.9.6:** Illustration of the format reticule overlaying a lung tissue sample. Arrows indicate alveolar macrophages that lies within the demarcated area of 80 $\mu m^2$ used for counting.

For each sample at least 5 fields were counted so that at least 25 fields were counted per lung sample. The averages of the 25 fields counted for each lung was used to determine the average AM count for that lung. The averages for the lungs of the 5 animals were used to calculate the average for each experimental group. AM that touched the upper and left hand lines were considered to be within the field and thus counted. Those AM touching the bottom and right hand lines were considered to be outside the field and not counted. Fields for counting was randomly selected.

## 2.10 Statistical Analysis

One-way ANOVA with Dunnett's post-test was performed using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com using the Tukey-Kramer test, which includes the extension by Kramer to allow for unequal sample sizes. A probability level of $P<0.05$ was chosen as significant to the study and the values were recorded as means ± standard error of means.

## 2.11 References


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# CHAPTER THREE
# *Results*

## 3.1 Liquid intake

Figure 3.1 shows the liquid intake of the mothers during gestation and lactation. From the data, it can be deduced that the liquid intake of the control mothers was similar to that of the mothers exposed to nicotine, those that received tomato juice only, as well as those that were exposed to both nicotine and tomato juice ($P>0.05$) during gestation (week 1 to 3) and lactation (week 4 to 6).

## 3.2 Intake of lycopene

The lycopene intake of the pregnant rats that received tomato juice only, and those that received both nicotine and tomato juice was calculated based on the lycopene content of the tomato juice (mg/ml) and the volume of the tomato juice consumed by the animals during gestation and lactation. The lycopene intake of the pregnant mothers that received tomato juice only during week one of gestation (first week of pregnancy) was 20.03±1.34 mg/g body weight/week (mg/wk), and 22.5±1.77 mg/g/wk ($P>0.05$) for the pregnant mothers that received a combination of nicotine and tomato juice (Table 3.2). At week four (beginning of lactation), the lycopene intake of the mothers that received only tomato juice was 10.67±1.4 mg/g body mg/wk, and that of the mothers that received a combination of nicotine and tomato juice, this was 10.72±1.02 mg/g/wk ($P>0.05$).

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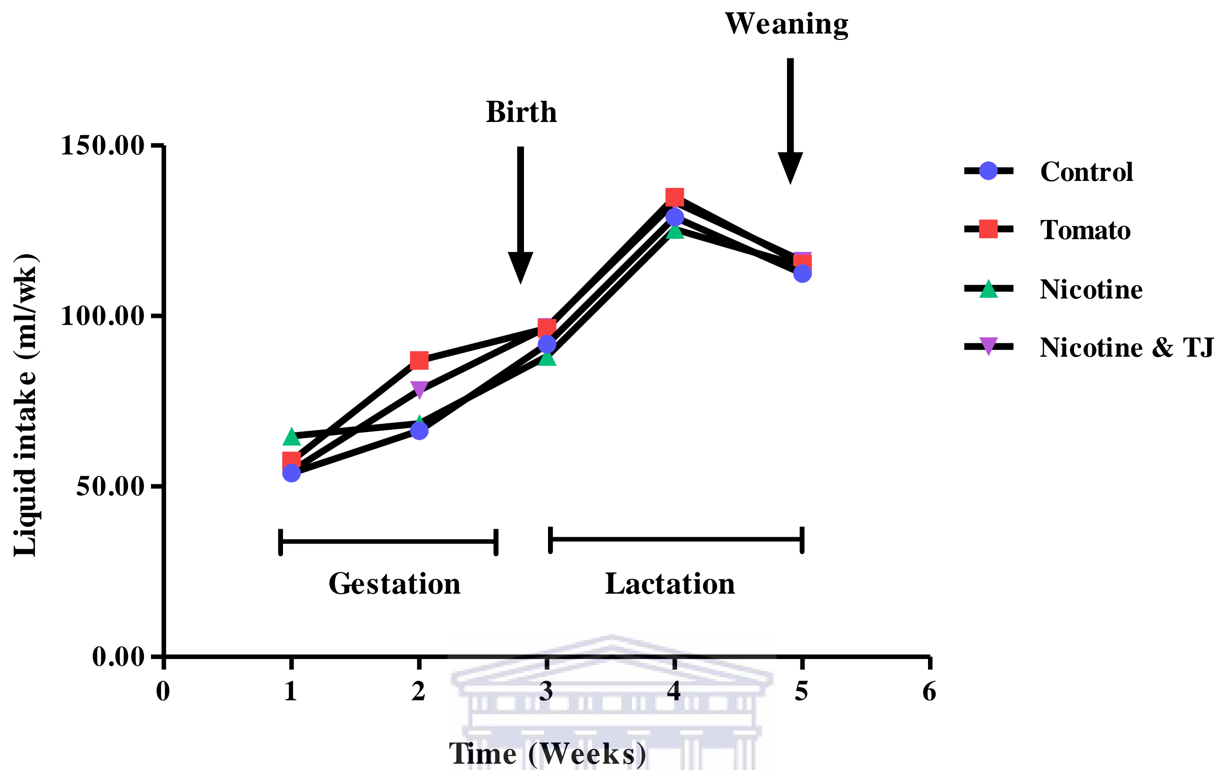


**Figure 3.1:** Liquid intake (ml/wk) of the control mothers (water), those that received tomato juice, the nicotine exposed (water), and those that received both nicotine and tomato during gestation and lactation. P-value: No differences in liquid intake within the control and experimental groups (P>0.05).

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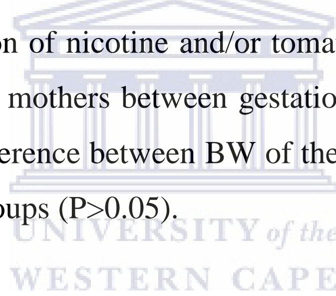
**Table 3.2:** Lycopene intake per 100 g body weight per week of the rats receiving tomato juice only, and the rats receiving both nicotine and tomato juice.

| Time (wks) | Tomato (T) | Nicotine & Tomato (Nic & TJ) | P: T vs. N & TJ |
|---|---|---|---|
| **Week 1** | 20.03±1.34 | 22.5±1.77 | >0.05 |
| **Week 4** | 10.67±1.4 | 10.72±1.02 | >0.05 |

## 3.3 Body weight increase

Figure 3.3 and Table 3.3 illustrates the weekly increase in body weight of the mothers during gestation and lactation when exposed to nicotine, tomato juice supplementation only, or exposure of the pregnant mothers to both nicotine and tomato juice. Week three marked the end of gestation. Between gestational week one and three, the weekly body weight increase of mothers that were exposed to nicotine only, increased by 54 g/wk so that at gestational week three it was at 354.50±8.31 g, 32.10% higher (P<0.002) than that of the control group which was 268.86±11.80 g at gestational week three and thus increased by 29.86 g/wk. The body weight increase up to gestational week three of the pregnant mothers that received tomato juice only (283.71±17.87 g) and those that received a combination of nicotine and tomato juice (267.50±20.30 g) increased at a similar rate (P>0.05). Between gestational week one and week three, the body weight increase of the control group was not different from that of the pregnant mothers that received tomato juice only, or those that were treated with both nicotine and tomato juice (P>0.05). Week six marked the end of lactation and the termination of nicotine and/or tomato juice treatment of the mothers. The weekly decrease in the BW of the mothers between gestational week four and week six was such that by week six, there was no difference between BW of the control group and that of the nicotine group or the other experimental groups (P>0.05).



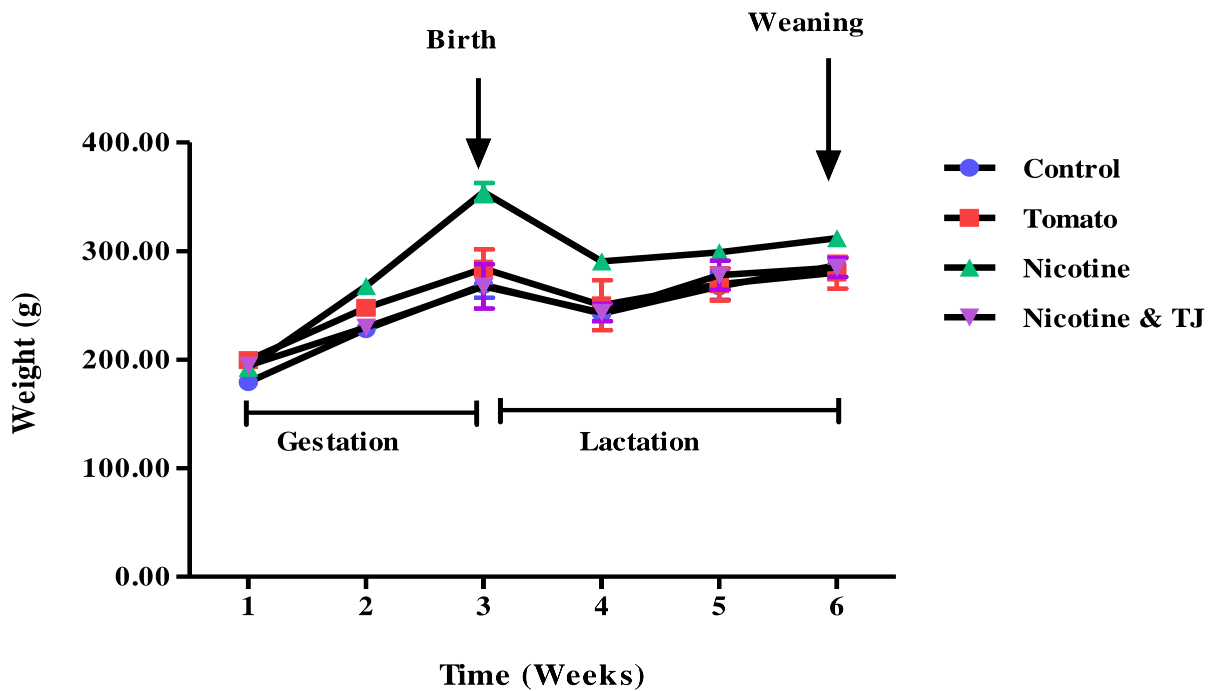



**Figure 3.3:** The effects of maternal exposure to nicotine or tomato juice, or the combination of both nicotine and tomato juice on the body weight of mothers during gestation (weeks 1 to 3) and (week 3 to week 6) lactation (Weeks 4 to 6). P-value: No differences (P>0.05) in BW within the experimental groups during gestation or lactation.

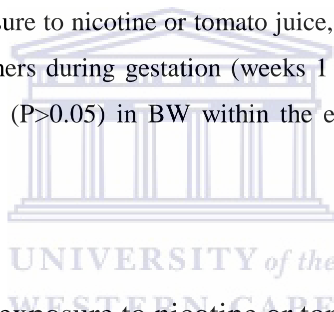

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**Table 3.3:** The effects of maternal exposure to nicotine or tomato juice, or of both nicotine and tomato juice on the increase body weight per week of the mothers during gestation (weeks 1 to 3) and lactation (weeks 3 to 6). P-value: No differences (P>0.05) in BW within the experimental groups during gestation and lactation.

| Time (wks) | Control | Tomato | Nicotine | Nic &TJ | C vs. N |
|---|---|---|---|---|---|
| **Week 1-3** | 29.86 g/wk | 28 g/wk | 54 g/wk | 24.33 g/wk | P<0.001 |
| **Week 4-6** | 14.81 g/wk | 10.05 g/wk | 7.05 g/wk | 13.78 g/wk | P>0.05 |

## 3.4 Litter size at birth

The data in Figure 3.4 shows that the litter size (pups/litter) at birth of the control mothers (12.86±0.59) was the same (P>0.05) as that of the mothers exposed to only nicotine (12.57±0.89), those that received tomato juice supplementation only (13.43±0.57), and those that were exposed to both tomato juice and nicotine (13.14±0.67).

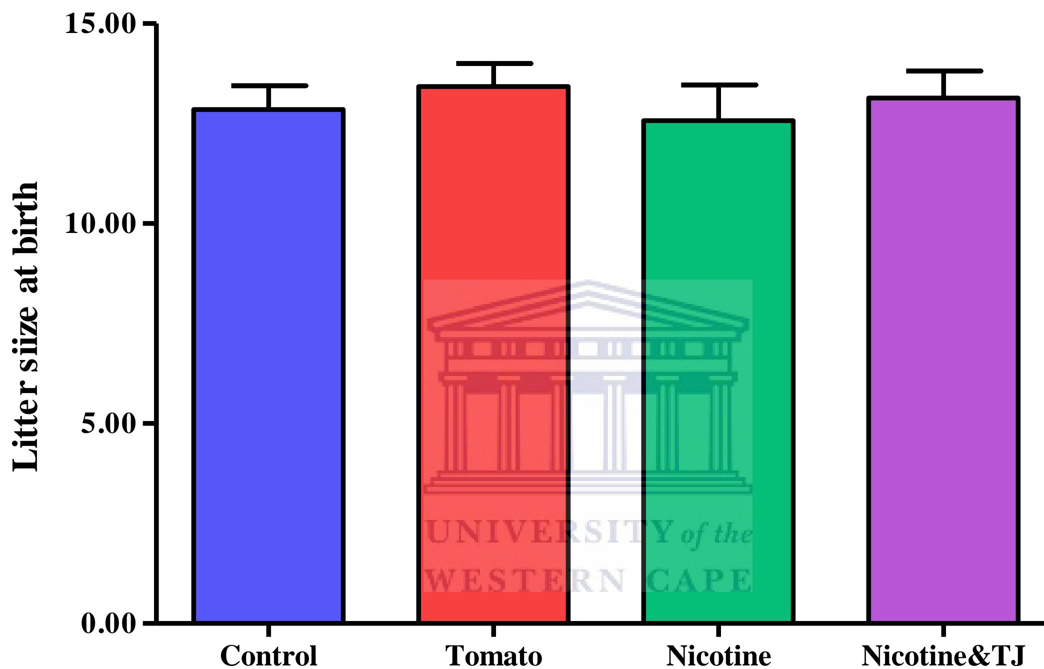


**Figure 3.4:** The effects of maternal exposure to nicotine or tomato juice, or to both nicotine and tomato juice, on the litter size at birth. P-value: No differences in litter size within the control and experimental groups (P>0.05).

## 3.5 The effect of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the growth of the F1 offspring.

### 3.5.1 Body weight (BW)

Figure 3.5.1 illustrates the daily body weight increase of the offspring treated during gestation and lactation. No significant differences were observed from a) postnatal day 14 through postnatal day 84 and b) the body weights of the offspring treated during gestation and lactation were the same as the control (P>0.05).

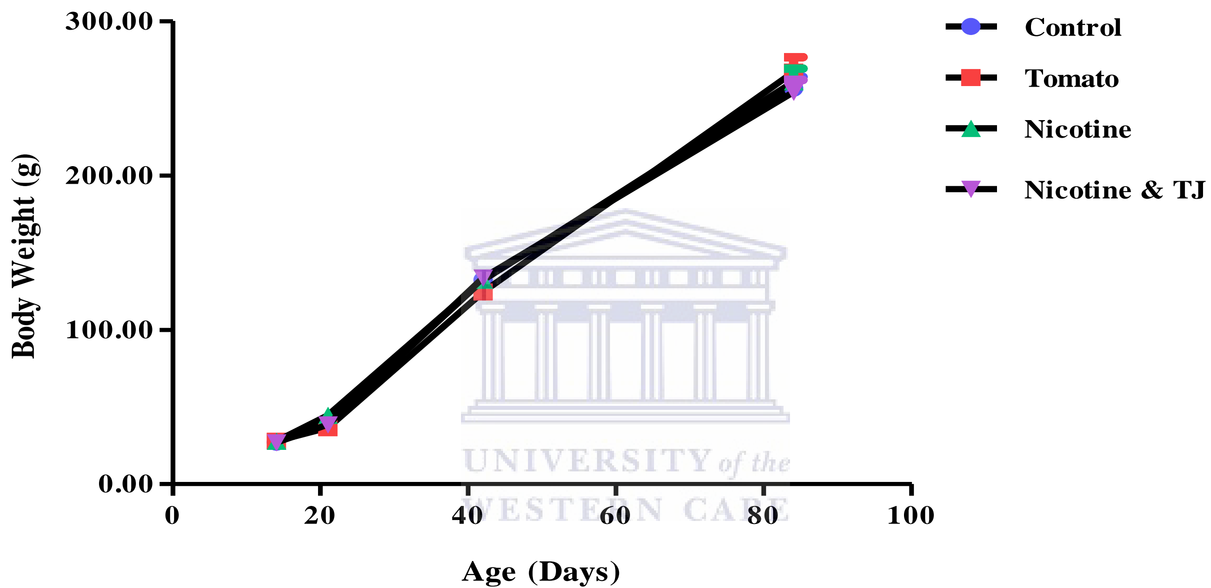


**Figure 3.5.1:** The effect of maternal exposure during gestation and lactation to nicotine, tomato juice, and both nicotine and tomato juice on the BW of the F1 offspring at day 14, 21, 42 and 84 postnatal. No differences (P>0.05) within experimental groups from postnatal day 14 up to postnatal day 84.

