Expression of Vascular Endothelial Growth Factor (VEGF) in the Developing Avian Lung; and the Effect of Efavirenz on Embryonic Development and Angiogenesis in Chick Chorioallantoic Membrane

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DECLARATION

I declare that this is my own work. It is hereby submitted for Doctor of Philosophy Degree in the Department of Anatomy, University of the Witwatersrand. The work contained here has not been submitted for any degree or examination at this or any other University. I also declare that this work was carried out in both the Department of Anatomy and the Department of Internal Medicine at the University of the Witwatersrand Medical School in Johannesburg under Animal ethics clearance number 2008/7/1.

Signature: _____ Date: _____ /_____ /2018

Rosaleen Thecla McNeil

DEDICATION

This work is dedicated to Almighty God Who preserved my life up to this point in time and has helped me to get to the end of this programme.

To God be all the Glory and Praise.

I also wish to remember my Mom Elizabeth Enanya Igomu (nee Ogwuche) who died early in life: (1944-1973).

PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS STUDY

MANUSCRIPTS PUBLICATION

1. Tropical Journal of Pharmaceutical Research: Rosie T McNeil, Clement B Penny and Margot J Hosie (2017). Antiretroviral Therapy and HIV-Associated Cancers: Anti-angiogenic Effect of Efavirenz on Chick Chorioallantoic Membrane. Tropical Journal of Pharmaceutical Research November 2017; 16 (11): 2741-2747 ISSN: 1596-5996 (print); 1596-9827
2. International Journal of Research in Medical and Health Sciences (IJRMHS): McNeil RT, Penny CB and Hosie MJ (2017). Developmental Angiogenesis: Expression of Vascular Endothelial Growth Factor (VEGF) in the developing lung. Int J Res Med Sci.13(1): 2307-2083

CONFERENCES PAPER PRESENTATION

1. MCNEIL RTO, PENNY CB, HOSIE MJ (2017). MCNEIL, R.T., PENNY, C.B., HOSIE, M (2017). Efavirenz suppresses angiogenesis in chick chorioallantoic membane. Oral Presentation, Research Day, *21-23 August 2017, Sefako Makgatho University Ga-Rankuwa*

2. MCNEIL RTO, PENNY CB, HOSIE MJ (2017). The role of VEGF in perineural angiogenesis in the domestic fowl (*Gallus gallus* variant *domesticus)*. Research Day, *21-23 August 2017, Sefako Makgatho University Ga-Rankuwa*
3. R.T. McNeil, M. J. Hosie and C.B. Penny (2013). Expression of vascular endothelial growth factor (VEGF) in the developing avian lung: the domestic fowl (*Gallus Gallus* variant *domesticus*). 51st Annual conference of the Microscopy Association of Southern Africa (MSSA, 2013), December 3-6, at Farm Inn, Pretoria, South Africa

4. MCNEIL RT (2012). VEGF expression in renal Development. ASSA conference, University of Namibia, Windhoek Namibia

ABSTRACT

The first part of this work characterized the pattern of endogenous VEGF expression in the developing avian lung while the second experiment evaluated the effect of Efavirenz on chick CAM (the domestic fowl, *Gallus gallus* variant *domesticus)*.

Introduction: Developmental angiogenesis is a complex process that requires a sequential interplay between embryonic cells, angiogenic factors such as VEGF, and extracellular matrix (ECM) components. VEGF has also been implicated in pathological angiogenesis such as neovascularization and carcinogenesis (e.g. Ferrara, 2000; Hagedorn *et al.* 2004; Losordo *et al.,* 2004; Maharaj, 2006; Makanya *et al.,* 2007). Anti-angiogenic drugs are therefore potential anti-cancer agents.

Spontaneous resolution of HIV-associated lung cancers has been observed in patients on "highly active antiretroviral therapy" (HAART).and Efavirenz, a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI), is a first line drug used in the management of HIV-1 infection in both adults and children. Efavirenz has not been adequately studied e.g. in patients with advanced HIV disease (CD4 (T- cell) counts < 50 cells/mm3), and on it use after failure of other regimens.

Thus the aim of this work is to evaluate he anti-angiogenic property of Efavirenz, marketed as Sustiva using Chick Chorioallantoic Membrane (CAM) of the domestic fowl (*Gallus gallus* variant *Domesticus*) as an *in vivo* vascular test environment.

Material and Methods: Animal ethics clearance was obtained (*Ethics clearance certificate number 2008/7/1*). Freshly laid, fertile eggs of the New Hampshire breed of chicken (*Gallus gallus* variant *domesticus*) and of the same clutches, were used for each aspect of the experiment. All the eggs were incubated in thermostatically regulated incubators and with humidity maintained using sponges soaked in water baths. The eggs were turned twice each day. In the first part 42 eggs were routinely processed in an automatic tissue processor (Shandon citadel 1000) and paraffin sections of 5 µm thickness were immunolocalized for VEGF using standard immunofluorescence technique (Polyclonal goat anti-rabbit antibodies 1:100 dilutions were detected with secondary

Alexa Fluor® 568 red Goat Anti-Rabbit IgG at 1:300 dilution, Santa Cruz). Nuclear staining was done with 1:1000 dilutions of DAPI. The sections were viewed on an Olympus IX71 inverted fluorescent microscope and images captured using Olympus analysis Software.

In the second aspect of the work, 30 Fertile eggs at the stage of onset for VEGF expression (day 3 of incubation; estimated Hamburger and Hamilton's stage 21) were used as *in vivo* vascular test environment. They were randomly allocated (n=6) to Human recombinant VEGF (R&D Systems), Thalidomide (Sigma-Aldrich) and Efavirenz, (Sequoia Research Products). Control 1 group had the diluent (70% ethanol) but Control 2 were not manipulated. The eggs were treated by a single injection into the air cells under sterile conditions. CAMs were captured on day 8 and 18 of incubation, using a Nikon microscope camera and compared. Results were analysed using one-way ANOVA and Fisher exact test.

Results: VEGF immune-reactivity was found in both airway epithelium and the cytoplasm of specific mesenchyme cells from as early as stage 22 (Day 3.5) of development. This expression gradually increased with lung maturation, peaking at stage 40 (Day 14).

In the second experiment, it was observed that exogenous VEGF caused vascular haemorrhage. Thalidomide-treated embryos had poorly patterned CAM blood vessel while Efavirenz had absolute anti-angiogenic effect on chick CAM, and also suppressed haematopoiesis. Embryonic viability was 80% for VEGF group, 20% in the thalidomide and 0% in the efavirenz group.

Discussion: VEGF is useful in the early processes of avian lung development and is particularly useful in pulmonary vasculogenesis. Its expression increased with lung maturation, structural complexity and function efficiency, in preparation for gas exchange and flight. Efavirenz showed more anti-angiogenic properties than thalidomide.

Conclusion: Since cancer cell growth and viability depends on neo-vascularization, the anti-angiogenic property of Efavirenz, a member of the HAART regimen, may cause spontaneous resolution of HIV associated cancers.

ACKNOWLEDGEMENTS

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I thank my children for their patience. I would not have made it without their understanding and support. Thank you, John-Studier, for keeping me company during my lab work. Thank you, Daniel, for taking care of John-Studier while I was away at school. Thank you Sisan for your emotional support; you encouraged me to finish this PhD. I thank my sister Vickie for her financial support when fees overwhelmed me and for our Skype chats. I thank my sister Julie for her prayers, Spiritual encouragement and your visits. I am grateful to my best friend Maria Ogbanje Otanwa for her friendship and support.

I am eternally grateful to Almighty God and Father for my life, my children, the opportunity to do the PhD programme and not giving up along the way. I give Him high praise for all the provisions, emotional, financial, physical support, and for the conclusion of the programme.

GLORY TO GOD!