**Table 3.5.1:** The effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the on the BW of the F1 offspring between postnatal day 14 and 84. Postnatal day 14-84; C vs. N: P>0.05.

| Age (Days) | Control (g) | Nicotine (g) | C vs. N | Tomato Juice (g) | C vs. TJ | Nicotine & TJ (g) | C vs. N & TJ |
|---|---|---|---|---|---|---|---|
| **14** | 27.08±0.85 | 27.85±0.82 | P>0.05 | 27.82±0.94 | P>0.05 | 26.95±0.93 | P>0.05 |
| **21** | 39.79±1.83 | 44.63±0.99 | P>0.05 | 36.68±0.85 | P>0.05 | 38.56±1.26 | P>0.05 |
| **42** | 132.36±2.36 | 132.02±2.81 | P>0.05 | 124.53±2.82 | P>0.05 | 133.56±2.95 | P>0.05 |
| **84** | 256.48±7.39 | 260.85±8.43 | P>0.05 | 266.99±9.60 | P>0.05 | 253.92±7.94 | P>0.05 |

### 3.5.2 Lung volume (Lv)

The data in Figure 3.5.2 demonstrates that the daily increase in lung volume (Lv) of the rat pups was the same between postnatal day 14 and 42. Following postnatal day 42, the daily increase in the Lv of the animals treated with nicotine surpassed that of the control animals and the animals treated with tomato juice and the combination of nicotine and tomato juice. Thus, at postnatal day 84 the daily increase in Lv of the rat pups treated with nicotine (12.83±0.30 g/ml) was approximately 20% higher (P<0.001) than that of the control (9.73±0.23 g/ml) rat pups, and the rat pups exposed to tomato juice (9.83±0.22 g/ml) and the combination of nicotine and tomato juice (9.83±0.28 g/ml).

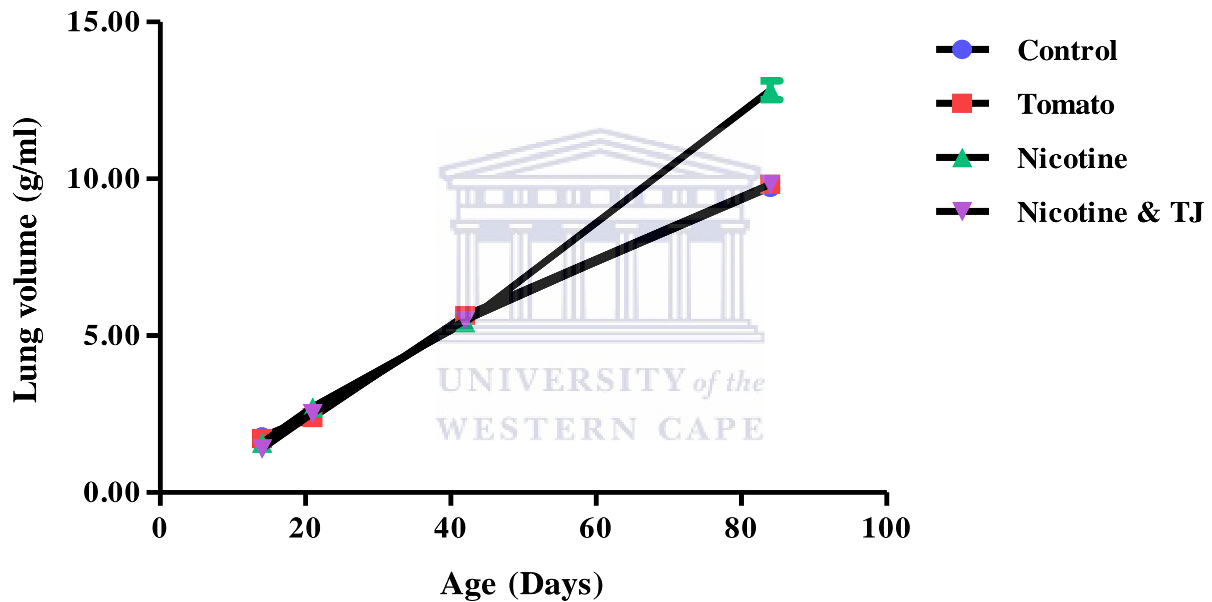


**Figure 3.5.2:** The effect of maternal exposure during gestation and lactation to nicotine, tomato juice, and both nicotine and tomato juice on the lung volume (Lv) of the F1 offspring. No dissimilarities exist between postnatal days 14 to 42 (P>0.001) of the control and experimental groups. P at postnatal day 84; control vs. nicotine: <0.001.

### 3.5.3 Chest circumference (CC) and Crown-rump length (CRL)

As for the Lv, the chest circumference (CC) (Figure 3.5.3.1) of the animals increased at a similar rate (P>0.05) between postnatal days 14 and 42 when comparing the control group to the experimental groups of the corresponding ages. Conversely, an increase in chest circumference between postnatal days 42 and 84 of the nicotine increased approximately 30% faster (P<0.05) than that of the control group as well as the other experimental groups. At postnatal day 84, a significant difference (P<0.001) was observed in the CC of the nicotine (170.76±2.77 mm) treated animals in comparison to that of the control (142.83±1.77 mm), tomato juice (145.88±1.89 mm), and both nicotine and tomato juice (141.98±1.61 mm) treated groups. As with the BW, a trend is observed in the crown rump length (CRL) (Figure 3.5.3.2) of the control and experimental animals and no significant differences were seen (P>0.05) between postnatal days 14 and 84.

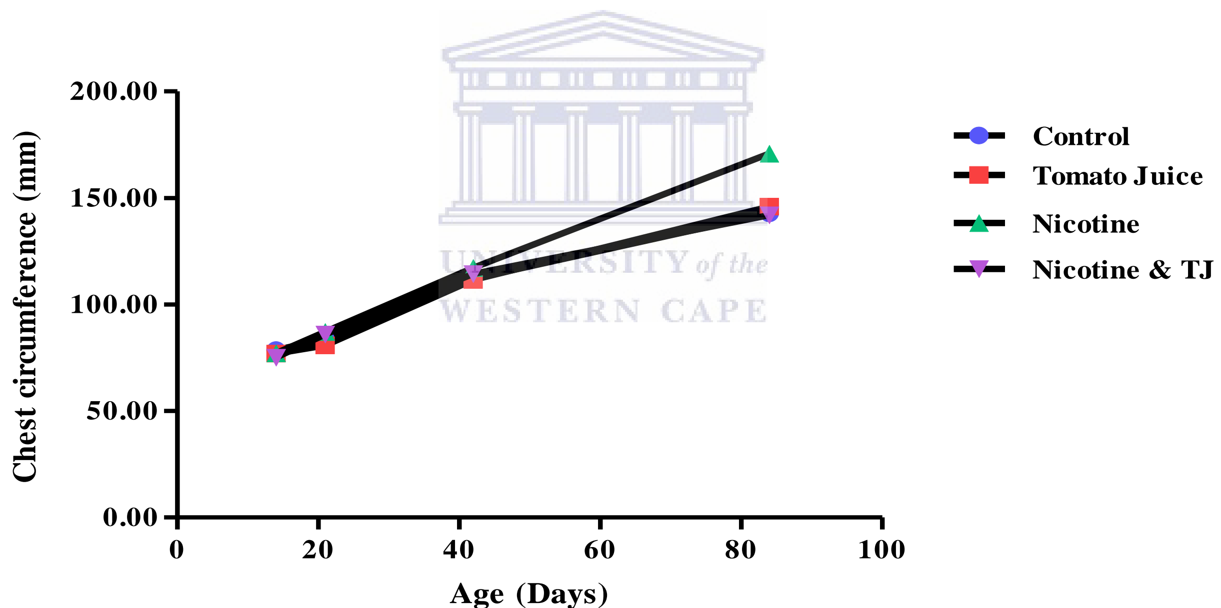

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**Figure 3.5.3.1:** The effect of maternal exposure during gestation and lactation to nicotine, tomato juice, and both nicotine and tomato juice on the chest circumference (CC) of the F1 offspring. No differences (P>0.05) were observed between postnatal days 14 and 42 of the control and experimental groups. P between postnatal day 42 and 84; control vs. nicotine: <0.05. Marked increase in CC at postnatal day 84 (P<0.001).

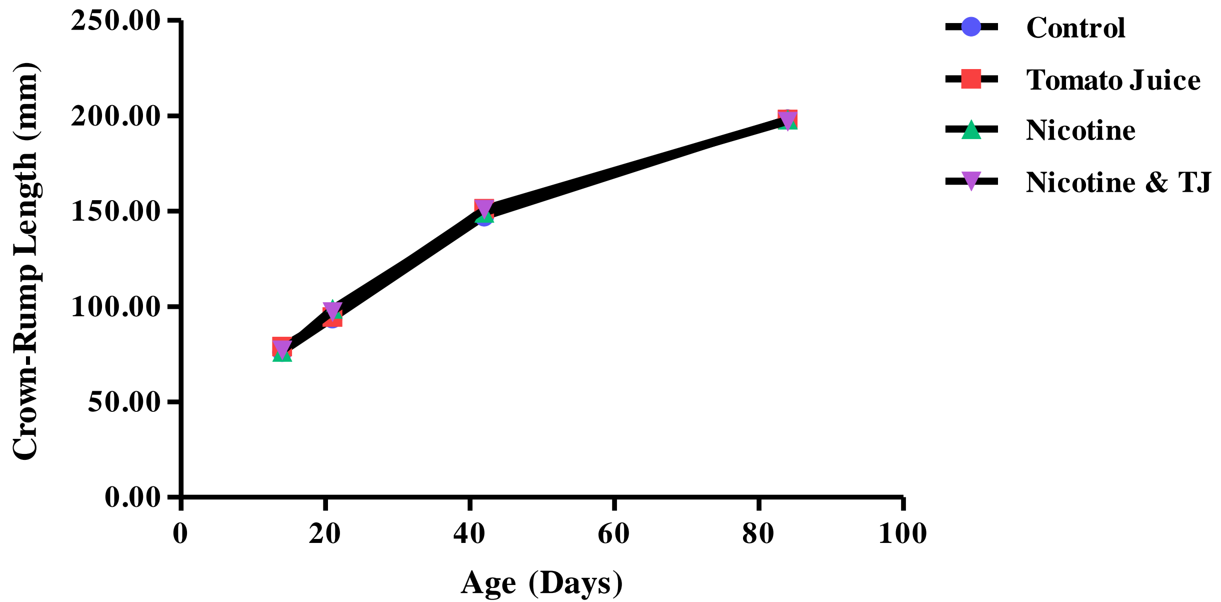


**Figure 3.5.3.2:** The effect of maternal exposure during gestation and lactation to nicotine, tomato juice, and the combination of nicotine and tomato juice on the crown rump length (CRL) of the F1 offspring. No differences ($P>0.05$) in the CRL within the experimental and control groups between postnatal days 14 through 84.

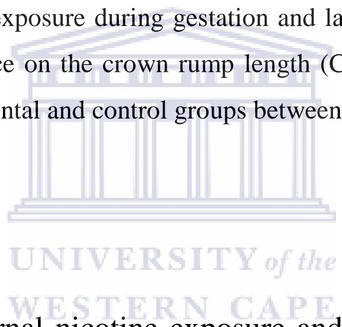
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**Table 3.5.3.1:** The effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the CRL of the F1 offspring between postnatal day 14 and 84. Postnatal day 14-84; C vs. N: $P>0.05$.

| Age (Days) | Control (mm) | Nicotine (mm) | C vs. N | Tomato Juice (mm) | C vs. TJ | Nicotine & TJ (mm) | C vs. N & TJ |
|---|---|---|---|---|---|---|---|
| **14** | 76.11±1.13 | 76.38±1.23 | P>0.05 | 79.00±1.82 | P>0.05 | 77.13±1.54 | P>0.05 |
| **21** | 93.71±1.96 | 98.81±1.03 | P>0.05 | 94.52±1.01 | P>0.05 | 97.14±1.34 | P>0.05 |
| **42** | 147.10±1.19 | 149.26±1.16 | P>0.05 | 151.29±1.47 | P>0.05 | 150.95±1.48 | P>0.05 |
| **84** | 198.37±1.06 | 198.16±1.29 | P>0.05 | 198.14±1.55 | P>0.05 | 196.98±1.46 | P>0.05 |

### 3.5.4 Chest circumference to body weight (CC/BW) ratio and chest circumference to crown rump length (CC/CRL) ratio

The chest circumference to body weight ratio (CC/BW) (Figure 3.5.4.1) and the chest circumference to crown rump length ratio (CC/CRL) (Figure 3.5.4.2) changed with an increase in age for the control and experimental rat pups. Although a trend can be seen in Figure 3.5.4.1, no statistical significance (P>0.05) exists between that of the control and experimental animals or the respective age groups. Similarly, the CC/CRL ratio show shown in Figure 3.5.4.2 show no differences (P>0.05) between the experimental rat pups and that of the control rat pups from postnatal day 14 through 84. This is indicative that growth was proportional for both the CC/BW and CC/CRL ratios of the rat pups exposed during gestation and lactation.

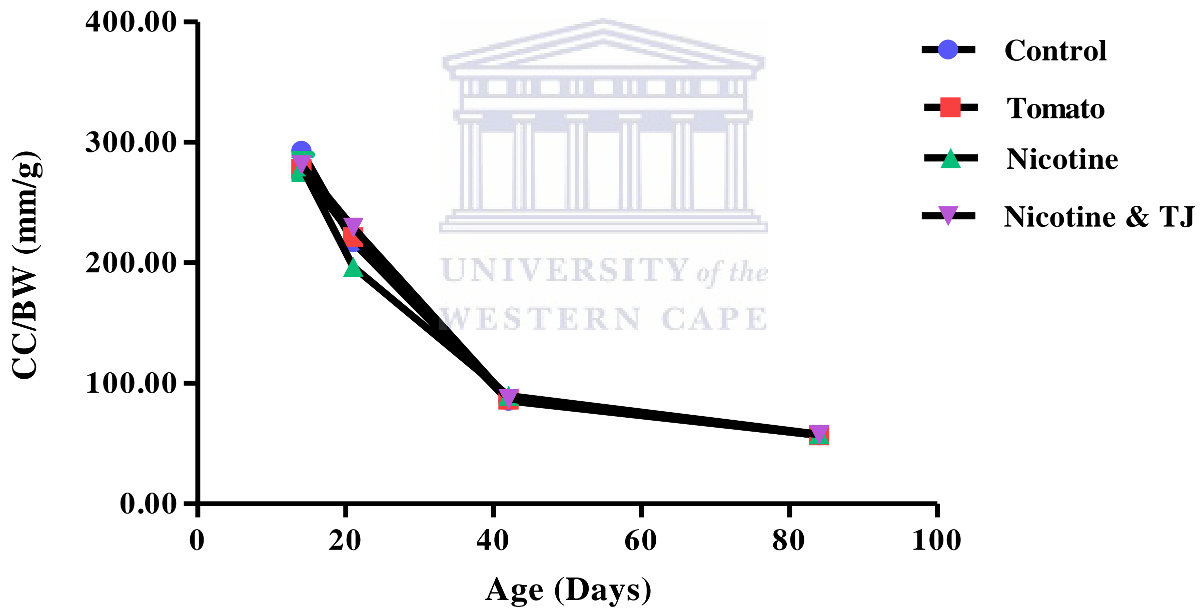

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**Figure 3.5.4.1:** The effect of maternal exposure during gestation and lactation to nicotine, tomato juice, and nicotine together with tomato juice on the CC/BW ratio of the F1 generation. P between postnatal days 14 and 84; control vs. nicotine: >0.05.

**Table 3.5.4.1:** The effect of maternal exposure to nicotine and the intake of tomato juice during gestation and lactation on the CC/BW ratio of the F1 progeny between postnatal day 14 and postnatal day 84. Postnatal day 14-84; C vs. N: P>0.05.

| Age (Days) | Control (mm/g) | Nicotine (mm/g) | C vs. N | Tomato Juice (mm/g) | C vs. TJ | Nicotine & TJ (mm/g) | C vs. N & TJ |
|---|---|---|---|---|---|---|---|
| **14** | 293.18±5.88 | 280.03±9.65 | P>0.05 | 278.07±6.86 | P>0.05 | 281.66±6.64 | P>0.05 |
| **21** | 217.08±7.66 | 196.71±3.87 | P>0.05 | 221.56±3.56 | P>0.05 | 229.55±7.48 | P>0.05 |
| **42** | 85.48±1.62 | 89.75±1.64 | P>0.05 | 86.65±1.63 | P>0.05 | 87.00±1.58 | P>0.05 |
| **84** | 56.86±1.06 | 57.80±1.32 | P>0.05 | 56.84±1.54 | P>0.05 | 57.40±1.21 | P>0.05 |

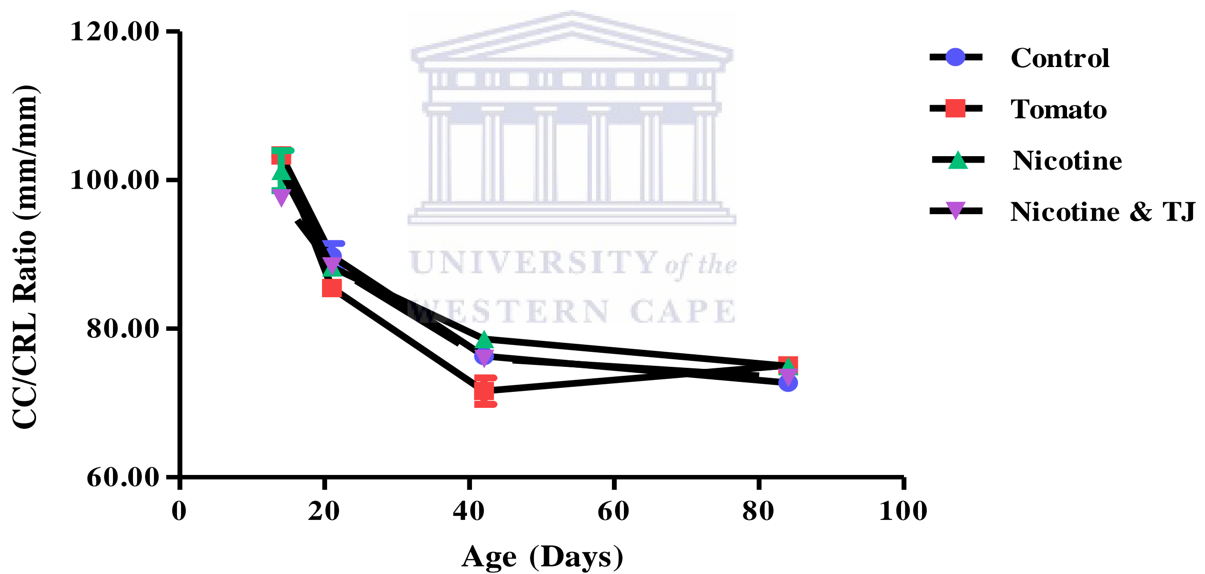


**Figure 3.5.4.2:** The effect of maternal exposure during gestation and lactation to nicotine, tomato juice, and both nicotine and tomato juice on the CC/CRL ration of the F1 offspring. No variations (P>0.05) in the CC/CRL ratio of the control and experimental groups between postnatal days 14 and 84.

**Table 3.5.4.2:** The effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the CC/CRL ratio of the F1 progeny between postnatal day 14 and 84. Postnatal day 14-84; C vs. N: P>0.05.

| Age (Days) | Control (mm/mm) | Nicotine (mm/mm) | C vs. N | Tomato Juice (mm/mm) | C vs. TJ | Nicotine & TJ (mm/mm) | C vs. N & TJ |
|---|---|---|---|---|---|---|---|
| **14** | 103.30±0.78 | 101.28±2.68 | P>0.05 | 103.30±0.78 | P>0.05 | 97.55±1.04 | P>0.05 |
| **21** | 89.77±1.69 | 88.28±1.05 | P>0.05 | 85.46±0.78 | P>0.05 | 88.40±1.09 | P>0.05 |
| **42** | 76.34±1.14 | 78.64±1.01 | P>0.05 | 71.61±1.77 | P>0.05 | 75.98±0.71 | P>0.05 |
| **84** | 72.74±0.70 | 74.97±0.60 | P>0.05 | 75.05±0.70 | P>0.05 | 73.40±0.77 | P>0.05 |

### 3.5.5 Lung volume to body weight (Lv/BW) ratio and chest circumference to lung volume (CC/Lv) ratio

The lung volume to body weight ratio (Lv/BW) (Figure 3.5.5.1) of the experimental and control rat pups were the same (P>0.05) between postnatal days 14 and 21. Also shown is that between postnatal day 21 and 42, no significant differences (P>0.05) were observed between the Lv/BW ratios and that the growth of the control animals versus the tomato juice and both nicotine and tomato juice experimental groups respectively, were proportional. However, following postnatal days 42 through to postnatal day 84, a significant dissimilarity in ratios (P<0.05) is illustrated between the control animals and that of the nicotine experimental animals. At postnatal day 84, the Lv/BW ratios of the nicotine (5.02±0.13 ml/g) animals were roughly 20% higher (P<0.05) than those of the control (3.86±0.10 ml/g), tomato juice (3.82±0.11 ml/g), and both nicotine and tomato juice (3.91±0.08 ml/g) groups. This is suggestive of disproportional growth. The data shown in Figure 3.5.5.2 illustrates a trend in the chest circumference to lung volume (CC/Lv) ratio. Growth was proportional and no differences (P>0.05) are present between the control, tomato juice and the combination of nicotine and tomato juice from postnatal day 14 through to 42. It should be noted that at postnatal day 84, the CC/Lv ratio of the nicotine (11.61±0.23 mm/ml) animals were significantly lower (P<0.05) as opposed to that of the control (14.94±0.30 mm/ml), tomato juice (15.07±0.31 mm/ml), and the combination of nicotine and tomato juice (14.80±0.34 mm/ml) groups.

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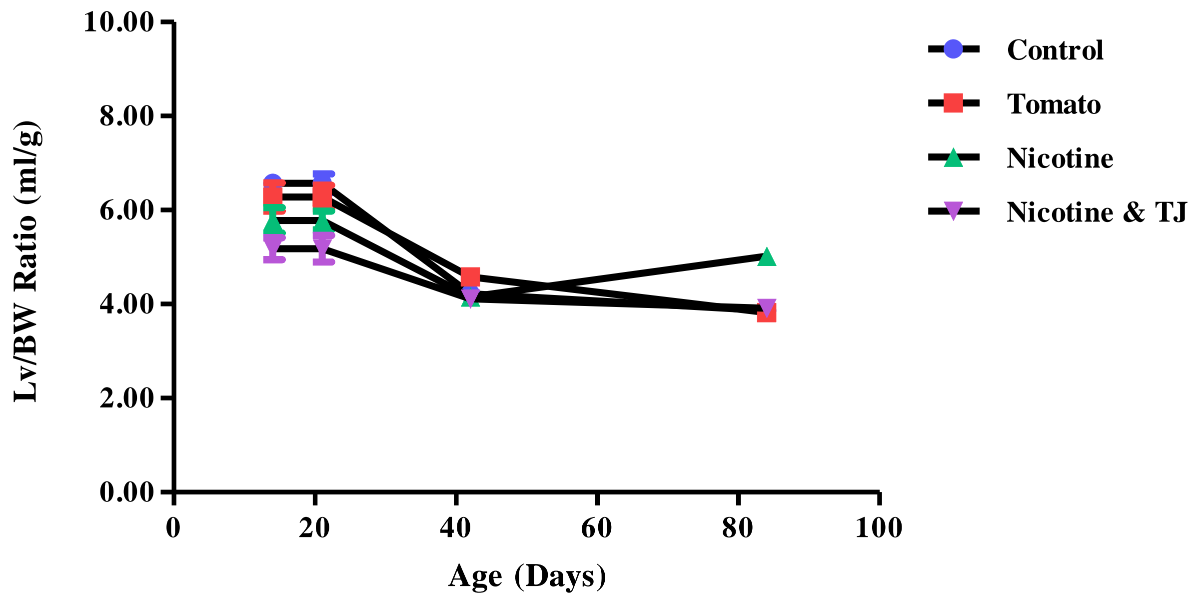
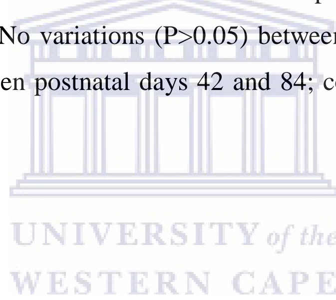



**Figure 3.5.5.1:** The effect of maternal exposure during gestation and lactation to nicotine, tomato juice, and nicotine together with tomato juice of the Lv/BW ratio of the F1 offspring. P between postnatal days 14 and 21; control vs. experimental groups: >0.05. No variations (P>0.05) between postnatal days 21 and 42 of the control and experimental animals. P<0.05 between postnatal days 42 and 84; control vs. nicotine. P at postnatal day 84; control vs nicotine: <0.05.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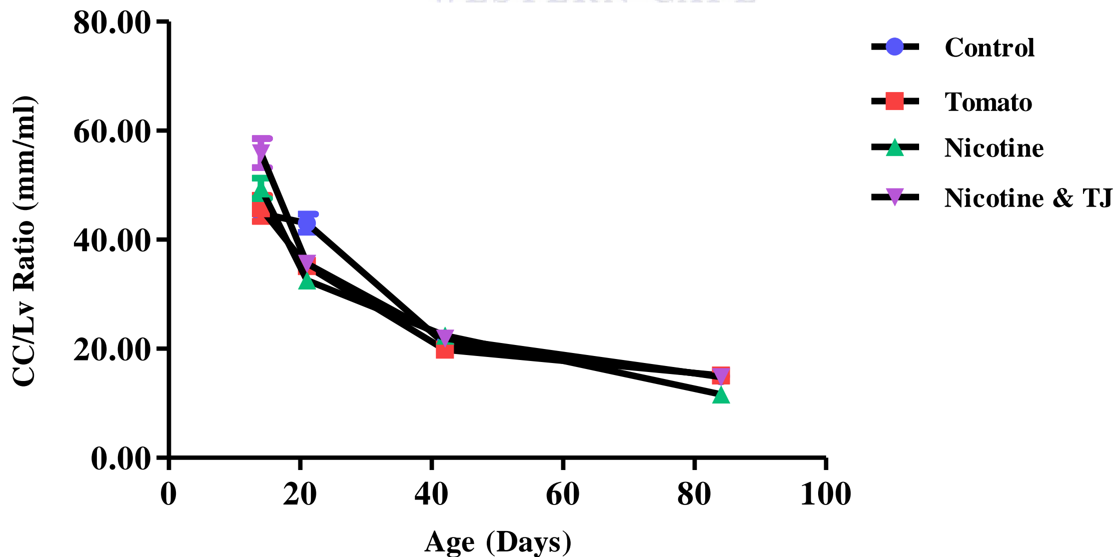


**Figure 3.5.5.2:** The effect of maternal exposure during gestation and lactation to nicotine, tomato juice, and both nicotine and tomato juice on the CC/Lv ratio of the F1 generation. No dissimilarities (P>0.05) between postnatal days 14 and 42 of the control and experimental animals. P at postnatal day 84; control vs. nicotine: <0.05.

## 3.6 The effect of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the lung morphometry and morphology of the F1 offspring.

### 3.6.1 Tissue and Air volumes ($V_t$ and $V_a$)

Figure 3.6.1.1 clearly illustrates the marked increase ($P<0.001$) in lung tissue volumes ($V_t$) of all the groups between postnatal days 21 and 84. The lung tissue volumes of the control animals increased linearly from 0.73±0.04 ml at postnatal day 21 to 4.03±0.095 ml at postnatal day 84; the animals that received tomato juice increased from 0.60± 0.04 ml at postnatal day 21 to 3.72±0.14 ml at postnatal day 84, while the tissue volumes of those exposed to the combination of nicotine and tomato juice increased from 0.58± 0.05 ml at postnatal day 21 to 3.95±0.13 ml at postnatal day 84 in the same amount of time. Concomitantly, the $V_t$ of the nicotine group increased from 0.60±0.04 ml to 2.93±0.065 ml between postnatal day 21 and 84 respectively. The $V_t$ of the nicotine exposed animals was lower than that of the control, tomato juice and both nicotine and tomato juice exposed animals ($P<0.05$). Interestingly, up to postnatal day 42, which is 3 weeks after nicotine withdrawal, the $V_t$ of the nicotine exposed rats resembles that of the control and other experimental groups. Therefore, the reduction in $V_t$ is only evident after postnatal day 42. An alteration in $V_t$ is related to changes in $V_a$. This indicates that when a decrease in $V_t$ occurs, as observed in nicotine exposed animals at postnatal day 84, a simultaneous increase in $V_a$ will be present in nicotine exposed rats of the same age. Additionally, this data further infers that tomato juice consumption by the mother prevented the effect of nicotine on tissue growth in the lungs of the offspring.

The data also shows a distinct increase in the $V_a$ between postnatal days 21 and 84 of the control and the animals exposed to tomato juice only and the combination of nicotine and tomato juice (Figure 3.6.1.2). The detrimental effects of nicotine on the lung parenchyma are demonstrated by the low values in the nicotine exposed rats and become more apparent as the animals' age.

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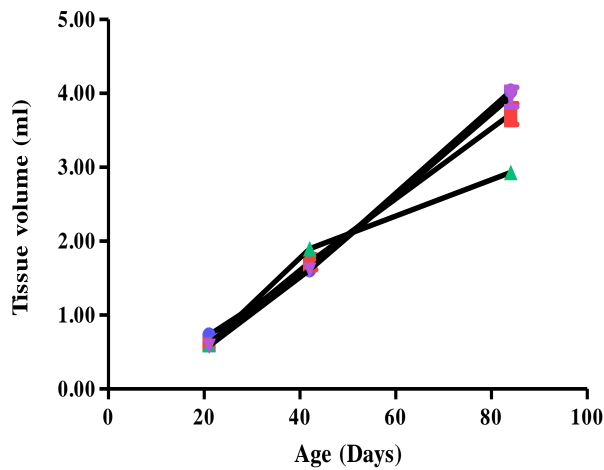


**Figure 3.6.1.1:** Tissue volume ($V_t$)

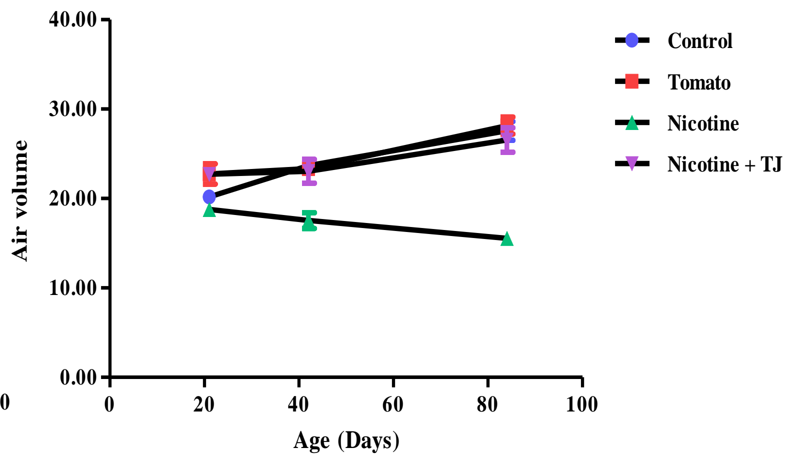


**Figure 3.6.1.2:** Air volume ($V_a$)

**Figure 3.6.1.1:** The effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the tissue volume ($V_t$) of the F1 offspring. $V_t$ at postnatal day 84; C vs. N: P<0.05.

**Figure 3.6.1.2:** The effect of maternal nicotine exposure and tomato juice during gestation and lactation on the air volume ($V_a$) in the lungs of the F1 offspring. P at postnatal day 84; C vs. N: <0.05.

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**Table 3.6.1:** The effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the air volume ($V_a$) in the lungs of the F1 offspring. P at postnatal day 84; C vs. N: <0.001. No significant differences (P>0.05) C vs. T or C vs. N & TJ for all age groups.

| Age (Days) | Control | Nicotine | C vs. N | Tomato Juice | C vs TJ | Nicotine & TJ | C vs. N & TJ |
|---|---|---|---|---|---|---|---|
| **21** | 20.19±0.29 | 18.77±0.47 | P<0.05 | 22.76±1.13 | P>0.05 | 22.73±0.75 | P>0.05 |
| **42** | 23.69±0.54 | 17.54±0.89 | P<0.01 | 23.34±0.50 | P>0.01 | 23.06±1.33 | P>0.05 |
| **84** | 27.57±1.05 | 15.55±0.18 | P<0.001 | 28.17±0.96 | P>0.01 | 26.56±1.37 | P>0.05 |

### 3.6.2 Mean linear intercept (Lm)

The findings shown in Figure 3.6.2 illustrate that at postnatal day 21, the Mean Linear Intercept (Lm) of the control animals were similar to that of the experimental animals of the same age (P>0.05). Conversely, between following postnatal day 21 up to postnatal day 84, the Lm of the nicotine exposed animals increased significantly so that at postnatal day 84 it was 30% higher (P<0.001) in comparison to the control animals of the same age. Between postnatal days 21 and 84, the Lm of the control animals decreased from 35.14±0.73 µm to 33.31±1.40 µm, the tomato juice exposed animals values decreased from 35.23±1.63 µm to 32.35±1.50 µm, and the Lm of the group exposed to the combination of nicotine and tomato juice decreased from 38.90±1.47 µm to 33.99±0.65 µm (P<0.05). In contrast, the Lm of the nicotine exposed rats increased by 23% from 36.87±0.75 µm to 48.07±1.88 µm (P<0.001) between postnatal days 21 and 84. No differences (P>0.05) were observed between the control animals and those exposed to tomato juice and both nicotine and tomato juice of the same age.

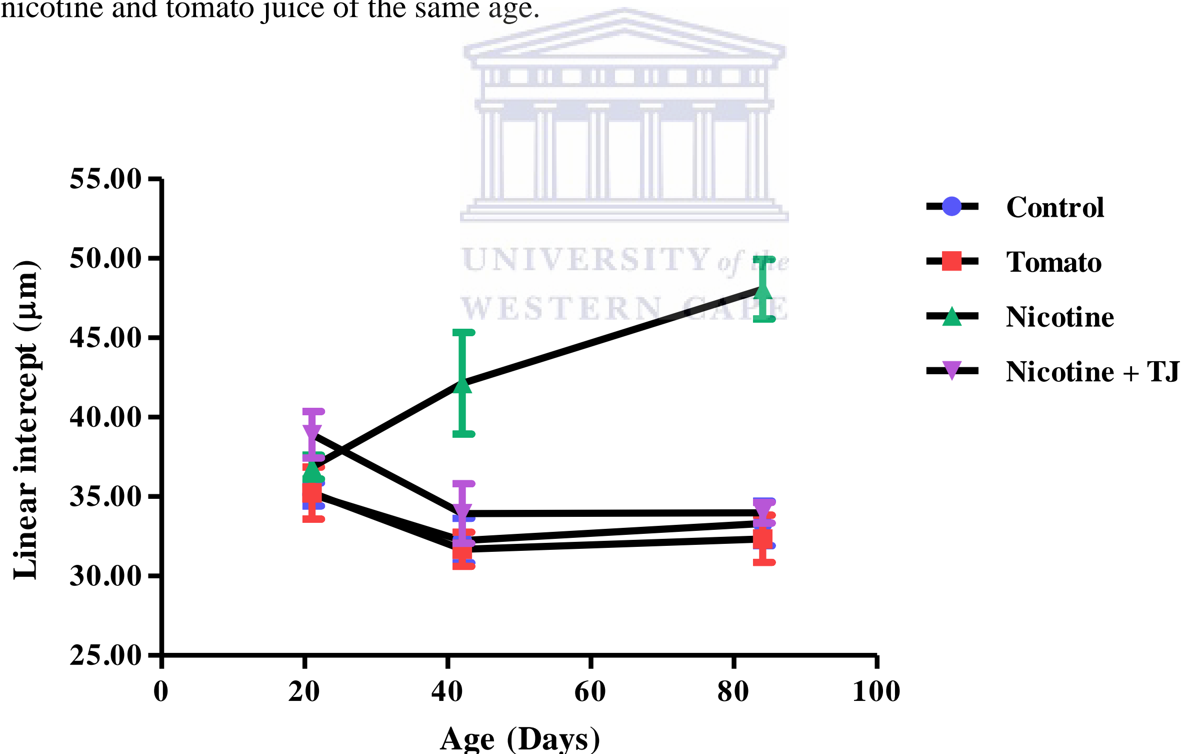

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**Figure 3.6.2:** The effect of maternal nicotine exposure and tomato juice during gestation and lactation on the Mean Linear intercept (Lm) in the lungs of the F1 offspring. Lm between postnatal day 21 and 84; C vs. N: P<0.001).

### 3.6.3 Interalveolar septal thickness (Tsept)

Up to postnatal day 42 Tsept (Figure 3.6.3) of the control rats was similar to that of the rats in the experimental groups (P>0.05). The increase in the Tsept of the nicotine exposed animals only became evident between postnatal day 42 and 84.The increase was such that at postnatal day 84, the alveolar wall thickness of the nicotine exposed animals (13.76±0.92 µm) animals was 34 % higher (P<0.001) than that of the control animals (9.04±0.67 µm). Furthermore, at postnatal day 84 the Tsept of the controls was the same (P>0.05) as those that received tomato juice only (9.24±0.56 µm), and those exposed to both nicotine and tomato (8.52±0.28 µm) juice.

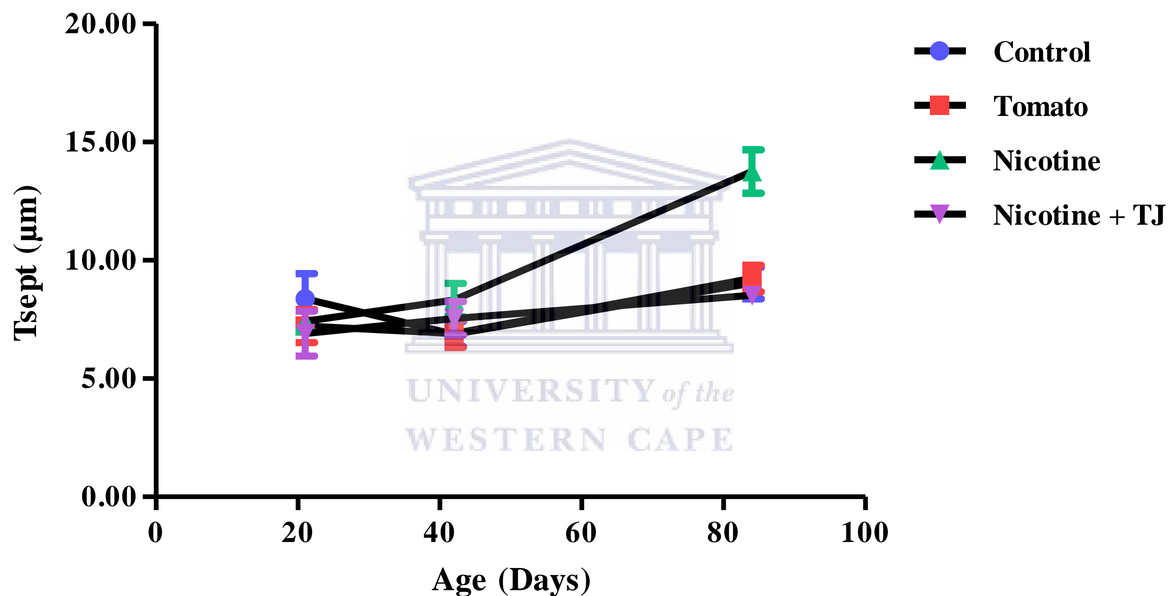

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**Figure 3.6.3:** The effect of maternal exposure to nicotine, tomato juice, and the combination of nicotine and tomato juice during gestation and lactation on the Interalveolar septal thickness (Tsept) in the lungs of the F1 offspring. Postnatal days 21 to 42; no differences (P>0.05) between control and experimental groups. P at postnatal day 84, C vs N: <0.001.

### 3.6.4 Static lung compliance ($C_{St}$)

Figure 3.6.4 illustrates an increase in static lung compliance ($C_{St}$) as a result of 1) aging 2) maternal exposure to nicotine during gestation and lactation. No significant differences ($P>0.001$) were observed between the control, tomato juice only, nicotine only, and nicotine together with tomato juice between postnatal days 21 and 42. However, a significant increase ($P<0.001$) was observed between postnatal day 42 and 84 in lung compliance in the lungs of the nicotine exposed offspring in contrast to that of the control rats. The $C_{St}$ of the nicotine rats increased from 1.66±0.06 ml/cm $H_20$/kg at postnatal day 42 so that at postnatal day 84 it was 17% higher (2.01± 0.05 ml/cm $H_20$/kg).

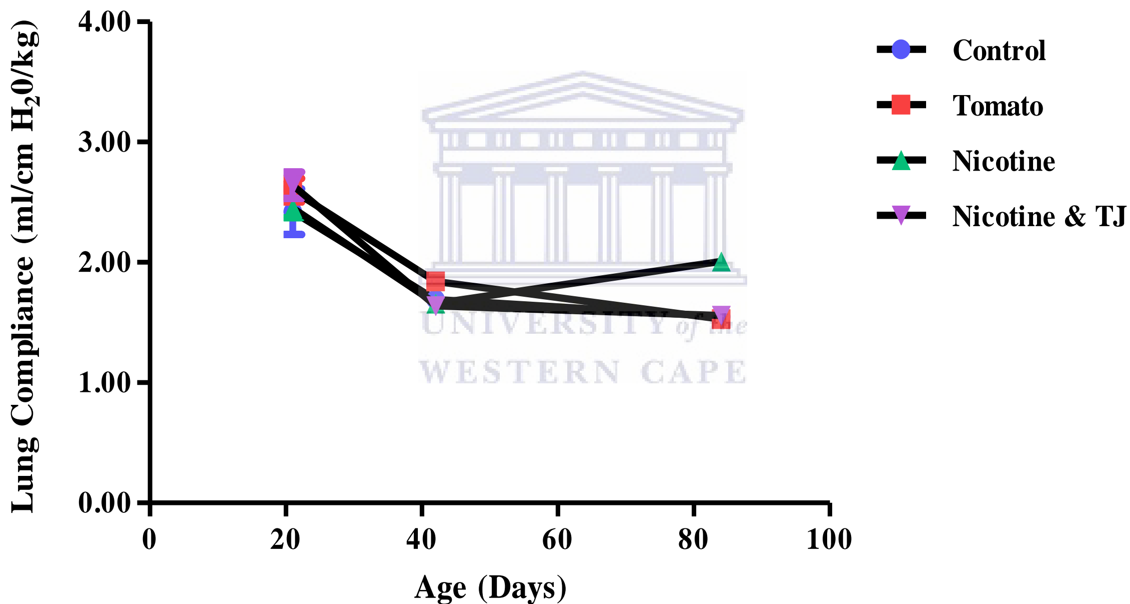


**Figure 3.6.4:** The effect of maternal exposure during gestation and lactation to nicotine, tomato juice, and the combination of nicotine and tomato juice on the $C_{st}$ of the F1 offspring. No variations ($P>0.001$) within the control and experimental rat pups between postnatal days 14 and 42. P at day 84; control vs. nicotine: <0.001.

## 3.7 The effect of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the cellularity in the lungs of the F1 offspring.

### 3.7.1. Lung structure

Figure 3.7.1 illustrates lung structure of control rats as well as that of rats that were exposed to nicotine, tomato juice, or to both nicotine and tomato juice. The administration of nicotine as well as the treatment of tomato juice or the combined use of nicotine and tomato juice to the offspring was only via the placenta and the mother's milk. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice. From Figure 3.7.1 it is clear that the lung structure of those rats that were exposed to nicotine during gestation and lactation display breakdown of alveolar walls which result in enlarged alveolar spaces that resembles microscopic emphysema (EM). The site of action in the alveolar walls and mechanism of action of nicotine will be investigated and data below illustrates the data generated in the study by investigating the cellular characteristics of the alveolar wall and alveoli.

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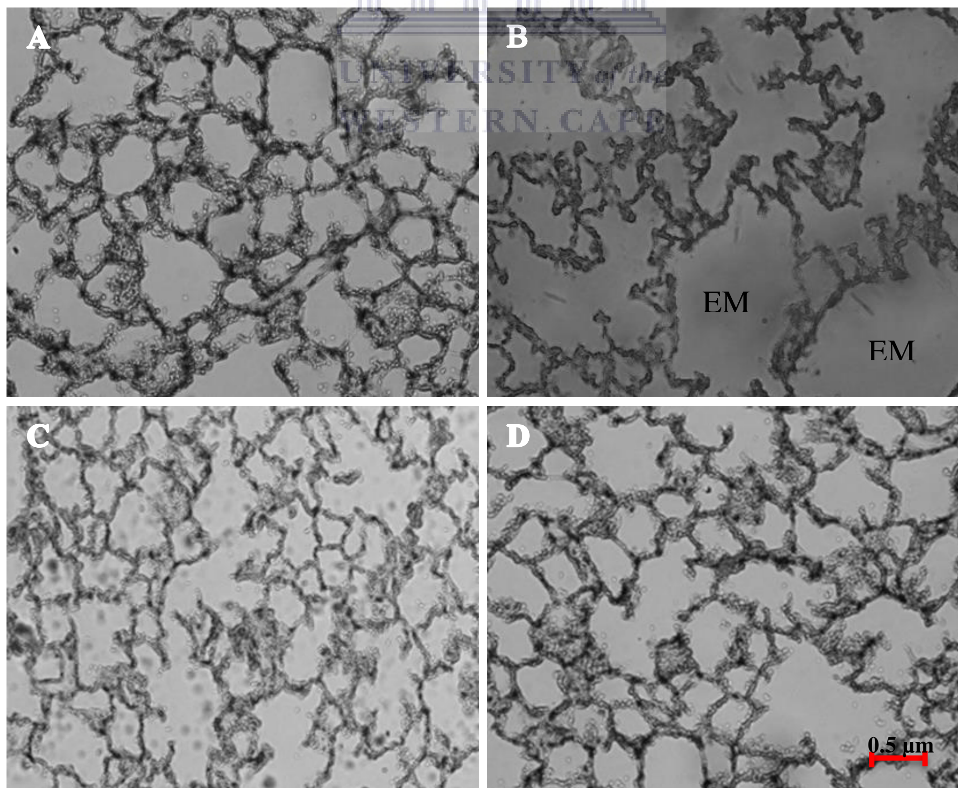


**Figure 3.7.1:** The effect of maternal exposure to nicotine, tomato juice and a combination of nicotine and tomato juice on lung parenchyma of the 84-day-old offspring. A. = Control; B= Nicotine; C. = Tomato juice; D. = Nicotine + tomato juice. EM = Emphysema-like lesion. (Bar = 0.5µm)

### 3.7.2 Cellular senescence in the alveolar wall

Figure 3.7.2 shows the effect of maternal exposure during gestation and lactation to nicotine, the supplementation of the mother's diet with tomato juice and the combination of nicotine and tomato juice supplementation on the senescent cell number per 100µm of alveolar wall in the lungs of the F1 offspring at postnatal day 84. The number of senescent cells per 100 µm alveolar wall in the animals that were exposed to nicotine (1.16±0.07 cells/100 µm) was 41% higher (P<0.01) than that of the control animals (0.69±0.69 cells/100 µm). The animals exposed to tomato juice (0.73±0.03 cells/100 µm) and the combination of nicotine and tomato juice (0.78±0.04 cells/100 µm) had a comparable senescent cell number per 100 µm length of alveolar wall to that of the control group (P>0.05).

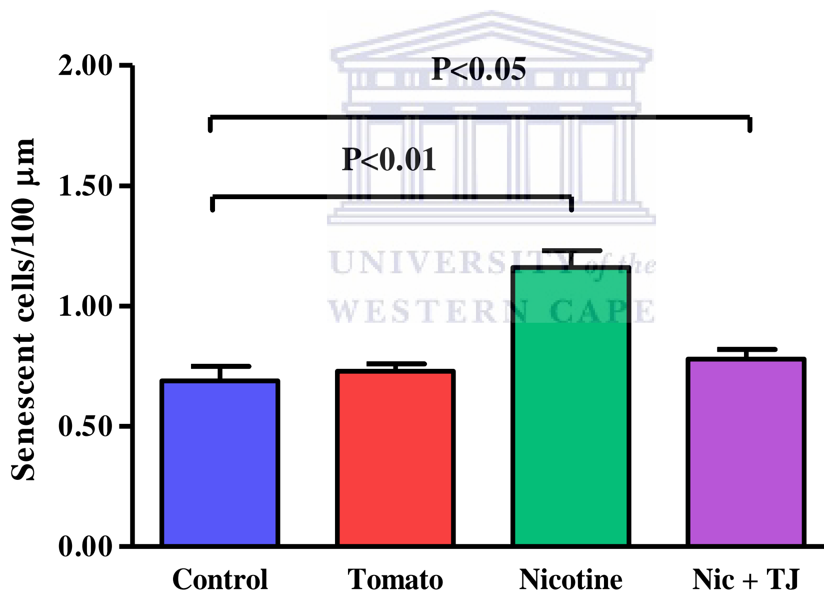


**Figure 3.7.2:** The effect of maternal exposure of the F1 offspring during gestation and lactation to nicotine, tomato juice and nicotine together with tomato juice of the on the number of senescent cells per 100µm of the alveolar wall at postnatal day 84. No differences (P>0.05) were observed between the control, tomato juice and combination of nicotine and tomato juice. Control vs. nicotine: P<0.05.

### 3.7.3 Cellular proliferation in the alveolar wall

The data in Figure 3.2.3 shows the number of proliferating cells per 100μm alveolar wall length in the control animals (1.43±0.06 cells/100 μm) was 21% higher (P<0.001) than the number of proliferating cells in the group exposed solely to nicotine (1.12±0.03 cells/100 μm). The amount of proliferating cells in the group who received tomato juice (1.50±0.06 cells/100 μm) only and the offspring of the mothers who were exposed to both nicotine and tomato juice (1.34±0.04 cells/100 μm) was the same (P>0.05) to that of the control rats.

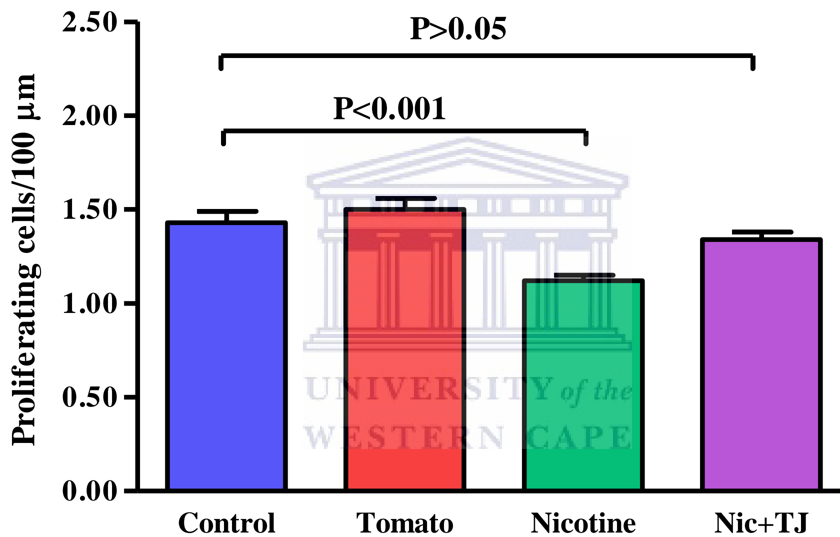

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**Figure 3.7.3:** The effect of maternal exposure of the F1 offspring during gestation and lactation to nicotine, tomato juice, and nicotine together with tomato juice on the number of proliferating cells per 100μm of the alveolar wall at postnatal day 84. Control vs. nicotine: P<0.001. Similarities (P>0.05) existed between the control, tomato juice and the combination of nicotine and tomato juice.

### 3.7.4 Cellular proliferation to Cellular senescence (P/S) ratio

Figure 3.7.4 shows that the P/S ratio of the control (2.07±0.12) animal lungs was significantly higher (P< 0.001) in comparison to the rats that were exposed to nicotine (0.97±0.10) only. Furthermore no differences (P>0.05) were observed between the P/S ratio of the control (2.07±0.12) rats when compared to the animals exposed to tomato juice (2.05±0.09) only nor those who were exposed to both nicotine and tomato juice (1.72±0.08).

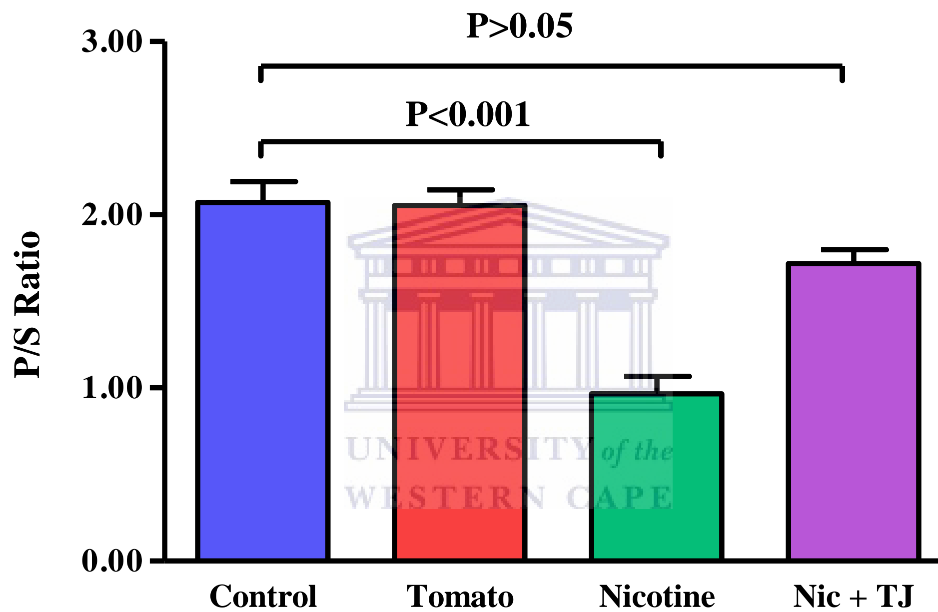


**Figure 3.7.4:** The effect of maternal exposure of the F1 off spring during gestation and lactation to nicotine, tomato juice, and nicotine together with tomato juice on the P/S ratio at postnatal day 84. Control vs. nicotine: P<0.001. No differences between the control, tomato juice and the combination of nicotine and tomato juice (P>0.05).

### 3.7.5 Alveolar fibroblasts

The data shown in figure 3.7.5 illustrates the fibroblast count (cells/100 μm) of the alveolar wall together with lesions resulting from exposure to nicotine and those exposed to nicotine with no emphysematous (NE) lesions. At postnatal day 84, whilst in direct comparison with the control animals, approximately a 50% (P<0.001) reduction in alveolar fibroblast number was observed between the control (15.94±1.07 cells/100 μm) animals and nicotine (7.05±0.62 cells/100 μm) animals with emphysematous lesions. Concomitantly, a marked decrease (P<0.001) was observed between the control (15.94±1.07 cells/100 μm) group and that of the nicotine (9.98±0.54 cells/100 μm) exposed group without any emphysematous lesions (NE). Similarly when comparing the nicotine (9.98±0.54 cells/100 μm) animals with no emphysematous lesions to the nicotine (7.05±0.62 cells/100 μm) animals with emphysematous lesions, a significant variation (P<0.05) between the respective groups was evident.

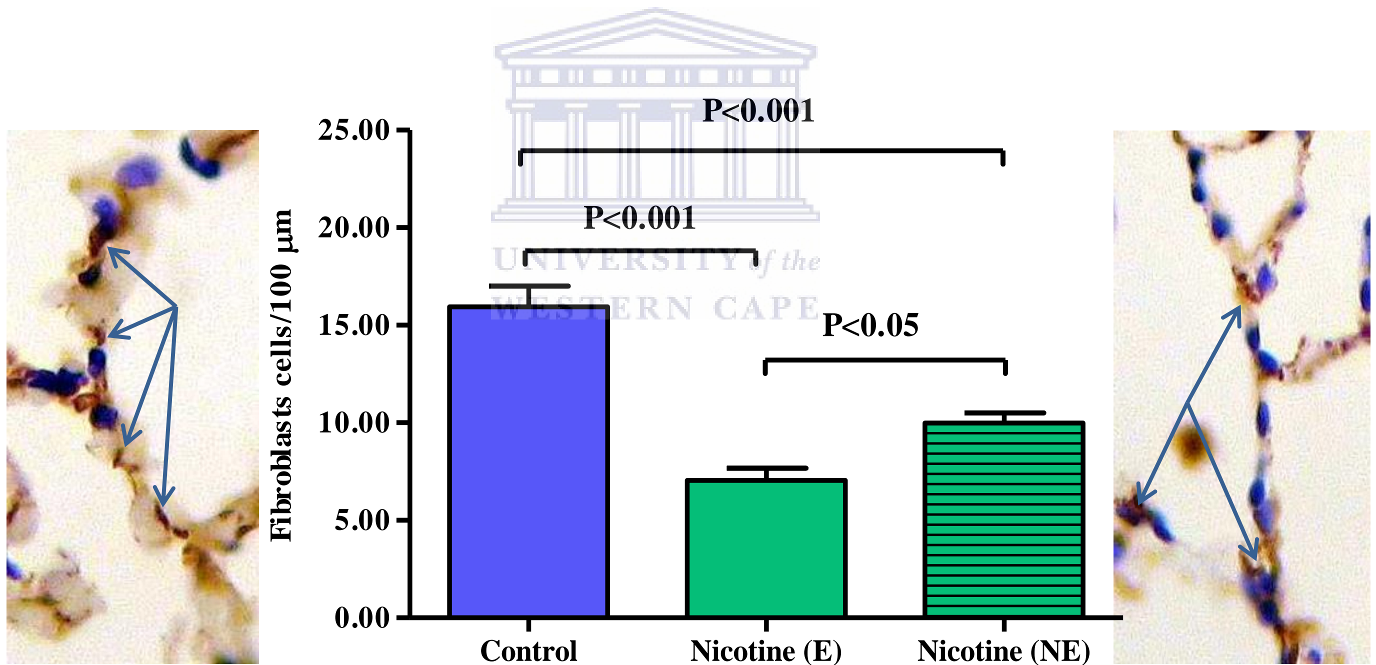


**Figure 3.7.5:** The effect of maternal nicotine exposure during gestation and lactation on the alveolar fibroblast number of the F1 offspring at postnatal day 84. Arrows= Fibroblasts; NE= Areas in the lung of the animals exposed to nicotine where no emphysema is evident. P at postnatal day 84; C vs. N (E): <0.001. Control vs. NE: P<0.001. NE vs. E: P<0.05.

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### 3.7.6 Alveolar Macrophage (AM) Number

Shown in Figure 3.7.6 are the alveolar macrophage counts (cells/80 $\mu m^2 \pm$SEM) in the alveoli at postnatal day 84. Although a trend is observed, no significant differences (P>0.05) was observed in the AM count between the control (0.20±0.02 cells/80 $\mu m^2$) and the nicotine (0.26±0.20 cells/80 $\mu m^2$) groups.

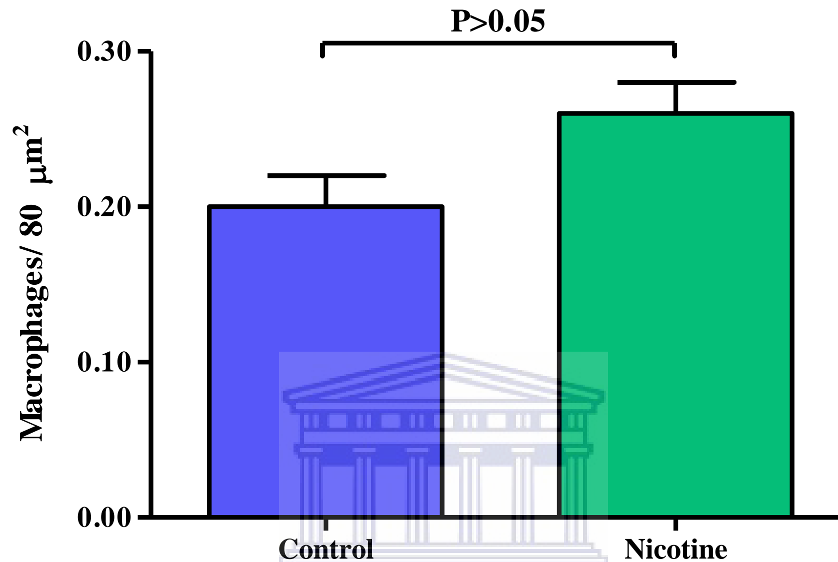


**Figure 3.7.6.1:** The effect of maternal nicotine exposure during gestation and lactation on the alveolar macrophage (AM) number in the lungs of the F1 offspring. C vs. N: P>0.05.

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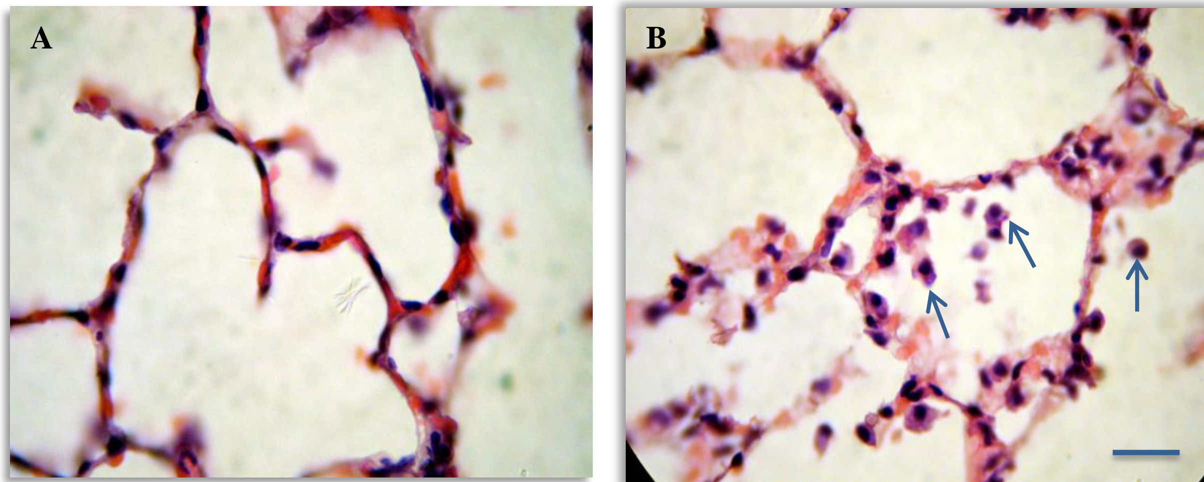


**Figure 3.7.6.2:** H & E stain of A) Control and B) nicotine exposed lung of 84 day old rats. Numerous free cells (Blue arrows) occur in the alveolus of the lungs that were exposed to nicotine via the placenta and mother's milk. (Bar = 2 μm)

### 3.7.7 Type II alveolar epithelial (ATII) cell number

The quantification of the number of Type II alveolar cells in the alveolar wall (Figure 3.7.7.1) showed that the number of Type II cells in the lungs of the control animals (0.38±0.02 cells/100 µm) was significantly ($P<0.001$) lower than that of the nicotine exposed lungs (0.57±0.03 cells/100 µm). This confirms the morphological observation in Figure 3.7.7.2.

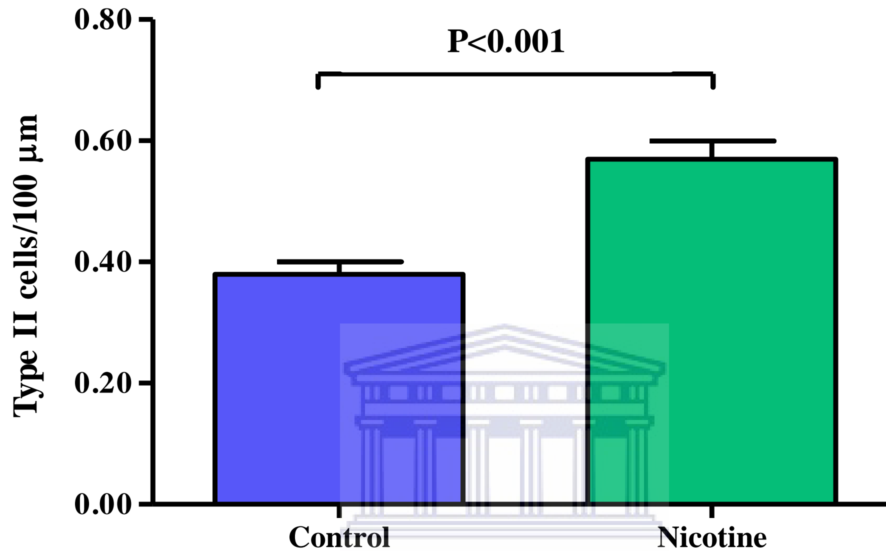


**Figure 3.7.7.1:** The effect of maternal nicotine exposure during gestation and lactation on the number of alveolar Type II cells in the lungs of the F1 offspring. P at postnatal day 84; C vs. N: <0.001.

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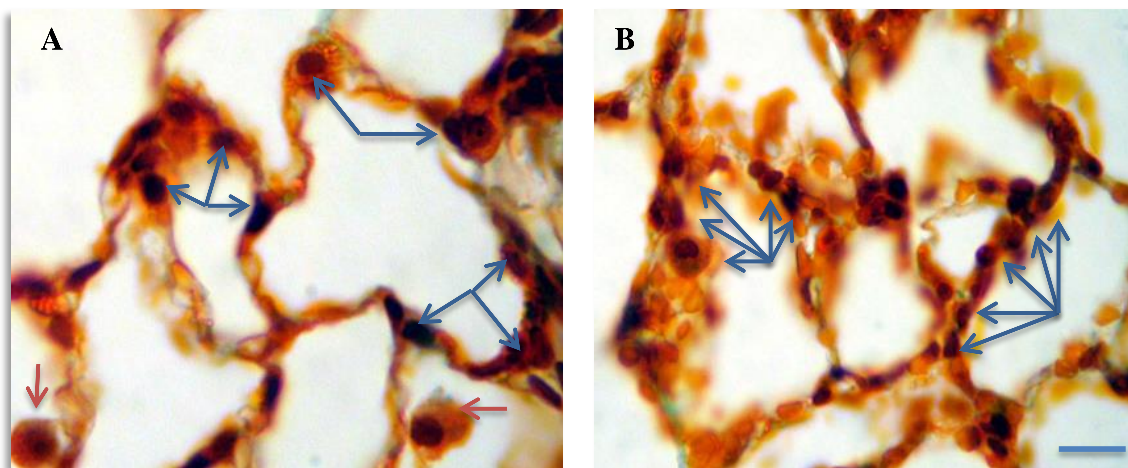


**Figure 3.7.7.2:** Type 2 alveolar epithelial cells (Blue arrows) in the lungs of control (A) and nicotine exposed (B) 84-day-old rats. Alveolar macrophages (Red arrows). The density of the T2 cells in the lungs of the nicotine exposed animals appears to be higher than in control rats. (Bar = 2 µm)

### 3.7.8 Type I alveolar epithelial cell integrity

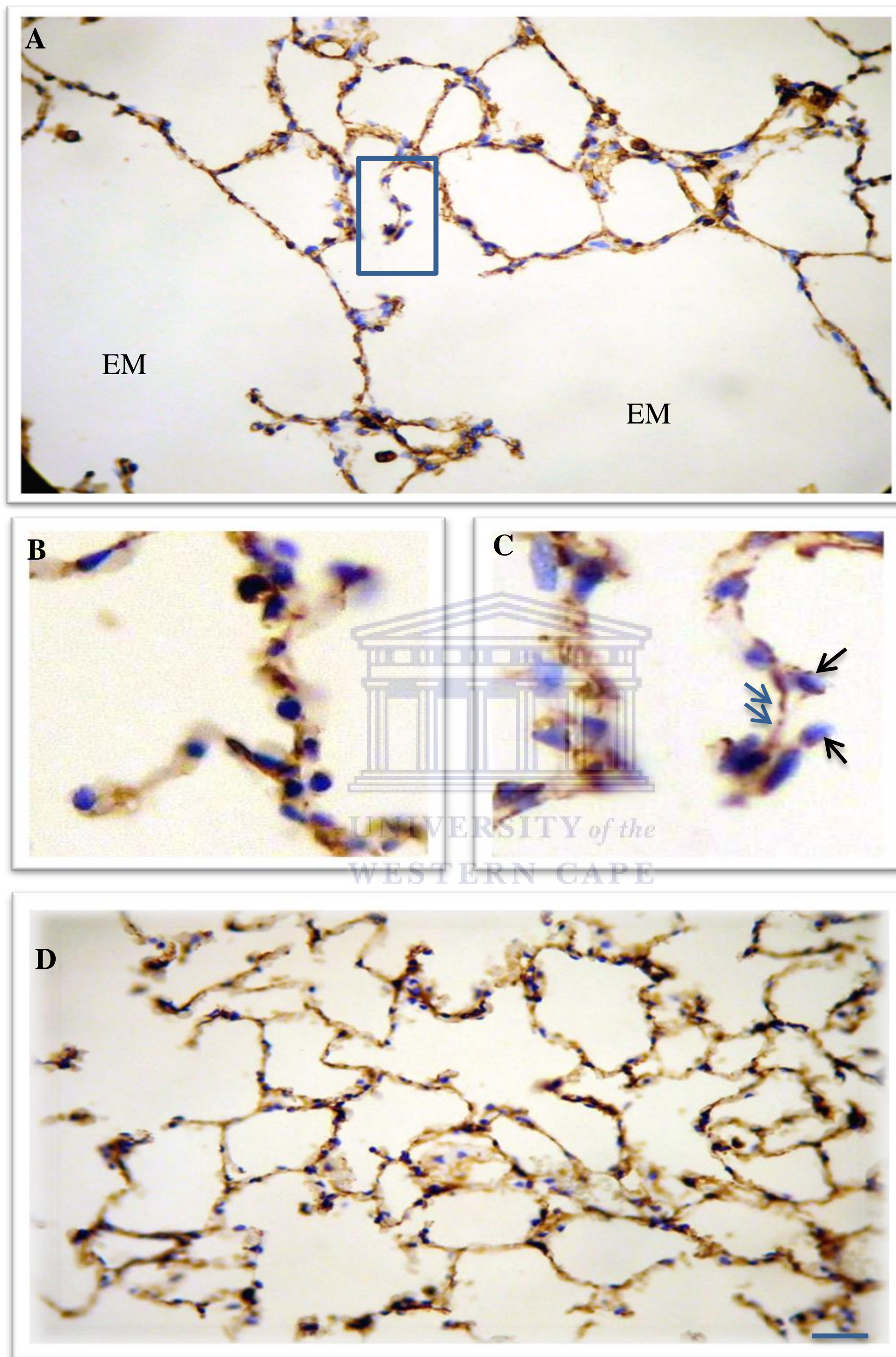


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**Figure 3.7.8:** Alveolar wall of a day-84-old nicotine exposed lung (A) with emphysema-like lesions (EM). All cells are in close contact with adjacent epithelial cells and the basement membrane. B) Alveolar wall of 84-day-old control lung showing an alveolar wall with intact cells whereas in the alveolar wall of the nicotine exposed rats (C) shows denudation (Black arrows) and exposure of the underlying connective tissue (Blue arrows). Cell tight junctions and linking to the basement membranes is compromised due to cells sloughing off. C) Block indicated where alveolar wall seen in C is taken from. (D) No EM lesions occur in the control lung.

### 3.7.9 Apoptotic cells

Figure 3.7.9.1 illustrates the number of apoptotic cells per 100 µm of the alveolar wall in the nicotine exposed, postnatal day 84 offspring. The number of apoptotic cells observed in the nicotine exposed offspring (0.29±0.05 cells/100 µm) was not significantly higher (P>0.05) than that of the control animals (0.21±0.02 cells/100 µm). This is evident from the micrograph in Figure 3.7.9.2

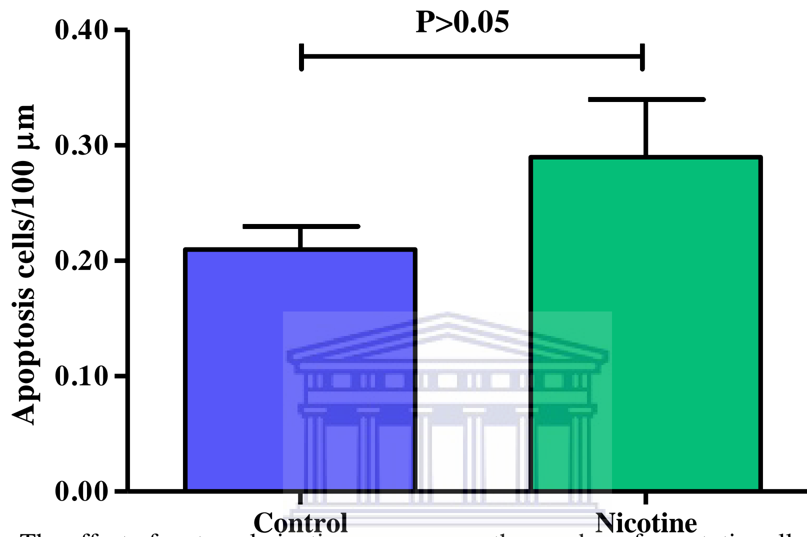


**Figure 3.7.9.1:** The effect of maternal nicotine exposure on the number of apoptotic cells in the alveolar walls of the F1 offspring at postnatal day 84. C vs. N: P>0.05.

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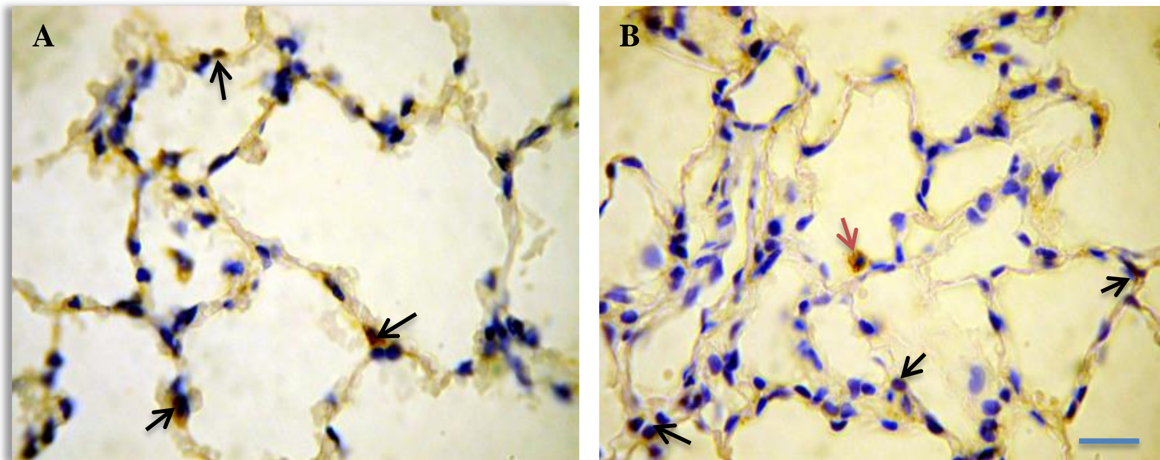


**Figure 3.7.9.2:** Apoptotic cells (Arrows) in the alveolar wall of 84-day-old control (A) and nicotine exposed (B) rats. Red arrow indicates an alveolar macrophage undergoing apoptosis. (Bar = 2 µm)

# CHAPTER FOUR
# *Discussion*

## 4.1 Introduction

Epidemiological studies have shown that cigarette smoking is the foremost cause of COPD such as emphysema and chronic bronchitis and is one of the leading causes of death worldwide (W.H.O., 2011, Zwerschke et al., 2003). Maternal smoking during pregnancy and lactation accounts for a significant percentage of fetal morbidity and mortality (Hammoud et al., 2005). Despite all the supporting evidence that maternal smoking is harmful to the unborn child (Schwartz et al., 1972), studies show that around 21% of all pregnant women in South Africa smoke during pregnancy (Steyn et al., 1997) and 17% of pregnant smokers were found to be in the Western Cape region (Chopra et al., 2007). As a result, a staggering 20 to 30% of all newborns exposed to tobacco smoke components either before or after birth, increases their susceptibility to respiratory diseases (Hofhuis et al., 2003a). Additionally, intrauterine exposure to tobacco smoke is the primary cause for a number of unpleasant outcomes including intrauterine growth retardation (IUGR), spontaneous abortion, sudden infant death syndrome (SIDS), preterm delivery and low birth weight (LBW) (Lieberman et al., 1994, Stocks and Sonnappa, 2013). Studies continue to show that nicotine on its own is harmful not only to the fetal lung, but other organs a well, including heart and nervous system (Argentin and Cicchetti, 2004, Kleinsasser et al., 2005). Numerous studies have indicated that diminished lung function owing to maternal exposure to tobacco smoke is prevalent in the offspring of smoking mothers. Consequently, the effects of maternal smoking during gestation and lactation on the offspring may even persist into later stages in life (Cunningham et al., 1994).

Nicotine is the chief psychoactive component found in tobacco smoke and owing to its addictive nature, results in the relapse of many of those who attempt to quit the habit. Nicotine readily crosses the placenta and interacts with the nicotinic receptors in the lungs of the developing fetus (Sekhon et al., 1999). Page-Sharp et al. (2003) have demonstrated that nicotine has been found to occur in considerable amounts in the milk of smoking mothers and more importantly, the concentration found in the human mothers' milk is 2 to 3 times higher than that of the plasma (Luck and Nau, 1984b, Dahlstrom et al., 1990). This leads to the interference of normal fetal lung development (Maritz et al., 1993). Additionally, nicotine and cotinine has been shown to be present

in the urine of 10 month-old non-suckling infants (Rylander et al., 1995) which suggests a passive uptake of nicotine. The uptake of nicotine in the present study was passive given that the offspring had received nicotine only via the placenta and mothers' milk.

## 4.2 Motivation for the use of 1 mg of nicotine/kg body weight/day

A regular pack of cigarettes contains 8 to 20 mg of nicotine and from that only 1 mg is absorbed when smoked. This means that from every cigarette stub containing approximately 1.2 mg of nicotine, around 0.1 mg of nicotine will be absorbed by the smoker. Human males and females who smoke have a daily nicotine intake ranging between 10.5 and 78.6mg (Kurtoglu et al., 2007). Assuming that 90% of nicotine is absorbed via inhalation (Duan et al., 1991), the nicotine intake of a 60kg female will vary between 0.16 and 1.18mg nicotine/kg body weight/day. Studies done by Benowitz et al. (1982) and Murrin et al. (1987) respectively demonstrated that when pregnant rats are treated with 1.5 mg of nicotine per day, the maternal plasma nicotine concentration is similar to the values observed in human smokers. In the present study 1 mg nicotine/kg pre-pregnancy weight/day was used and was thus within the scope of the nicotine intake of routine smokers. Since nicotine readily transcends the placenta and occurs in the milk of both the human and rat mother (Maritz and Harding, 2011a), like in humans, the fetal rats will be exposed to nicotine via the placenta and therefore it is conceivable that the nicotine content of the female rats was of the same magnitude as it is for humans. This indicates that in all likelihood, both perinatal and postnatal exposure to nicotine of the rat pups resembled that of the human offspring. Assuming that rats respond to nicotine in the same manner as humans, the data produced in the present study can be expected to be similar as the effects of nicotine, inhaled by smokers, on the offspring. It should also be noted that pregnancy has several variable effects on drug metabolism; hence the plasma nicotine concentration determined subsequent to birth may not mirror the actual concentrations throughout different periods of pregnancy, specifically during critical windows of organogenesis. The juvenile antioxidant capacity of the fetal rat lung renders it highly susceptible to the effects of nicotine exposure via the placenta resulting in radical-mediated cell injury (Ziegler et al., 2014, Yoon et al., 2006). Owing to the slow metabolism of the developing fetus, the half-life of nicotine will be much longer in the lungs of the offspring (Maritz and Harding, 2011b). This suggests that the metabolic processes affected by nicotine will be extensive in comparison to that of the adult.

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## 4.3 The effect of nicotine, tomato juice only, and of both nicotine and tomato juice on the body weight and litter size of the pregnant and lactating mothers.

Smoking during pregnancy is the main cause of increased prenatal morbidity and mortality (Kramer, 1987, Hammoud et al., 2005). The harmful constituents found in cigarette smoke, such as nicotine, and a number of other harmful compounds, limit the supply of oxygen and vital nutrients via the placenta. This results in several undesirable outcomes such as spontaneous abortion, preterm birth, sudden infant death syndrome (SIDS), intrauterine growth retardation (IUGR) and as a consequence, low birth weight (Lieberman et al., 1994, Salihu and Wilson, 2007, Gluckman et al., 2008). Studies indicate that a positive correlation exists between low birth weight and fetal onset of adult disease including coronary heart disease, type 2 diabetes, and adiposity (Siu and Tyndale, 2007). As a result, smoking mothers have been strongly encouraged to quit the habit. Several pharmacotherapies such as nicotine replacement therapy (NRT) have been advised by health professionals to treat nicotine dependence. Although claims exist that NRT is harmless (Zwar et al., 2006), numerous studies have demonstrated that insistent use of nicotine promotes lung carcinogenesis unimpeded by other associated tobacco alkaloids (Dasgupta and Chellappan, 2006, Ruiz, 2006). Furthermore, it is not desirable to use nicotine in any form as it crosses the placenta and accumulates in the developing fetus (Maritz, 2009).

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It has been suggested that IUGR is a consequence of restricted placental blood flow and thus nutrient supply to the developing fetus (Abrams et al., 2000). If so, smoking or NRT will result in IUGR and low birth weight: it is therefore conceivable that the litter sizes of the nicotine offspring will be smaller than the control animals. However, in this study the number of rat pups per litter was the same for the control and experimental groups. Furthermore, as demonstrated in the present study, maternal nicotine exposure had no effect on the body weight of the offspring. This implies that blood and thus nutrient and oxygen supply to the growing fetuses was sufficient and met the demands of the growing fetuses.

Maternal weight gain during pregnancy is a cardinal indicator of both maternal and infant health (Abrams et al., 2000) and deficient gestational weight gain may result in short, mid, or long term implications on maternal and pediatric health (Strauss and Dietz, 1999, DeVader et al., 2007, Langford et al., 2011). Gestational weight that is well within the recommended range plays a crucial role in reducing adverse outcomes such as preterm birth, low birth weight, and IUGR

(DeVader et al., 2007, Frederick et al., 2008, Langford et al., 2011). Contrary to this, excessive gestational weight gain has been associated with several complications including fetal macrosomia, hypertensive syndromes during pregnancy, cesarean section birth, and hemorrhages (Rush, 1974, Davies et al., 1976, Rantakallio and Hartikainen-Sorri, 1981).

The findings in the present study showed that the exposure of pregnant rats to nicotine, tomato juice, or the combination of nicotine and tomato juice had no effect on the body weight of the pregnant rats during weeks 1 and 2 of gestation. However, the BW of the pregnant rats administered with nicotine increased much faster than the control and other experimental groups so that at gestational week 3, it was significantly higher. Additionally, the increase in the BW of the mother treated with nicotine cannot be due to an increase in amniotic fluid volume. Maternal smoking, and therefore by implication, nicotine intake, seem not to affect the amniotic fluid volume nor fetal urine output (Burn et al., 1945). Although no evidence exists for the direct effect of this, it appears that cigarette smoke and nicotine stimulates the hypothalamus to release antidiuretic hormone (ADH) from the posterior lobe of the pituitary gland, causing the inhibition of diuresis (Dalessio, 1969) and consequently a shift in favour of an increase in water accumulation in the body of the mother. Furthermore, the relationship of nicotine intake and idiopathic edema has also been documented (Finch et al., 2004). It is therefore plausible that the continuous exposure to nicotine may result in the constant overproduction of ADH and a gradual buildup of water in the bodies of the pregnant rats and therefore an increase in BW. Given that the fluid intake of the pregnant rats treated with nicotine was the same as the other groups, it could not have influenced the increase in BW. Following birth, the persistent effects of ADH is withdrawn and subsequently control of the water balance to normal in the bodies of these animals. This is evident from the fact that the BW of the nicotine treated mothers resembled that of the control and other experimental groups after birth. Notably, the supplementation of tomato juice in the pregnant rats' diet averted the increase in BW at week three of gestation; however, this mechanism whereby it countered the effect of nicotine is not known. Since the litter sizes were unaffected by maternal nicotine intake and was thus is not implied in the BW increase of the nicotine treated pregnant mothers.

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## 4.4 The effect of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the development of the F1 offspring.

### 4.4.1 Body weight (BW)

Studies have reported that maternal nicotine exposure causes a lower birth weight and body fat of the offspring (Grove et al., 2001). This indicates that nicotine exposure during pregnancy may result in the interference with the growth of the fetus and offspring leading to intrauterine growth retardation (IUGR). Therefore, maternal nicotine exposure during pregnancy is expected to suppress fetal growth resulting in lower birth weight of the offspring (Birnbaum et al., 1994, Philipp et al., 1984). In the present study, the body weights (BW) of the offspring of mothers who were exposed to nicotine during gestation and lactation were not different from that of the control animals. This suggests that growth and development were the same for at least up to postnatal day 84. This suggests that all metabolic and growth control mechanisms were alike. This, in all likelihood, explains why maternal exposure to nicotine during gestation and lactation had no effect on the body weight of the offspring.

Contrary to the present study's findings, Andres and Day (2000) have previously suggested that smoking during pregnancy induces intrauterine growth restriction (IUGR). Given that the risk of IUGR is significantly greater in females who smoke cigarettes with a higher nicotine content (Bainbridge and Smith, 2006), it is conceivable that nicotine is the causative factor. Furthermore, it is plausible that placental perfusion during pregnancy will be compromised by maternal smoking as a result of nicotine-induced vasoconstriction in the placenta (Economides and Braithwaite, 1994). However, contrary to this, Bainbridge and Smith (2006) demonstrated that nicotine does not influence the feto-placental blood supply. Hence, it is questionable that the nutrient supply to the developing fetus will be compromised by nicotine. In support of the latter, studies done by Maritz and Windvogel (2003) showed that the exposure of pregnant rats to nicotine had, like in the present study, no effect on the body weight of the offspring. In fact, any contribution to IUGR in smokers is not at the level of the feto-placental perfusion but rather at the level of placental function and/or uterine function (Maritz and Windvogel, 2003).

Moreover, this is well substantiated by the fact that maternal smoking alters critical characteristics of placental function including, progesterone production (Piasek et al., 2001); estrogen metabolism (Zhu et al., 2002); amino acid transport (Pastrakuljic et al., 1999); and the location and activity of

drug metabolizing enzymes (Zdravkovic et al., 2005). However, in the present study, and at the concentration used, nicotine had no such effect as tobacco smoke in addition to nicotine; also contains a variety of other foreign substances.

On the other hand, Philipp and co-workers (1984) and Birnbaum et al. (1994) demonstrated that with the use of relatively high concentrations of nicotine, namely 3 and 6 mg nicotine/kg body weight/day respectively, causes vasoconstriction in the placenta and may result in a decrease of the nutrient supply to the fetus. Consequently this will result in a lower birth weight of the offspring. This is unlike the effect of 1 mg nicotine/kg BW/day. This suggests that uterine vasoconstriction occurs at higher nicotine concentrations with a consequent lower BW at birth. In the present study, maternal exposure to nicotine during gestation and lactation had no effect on the body weight of the offspring.

As shown by Benowitz et al. (1982) that when pregnant rats received 1.5mg nicotine/kg body weight/day, the plasma nicotine concentration values were similar to that of human smokers. It is possible that perhaps the treatment of 6 and 3mg of nicotine/kg body weight/day may have been too steep. Benowitz et al. (1982) and Murrin et al (1987) established that the birth weight of the offspring, litter size, and postnatal weight gain was unaffected. Thus, the present study is consistent with the findings of Benowitz et al (1982) and Murrin et al (1987), as the nicotine dosage used in the present study was in accordance with that consumed by human smokers and thus had no effect on the body weight of the offspring. Given that nicotine was only dispensed once per day, it is possible that the amount of time placental vasoconstriction lasted might not have been long enough to cause a decrease in nutrient supply, and therefore no significant influence on the growth of the fetus. On the other hand, nicotine accumulates in the fetus due to a slow degradation thereof by the fetal tissue (Maritz and Harding, 2011b) and it is therefore conceivable that it will still impact on the development of the fetus despite a short period of placental vasoconstriction and subsequently nutrient supply for that period of time.

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These results stand agreement with previous studies in which it was also showed that maternal nicotine exposure did not affect growth parameters such as chest circumference, crown-rump length, and body weight of the offspring (Maritz et al., 2011b, Maritz and Windvogel, 2003, Pausová et al., 2003).

Studies done by Fowden and colleagues (2006) revealed that the duration, severity, and type of insult can affect intrauterine growth. This supports the study done by Philipp et al. (1984) and Birnbaum et al. (1994) namely that by increasing the nicotine intake will result in low birth weight of the offspring due to the high nicotine concentrations. However, as shown by the present study, although proportional growth in the offspring was not affected by maternal nicotine exposure during gestation and lactation, it does not suggest that maternal nicotine had no effect at a cellular level and on the homeostatic mechanisms of the offspring.

The present study also established that the CC/CRL ratios as well as the CC/BW ratios were unaffected by the maternal exposure to nicotine during pregnancy and lactation. This implies that nicotine had no effect on the proportional growth of the offspring. It is interesting that the CC, which is an indicator of lung volume (Borkan et al., 1981), of the nicotine exposed rats were increased at day 84 which suggest that the lung volume in the nicotine exposed offspring was increased as well.

### 4.4.2 Lung volume (Lv)

Despite the fact that the BW and growth parameters of the offspring was unaffected by maternal nicotine exposure during gestation and lactation, the lung volumes (Lv) of the 84-day-old animals that were exposed to nicotine were indeed higher than the control and the other experimental groups. This increase in lung volume was evident after postnatal day 42 and gradually became more significant as it reached postnatal day 84. Approximately a 20% increase in Lv was observed at postnatal day 84. Gehr et al. (1981) showed that bigger animals require a larger internal surface area in the lungs to adequately supply oxygen to meet the higher demand for oxygen by the larger body. The increase in CC and lung volume at postnatal day 84 was not a response to an increased demand for oxygen due to an increase in BW because the BW was not different from that of the controls of the same age. The larger lung volumes were in all likelihood due to a gradual deterioration of the connective tissue framework of the lung later in the life of the offspring. The gradual increase in lung volume later in the life of the nicotine exposed rats can possibly be the reason for the gradual increase of the chest circumference of these animals as seen in emphysema (Stanley J. and Swierzewski, III, MD., 2000). A gradual deterioration of the connective tissue framework may result in an increase in the compliance of the lungs of the offspring that were exposed to nicotine via the placenta and mothers' milk.

The present study also demonstrated that between postnatal days 14 through 42 the Lv/BW ratios of the control and experimental animals were the same. However, following postnatal day 42 through 84 the Lv/BW ratios of the nicotine exposed offspring were higher than that of the control animals. The increase in Lv/BW ratios of the postnatal 42 and 84 day old rats exposed to nicotine is a clear indication of the disproportionate increase in lung volume of these animals. However, even though the CC of the 84-day-old nicotine exposed rats was higher than that of the control rats of the same age, it is unlikely that the difference in CC/Lv can be attributed to disproportional growth, but rather to a change in the compliance of the lungs of these animals. The supplementation of tomato juice in the mother's diet prevented the faster increase in Lv of the lungs of the nicotine exposed rats. The increase in Lv of the nicotine exposed rats was more evident as they aged suggesting that it was programmed during the fetal and early neonatal stages to increase faster later in life.

## 4.5 The effect of maternal exposure to nicotine and tomato juice on lung morphometry and morphology of the F1 offspring

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### 4.5.1 Lung structure

In the present study, nicotine administration to the F0 generation commenced 24 hours after mating. The F1 generation therefore only received nicotine via the placenta and mothers' milk. Nicotine exposure occurred during all phases of lung development *in utero*, that is during the phases of rapid alveolarisation (postnatal days 4-13) (Windvogel, 2006a), the phase of equilibrated lung growth (postnatal days 14-21) (Schittny and Burri, 2008), and terminated at weaning which is postnatal day 21. During rapid alveolarisation, the number of alveoli increases with a concomitant decrease in the size of the alveoli. This means that the tissue volume ($V_t$) of the lung will increase as it matures and air volume ($V_a$) is not equated to lung volume (LV). The $V_t$ calculated in this study demonstrated that the lungs of all the groups in fact did increase between postnatal days 21 and 84. However, between postnatal days 42 and 84, the $V_t$ of the nicotine exposed animals was considerably lower than that of the control animals. Typically, when a decrease in $V_t$ occurs, a concomitant increase in $V_a$ will be observed; the late onset of the decline in tissue density with a simultaneous decrease in $V_a$ is an indication that the lungs of these animals were programmed to deteriorate later in life. This is also an indication of an increased susceptibility to stress damage later in the life of these animals. In contrast to the control, tomato juice only and both nicotine and tomato juice; the alveoli observed

in the nicotine exposed progeny appeared to be larger in size but fewer in number. This marked reduction in $V_t$ seen in the nicotine exposed rats can be ascribed to a gradual destruction of the alveoli and the formations of emphysema-like lesions.

The breakdown of the alveolar walls and the increase in static compliance suggests that the connective tissue framework was compromised later in the life of the rats that were exposed to nicotine via the placenta and mother's milk. Hence, it follows that the alveolar volume of the nicotine exposed animals will be greater in comparison to the control rats. This is supported by the higher Mean Linear Intercept (Lm) of the nicotine exposed progeny. It should especially be noted that the decrease in $V_t$ accompanied by an increase in Lm only became evident after postnatal day 42 which suggests that the lungs of the nicotine exposed offspring was programmed to start deteriorating later in the life of the offspring. This suggests that up to postnatal 42, the tissue volume as well as the alveolar volume and number in the lungs of the F1 progeny was not significantly affected by nicotine exposure during gestation and lactation.

Additionally, the destruction of alveoli is shown by the Lm which in fact gradually increased following postnatal day 21. This means that even though the difference in $V_t$ only became apparent after postnatal day 42, the diameter of the alveoli had already increased and thus showing early onset of microscopic emphysema (Collins et al., 1985, Elliot et al., 2001). Light micrographs have indeed demonstrated emphysema-like lesions in the lungs of the nicotine exposed rats following postnatal day 42. Furthermore, one should note that tomato juice intake by the mother inhibited the effect of nicotine on $V_t$ in the lungs of the offspring.

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Since the $V_t$ of the nicotine exposed offspring gradually decreased after postnatal day 42, a thinning of the alveolar wall was expected to ultimately give rise to alveolar wall destruction observed during parenchymal damage. Maritz and Windvogel (2005) indeed showed that at postnatal day 21, the alveolar walls or interalveolar septal thickness (Tsept) of the nicotine exposed animals were thinner than the control animals of the same age. However, contrary to these findings, in the current study an increase in Tsept of the postnatal day 84 nicotine exposed rats was observed and can be ascribed to an imbalance in the synthesis and degradation of the alveolar wall components. Since the thickening of the alveolar wall became apparent only later in the life of the nicotine exposed offspring, it is plausible that the homeostasis of the metabolic processes that are geared to maintain the integrity of the alveolar wall was compromised.

Since the $V_t$ of the nicotine exposed animals decreased despite the increase in Tsept, it is likely due to faster deterioration of the lung parenchyma, and thus $V_t$. Since the offspring only received nicotine via the placental blood and mothers' milk, these changes are unlikely as a result of the direct effect of nicotine or its metabolites. This is verified by the fact that the half-life of nicotine is around 90 minutes (Benowitz et al., 1982) and the primary metabolite, cotinine, is 16 to 19 hours (Jarvis et al., 1988). It is also unlikely to be as a result of an oxidant-antioxidant imbalance at this relatively late stage of lung growth seeing as these alterations in the alveolar region of the rats' lungs occurred 3 to 9 weeks post nicotine exposure, and all the metabolites were expelled from the lungs of the F1 progeny. These changes are rather due to a change in the factors that maintain lung integrity as the animals age.

Since nicotine is genotoxic (Kleinsasser et al., 2005) and has been shown that long-term exposure gives rise to an inclination towards genetic instability (Hartwell and Kastan, 1994, Sastry et al., 1998, Guo et al., 2005a), it is highly likely that maternal nicotine exposure during phases of rapid cell proliferation and differentiation may in fact result in changes in the "program" that controls lung development, maintenance of lung integrity, and aging of lung tissue which may render the lungs more susceptible to disease (Maritz et al., 2011b, Maritz and Windvogel, 2003). The increase in the alveolar volume as well as alveolar wall thickening indeed point to the development of emphysema-like lesions in the lung parenchyma. These collectively are all signs of premature lung aging (Bruin et al., 2008a) and negatively impacts the function of the lung as a gas exchanger.

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### 4.5.2 Tomato juice supplementation

The present study showed that by supplementing the diets of the pregnant rats with tomato juice during gestation and lactation inhibited the unfavourable effects of nicotine on the lungs of the F1 progeny. Since tomato juice is rich source of lycopene and other antioxidants, it is plausible that the daily intake of tomato juice augmented the antioxidant status of the mother as well as the offspring, and thereby the capacity to guard itself against exogenous substances such as nicotine. Even though lycopene is the primary bioactive component found in fresh tomatoes and tomato products (Takeoka et al., 2001), it is possible that other antioxidants and phytonutrients in tomato juice may have contributed in protecting the developing fetal lung against the deleterious effects of nicotine.

As for the exposure of nicotine, tomato juice supplementation took place during the phases of lung development when cell turnover is rapid as well as when the release of oxidants under the influence of nicotine (Zhao et al., 2006) was conceivably at its peak. From the present study, it is evident that tomato juice, and presumably lycopene and other phytonutrients found in tomato juice, safeguarded the lungs during phases of rapid lung growth when it is most sensitive to the adverse effects of foreign materials, such as nicotine. Studies done in humans demonstrated that lycopene alone prevented DNA damage (Neyestani et al., 2007) as well as cell membrane oxidation *in vivo* . It is therefore possible that the lycopene in tomato juice improved the protection mechanisms of the mother and fetus to such an extent that it resembled the lungs of the control animals. Furthermore, based on the data in the current study, and directly extrapolating from rats to humans, a daily intake of 250 ml (approximately one and a half glasses) of tomato juice will contain enough lycopene, phytonutrients, and other antioxidants such as vitamin C to protect the fetus in a 60 kg human female.

## 4.6 The effects of maternal nicotine exposure on the cellular integrity of the alveolar walls in the lungs of the F1 offspring.

Morphometric data showed that exposure of the fetus and neonate to nicotine via the placenta and mother's milk interfered with the homeostatic mechanisms that are essential to maintain the structural and functional integrity of the lungs of the offspring throughout life. Since these changes in the lung parenchyma were only evident later in the life of the nicotine exposed offspring, it was suggested that these changes were programmed in utero when the developing organs were most sensitive to the effects of the environment. Any changes in the metabolism, such as the disturbance of the balance between synthesis and breakdown of components of the extracellular matrix with the consequent thickening of the alveolar walls at postnatal day 84, implies that the cellular components of the alveolar wall, such as fibroblasts, were involved. It was thus important to investigate the cell integrity of the alveolar walls, such as fibroblasts, type I and –II cells and even the alveolar macrophages that occur free in the alveoli. This will help to establish whether the cellular composition of the parenchyma was affected. Any change in the composition will be an indication of the site of action of nicotine as well as of the consequences since each cell type plays a unique role in maintaining the parenchymal integrity of the lung in the long term. The impact of

maternal nicotine exposure on these cells was only investigated at postnatal day 84 since that is when most of the changes in lung structural integrity became evident.

### 4.6.1 Apoptosis and cell proliferation

Lung development and maintenance necessitates a very dynamic process of cellular replication and apoptosis (Aoshiba and Nagai, 2009), especially since the lung is exposed to many exogenous substances such as atmospheric pollutants particularly in smokers. Apoptosis, or programmed cell death, plays a crucial role in numerous physiological processes during fetal lung development and in adult tissue. This means that the relationship between cell death and cell replacement is tightly controlled to ensure maintenance of lung structure and function. Although many hypotheses for the development of emphysema have been put forward including the proteinase-antiproteinase theory, Calabrese and colleagues (2005) have proposed that an *apoptosis-proliferation imbalance* contributes to the development of emphysema. It was indeed shown that apoptosis and cell proliferation does not increase at the same rate (Imai et al., 2005). Consequently, this results in a loss of cells in the alveolar walls during emphysema (Segura-Valdez et al., 2000) and subsequent deterioration of parenchymal tissue. Banerjee et al. (2007) has indeed demonstrated that smoking increases apoptosis with a concomitant decrease in cell proliferation which contributes to emphysema as a smoker ages. Although it is said that nicotine deregulates essential biological processes like apoptosis and angiogenesis (Zeidler et al., 2007), it was evident from the present study that nicotine exposure via the placenta and mothers' milk had no significant effect on the apoptosis in alveolar walls of the F1 offspring however, has the tendency to be higher. It is therefore not likely that the destruction of alveolar walls and the consequent increase in alveolar volume could solely be ascribed to an *apoptosis-cell proliferation imbalance* in the lungs of the nicotine exposed progeny.

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### 4.6.2 Cellular proliferation and cellular senescence

Based on the findings of the present study it is apparent that maternal nicotine exposure during gestation and lactation reduced the rate of cellular proliferation in the alveolar wall of the F1 offspring. This was accompanied by an increase in the number of senescent cells in the alveolar walls of the nicotine exposed F1 progeny in comparison to that of the control animals. Furthermore,

this resulted in a P/S ratio in the lungs of the nicotine exposed offspring that was significantly lower than that of the control and other experimental groups. The skewed ratio in the lungs of the nicotine exposed animals during gestation and lactation indicates a *cell proliferation-cell senescence* imbalance. The shift in cell proliferation-cell senescence balance in favour of senescence will compromise the lungs ability to replace damaged and/or dead cells thus causing a gradual loss of alveolar wall integrity. Since the exposure of nicotine via the placenta and mothers' milk had not increased apoptosis in the lungs of the nicotine exposed F1 offspring, it follows that apoptosis did not influence the imbalance in cell death and cell replacement in the alveolar walls. However, due to accelerated cellular senescence accompanied by a slower cell turnover, it is inevitable that that alveolar wall destruction will occur. These findings furthermore suggest that exposure to nicotine during gestation and lactation caused the lungs of the F1 offspring to age prematurely.

The imbalance between cellular damage and repair became evident relatively late in the life of the F1 progeny and seems to be irreversible. It is therefore highly likely that maternal exposure during gestation and lactation at critical windows of lung development induced developmental modifications at a cellular level which contributed to perpetual physiological, structural, and epigenetic alterations in the lungs of the F1 offspring resulting in micro emphysema-like lesions later in life. As a result of impaired repair mechanisms in the lungs of the nicotine exposed animals, it is conceivable that the lungs of the offspring will be deemed more vulnerable to stress induced damage such as atmospheric air pollutants.

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The mechanism whereby the exposure of the F1 offspring to nicotine during pregnancy and lactation caused an imbalance in the *cell proliferation-cell senescence* is likely owing to oxidative damage stimulated during the early stages of lung growth. By treating the mothers' with nicotine, it reduces the antioxidant capacity of the mother and fetal lung. Therefore, it increases the oxidant load of both mother and fetus (Durak et al., 2002) and thus expose the developing lung to higher levels of oxidants. Furthermore, the increase in oxidant load and compromised antioxidant capability or low antioxidant levels will thus predispose the rapidly proliferating cells of the lungs of the offspring to programming. This is substantiated by the observation that the intake of tomato juice by the mothers' treated with nicotine averted the alterations in the cellular processes involved the alveolar walls of the offspring. This may be due to the maintenance of the mothers' antioxidant status and by implication, the offspring, giving rise to an augmented defense against oxidant induced programming in the lungs of the F1 offspring.

So far the data shows that overall cell proliferation was suppressed. At the same time the senescent cell numbers per length of alveolar wall increased. This implies that the accumulation of senescent cells will also contribute to fewer cells that can proliferate. The data so far represents an overall picture of cell senescence and proliferation of all parenchymal cells. It is important to establish what the response of specific individual cells in the alveolar walls to nicotine exposure is in order to determine the site of action of nicotine and the consequences on lung structure and function in the longer term. Fibroblasts, type I and –II cells density (numbers/length of alveolar wall) of the alveolar walls were determined, as well as that of macrophages in the alveoli since there is communication between all these cells.

### 4.6.3 Fibroblast Density

Cigarette smoke has shown to have cytotoxic effects on lung fibroblasts (Ishii et al., 2001) which have led to the induction of apoptosis of these cells (Carnevali et al., 2003, Baglole et al., 2006). Pulmonary fibroblasts are the primary cell type responsible for synthesis and secretion of the main components of the alveolar ECM (Koh et al., 1996) which plays a major role in the connective tissue framework of the lung. Moreover, pulmonary fibroblasts have been said to participate in alveolar growth and multiplication (Warburton and Bellusci, 2004).

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As shown in this study, the fibroblast density was markedly reduced in the alveolar walls of the lungs of the 84-day-old F1 offspring exposed to nicotine in utero and during lactation in comparison to that of the control animals. It is also interesting to note that while a significant decrease in the fibroblast density was seen between the control animals and the nicotine exposed offspring, the fibroblast density in areas where emphysematous-like lesions were present, was significantly lower than in the areas where no emphysematous-like lesions were observed. This implies that the lower fibroblast density is associated with emphysema-like lesions. It is plausible that the fibroblasts are sensitive to the effect of nicotine and develop premature senescence and become unable to proliferate as the lungs grows and develop. Since alveolar fibroblasts secrete growth factors that target both alveolar epithelial and endothelial cells (Plantier et al., 2007) one would anticipate that due to injury, the fibroblast activity would be increased in areas where alveolar damage has occurred. Nobukuni et al. (2002) demonstrated that cigarette smoke inhibits pulmonary fibroblast proliferation and migration. The data in the current study indicate that nicotine in tobacco smoke contributes to the suppressed fibroblast proliferation in the lungs of

smokers. It is therefore conceivable that this may be the reason as to why the fibroblast density was: 1) distinctly reduced in the lungs of the nicotine exposed F1 offspring, and 2) a further reduction of fibroblasts in areas with emphysematous-like lesions. Moreover, as for the effect of maternal nicotine exposure on other cell types, chronic exposure to cigarette smoke results in a senescent fibroblast phenotype (Nyunoya et al., 2006). This implies that maternal nicotine exposure during gestation and lactation probably induces a decrease in the number of metabolically active fibroblasts available for alveolar multiplication and growth via premature origins of these cells. This is especially evident later in the life of the nicotine exposed offspring and exacerbated in regions where emphysema-like lesions has developed. Due to financial constraints, we were unable to demonstrate whether the supplementation of tomato juice in the diet of the mothers' treated with nicotine would avert the deleterious effects on pulmonary fibroblasts. However, if directly extrapolated from previous results shown in the present study, we can safely assume that the supplementation of tomato juice in the mothers' diet may have prevented the reduction in fibroblast activity, but this would require further study.

### 4.6.4 Alveolar macrophages (AM)

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The findings in the present study show that maternal nicotine exposure during gestation and lactation had no effect on the alveolar macrophage (AM) number in the lungs of the nicotine exposed offspring at postnatal day 84. AM occurs free in the alveoli and plays a fundamental role in the protection of lung integrity. Although the number in AM were unaffected, it is likely that the metabolic activity of the AM were affected. In support of this are studies done by Wongtrakool and co-workers (2012) that demonstrated that nicotine suppresses the innate immune response of these cells to foreign particles that enter the alveoli. It is therefore plausible that although the alveolar macrophage numbers were not affected by maternal nicotine exposure during gestation and lactation, the innate immunity of these lungs might be affected since it is plausible that more alveolar macrophages are senescent than in the control lung and thus not effective in protecting the lung. This probably explains why in utero nicotine exposure, mediated in part via the alpha-7 nicotinic acetylcholine receptor (α-7 nAChR), and increases the risk of lower respiratory tract infections in neonates due to a lower phagocytic function (Wongtrakool et al., 2012).

### 4.6.5 Alveolar Type I (ATI) and Type II (ATII) cells

Exposing the fetus to nicotine via the placenta and mother's blood resulted in enhanced alveolar type II (ATII) proliferation in the lungs of the day 84 offspring. These findings are consistent with that of Maritz and Thomas (1995). It is however, important to note that the faster type II cell proliferation in the latter study took place while the mother was still exposed to nicotine and the offspring therefore still receiving it via mother's milk. In the present study the type II cell proliferation was still markedly faster in the 84-day-old nicotine exposed offspring than that of the control animals despite the fact that it was not exposed to nicotine since weaning on postnatal day 21. This implies that type II cell proliferation remains high in these rats even though there is no nicotine present in the lungs at this late stage. It is therefore highly likely that this is a programmed process and in all likelihood gave rise to a permanent faster type II proliferation.

Whether the rapid Type II proliferation in the alveolar walls of the nicotine exposed offspring can be attributed to a direct effect of nicotine during gestation and lactation on the type II cells is not known. There are however 2 possible mechanisms that may induce the observed proliferation of type II cells in this and other studies. Firstly, Rehan et al. (2007) reported that ATII cell proliferation in nicotine exposed animals is a result of the indirect effect of nicotine, that being a paracrine mechanism(s) secondary to its effects on adepithelial fibroblasts. Alveolar interstitial fibroblasts (AIF's) have been shown to express the peroxisome proliferator activated receptor (PPAR)-γ. The PPAR-γ is reported to have an antimitogenic role in other systems, where it is has been shown to impede cell proliferation by regulating the activation of cyclins and cyclin dependent kinases (Law et al., 2000). AIF's that are situated adjacent to ATII cells express PPAR-γ (Rehan et al., 2006) and nicotine downregulates PPAR-γ by these fibroblasts (Rehan et al., 2005). Furthermore, from the observations that nicotine exposure during gestation and lactation markedly reduced the fibroblast activity in the lungs of the F1 offspring, it follows that ATII cell proliferation in the nicotine exposed progeny can possibly be ascribed to a) the imbalance of fibroblast-derived epithelial cell growth stimulatory and –inhibitory paracrine mediators or b) the reduction in fibroblast numbers and therefore an absence in the expression of PPAR-γ resulting in ATII cell proliferation.

Secondly, although the alveolar type I cells were not quantified in the present study; micrographs depicted the sloughing of these cells from the alveolar basement membrane in the lungs of the 84-day-old nicotine exposed offspring. It is known that when type I cells are damaged it is replaced by

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the type II cells; a process that requires type II cell proliferation and the differentiation of one of the daughter cells into a type I cell to replace the damaged type I cell (Crapo et al., 1980). These 2 suggested mechanisms are indirect and require a type II cell that is responsive to the external stimuli, such as type I cell death.

The cell-cell junctions of the ATI cells were damaged as part of the cells sloughing off the basement membrane. The reason for the apparently shorter lifespan of the type I cell is not known. It is however, known that the type I cell is dependent on glycolysis for energy and thus survival. It is has been shown that maternal nicotine exposure suppress glycolysis irreversibly in the lungs of the nicotine exposed animals (Kordom et al., 2003). It is therefore plausible that the shorter lifespan of the type I cells are due to a lower energy supply via glycolysis. It was indeed recently shown that glycolytic enzymes can modulate the life span of early fibroblasts (Kondoh et al., 2005). An increase in glycolysis decreases oxidative damage of these cells and delay onset of senescence (Figure 4.6.5.1). Inhibition of glycolysis on the other hand, results in less protection against oxidative damage and is associated with the premature onset of senescence. Furthermore, it has been demonstrated that glycolytic flux declines during senescence in both murine and human fibroblasts (Zwerschke et al., 2003, Kondoh et al., 2005). This means that the inhibition of glycolysis could also contribute to premature aging of the type I cells. It is also plausible that the inhibition of glycolysis by nicotine in the long term also resulted in premature senescence of fibroblasts as well as type I cells and thus the role it plays in the maintenance of lung structural integrity and function as the animal ages. This might explain the lower number of fibroblasts in the alveolar walls of the nicotine exposed progeny as well as the sloughing from the basement membrane of the type I cells. It is therefore tempting to speculate that inhibition of glycolysis may induce premature senescence and degeneration of alveolar walls and the subsequent development of microscopic emphysema.

**Figure 4.6.5:** Illustrates that glycolysis suppress senescence and protect against oxidative stress. Inhibition of glycolysis compromises the role of glycolysis in controlling senescence and oxidant levels in the lung.

Apart from glycolysis mitochondria act as a major source of energy that is essential for normal cell function. However, perturbation of mitochondrial homeostasis may accelerate senescence (Ziegler et al., 2014). In a previous study it was found that exposure to nicotine resulted in mitochondrial swelling (Maritz and Thomas, 1994, Maritz and Thomas, 1995). In a study by Ziegler et al. (2015) it was shown that the mitochondria of senescent cells are abnormally enlarged. Some studies also showed that these mitochondria release more oxidants which may contribute to cellular senescence (Yoon et al., 2006). Although no data was generated in the present study regarding mitochondrial structure and function, the data generated in studies investigating the effect of maternal nicotine exposure on lung cell and mitochondrial integrity suggest that the changes induced in the mitochondria in the cells of the alveolar wall may contribute to cell senescence and premature aging. On the other hand, in a previous study it was shown that although maternal nicotine exposure induces mitochondrial swelling, it had no effect on the oxygen consumption of the lung tissue in vitro (Maritz, 1986). It is thus not likely that nicotine induced senescence is promoted via its impact on mitochondria. The swelling of the mitochondria appears not to be equal to enlargement of the mitochondria in senescent cells. It furthermore also implies that mitochondrial swelling is not a sign of cell senescence.

In addition, in a study by Zwerschke et al. (2003) it was found that an elevation of the intracellular AMP level occur during replicative senescence, which is sufficient to induce both growth arrest and properties of the senescent phenotype. Since maternal nicotine exposure during gestation and lactation increased the total AMP in the lungs of the offspring (Maritz and Burger, 1992), it is conceivable that this may contribute to premature senescence of the cells in the alveolar walls and thereby resulted in a gradual degradation of the alveolar walls over time.

Apart from being an integral part of the alveolar wall the alveolar epithelial cells also play a crucial role in the innate immunity of the lungs (Derscheid and Ackermann, 2013). Intact cell-cell junctions act as a barrier against foreign materials and in this way prevent it from damaging underlying cells and from entering the circulation. It also protects the underlying basement membrane against the harmful effects of, for example, inhaled oxidants. It is therefore clear that damage to these cells, as illustrated in this study, will not only compromise the structural integrity of the alveolus, but also its role in innate immunity which will consequently increases the vulnerability of the F1 offspring lungs to respiratory diseases.

### 4.6.6 Endothelial cells

The endothelial cells are also part of the alveolar wall and damage to these cells, or if the processes that are responsible for the maintenance of homeostasis of these cells, its role in the maintenance of the integrity of the alveolar wall may also contribute to a gradual breakdown of the alveolar wall with a concomitant increase in alveolar volume. Although these cells were not studied in this project it is plausible that these cells were also affected because the structure and function of the endothelial cells on both sides of the blood-air barrier requires fully functional fibroblasts (Borok et al., 2011).

In summary, the results from this study and information from the literature shows that maternal nicotine exposure to nicotine during gestation and lactation programmed the lungs of the offspring to develop senescence prematurely. A consequence of the premature aging of the lungs is the increased susceptibility to disease due to:

1. Alveolar walls that deteriorate faster due to the imbalance between cell replacement and cell death. This is reflected in slower cell proliferation, type I cell damage, and decreased fibroblast numbers (density) in the alveolar walls.

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2. Innate immunity is in all likelihood also compromised due to the damage to cell-cell junctions in the alveolar walls which exposed the underlying basement membrane to foreign materials, such as oxidants that are inhaled, and may play a role in damage to the 3D connective tissue framework. In addition the barrier function is compromised that will allow the penetration of the oxidants and other substances which may interfere with other cells such as the endothelial cells. The overall effect is a compromised innate immunity function.

The effect of maternal nicotine on the lung structure and function is independent from normal growth and development of the offspring since BW was normal at birth and growth was proportional after birth and not different from that of the control animals. It therefore follows that the effect of maternal nicotine on lung integrity is due to an imbalance in the oxidant-antioxidant ratio induced by nicotine because when restoring the mothers', fetus' and neonate's oxidant-antioxidant ratio by supplementing the diet with tomato juice rich in lycopene, all the adverse effects on the developing lung was averted.

In conclusion, the data suggests that maternal (F0) nicotine exposure during gestation and lactation programmed the lungs of the F1 progeny to develop emphysematous lesions as a consequence of alveolar wall destruction and therefore diminished lung integrity. The data in the present study strongly suggests that the effects of grand maternal (F0) nicotine exposure may be inherited by the F2, F3, and possibly the F4 generation. Therefore, it is imperative to further explore the effects of grand maternal nicotine exposure on the lungs of the F2 and subsequent generations as well as the mechanisms involved in the transfer of premature aging of the lungs.

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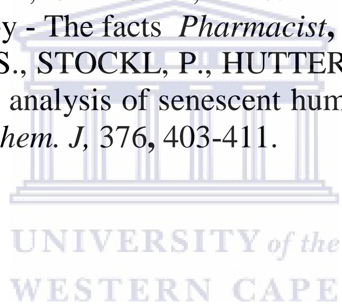
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