TABLE OF CONTENT

Table of Contents

DEDICATION iii

PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS STUDY iv

MANUSCRIPTS PUBLICATION iv

CONFERENCES PAPER PRESENTATION iv

ABSTRACT vi

ACKNOWLEDGEMENTS viii

LIST OF TABLES xvi

NOMENCLATURE/ LIST OF ABBREVIATIONS AND SYMBOLS xvii

1.0. CHAPTER ONE: THE AVIAN RESPIRATORY SYSTEM 1

1.1. Introduction 1

1.2. Structure of the Avian Respiratory System 2

1.2.1. The Lung 2

1.2.2. Air Sacs 3

1.2.3. The Avian Bronchial System 4

1.3. Flight 7

1.4. Altitude and Adaptation 8

1.5. Development of the Avian Lung 9

1.5.1. The Lung Primordium 10

1.5.2. Morphogenesis 11

1.5.3. Pulmonary Angiogenesis 12

1.6. Regulation of Respiration 13

2.0. CHAPTER TWO: MOLECULAR REGULATION OF LUNG DEVELOPMENT 15

2.1. Morphogenesis 15

2.2. Growth Factors Implicated in Lung Development 16

2.3. VEGF and Pulmonary Angiogenesis 18

2.4. Role OF FGF7 and FGF10 in Pulmonary Morphogenesis 20

2.5. Epidermal Growth Factor (EGF) 21

2.6. Transforming Growth Factor Beta (TGFß) 22

2.7. Bone Morphogenetic Protein (BMP) 24

2.10. Some Growth Factors That Regulation Animal Development 26

2.10.1. Hox genes 27

2.10.2. Transforming growth factors (TGF) 28

2.10.3. Retinoic acid 28

2.10.4. Sonic hedgehog 29

2.10.5. WNT Gene 30

2.10.6. Signal Transducer and Activator of Transcription (STAT) 31

2.10.7. Testis-determining factor (TDF) 32

2.10.8. SOX 33

2.10.9. Guanine nucleotide-binding proteins (G-proteins) 33

2.10.10. Platelet-derived growth factors (PDGFs) 34

2.10.11. Chicken Gonadal and Embryonic Development – MHM gene 34

2.10.12. Insulin-like growth factor-I (IGF-I) 34

2.11. Experimental Embryology 35

3.0. CHAPTER THREE: EXPRESSION PATTERN OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN THE DEVELOPING AVIAN LUNG OF THE DOMESTIC FOWL (*GALLUS GALLUS* VARIANT *DOMESTICUS*) 37

3.1. Ethics 37

3.2. Problem statement. 38

3.3. Justification 38

3.4. Aim: 38

3.5. Objectives 38

3.6. Materials- 39

3.7. Methodology 39

3.7.1. Care of the Eggs 39

3.7.4. Experimental Groups 41

3.8. Tissue Processing 41

3.8.1. Fixation 41

3.8.2. Paraffin sections 42

3.8.3. Haematoxylin and Eosin Staining 42

3.9. Standard Immunofluorescent Technique....43
3.10. Results....45
3.10.1. Results: H and E Sections....45
3.10.2. Results VEGF Staining....62
3.10.3. Results: Immunofluorescence....64
3.11. Discussion....92
3.12. Conclusion....92
4.0. CHAPTER FOUR: COMPARATIVE EFFECT OF EFAVIRENZ ON ANGIOGENESIS IN CHICK CHORIOALLANTOIC MEMBRANE....93
4.1. Pulmonary Angiogenesis and Lung Development....93
4.2. Role of VEGF in Carcinogenesis....94
4.3. Angiogenesis Inhibitors....95
4.4. Antiretroviral therapy: HAART....97
4.5. Statement of the Problem....98
4.6. Justification....98
4.6. Efavirenz....99
4.7. Ethics Clearance....101
4.8. Objectives....101
4.9. Materials and Methods....101
4.10. Results....105
4.11. Discussion....120
4.11.1. Control Embryos....120
4.11.2. Treatment with VEGF....121
4.11.3. Treatment with Thalidomide....122
4.11.4. Treatment with Efavirenz....122
4.12. Conclusion....123
5.0. CHAPTER FIVE: GENERAL DISCUSSION....124
5.1. Development of the avian lung....124
5.2. Development of the airway....125
5.3. Development of parabronchi....126

5.4. VEGF Expression in the developing avian lung....126
5.4.1. Stage 19 - 22....127
5.4.2. Stage 25....128
5.4.3. Stage 27....128
5.4.4. Stage 29....128
5.4.5. Stage 31....129
5.4.6. Stage 37....129
5.4.7. Stage 38....129
5.4.8. Stage 40....130
5.4.9. VEGF expression in other organs....130
5.5. Effects of angiogenesis inhibition in chick CAM....131
5.5.1. Five days of Angiogenesis inhibition....131
5.6. Use of Efavirenz in Pregnancy....132
5.7. The role of VEGF in carcinogenesis....135
5.8. Use of Efavirenz in Cancer treatment.....136
5.9. Conclusion....137
6.0. REFERENCES....138
7.0. APPENDIX SECTION....162
APPENDIX 1: ANIMAL ETHICS CLEARANCE....162
APPENDIX 2: IMMUNOHISTOCHEMISTRY DATA COLLECTION SHEET....162
APPENDIX 3: AUTOMATED TISSUE PROCESSING PROTOCOL....162
APPENDIX 4: RECIPES FOR SOLUTIONS AND REAGENTS....162
APPENDIX 5: IMMUNOFLUORESCENCE PROTOCOL....162
APPENDIX 6: RELATIONSHIP BETWEEN STAGES OF DEVELOPMENT....162
APPENDIX 7: STATISTICAL ANALYSIS OF RESULTS....162
APPENDIX 8: PUBLICATIONS AND PRESENTATIONS ARISING FROM....162
APPENDIX 1: ANIMAL ETHICS CLEARANCE....163
APPENDIX 2: DATA COLLECTION SHEET FOR IMMUNOHISTOCHEMISTRY....163
APPENDIX 3: AUTOMATED TISSUE PROCESSING PROTOCOL....165
(Using automated tissue processor (Shanden, Citadel 1000)....165
APPENDIX 4: RECIPES FOR SOLUTIONS AND REAGENTS....169
APPENDIX 5: IMMUNOFLUORESCENCE PROTOCOL....173

APPENDIX 6: RELATIONSHIP BETWEEN STAGES OF DEVELOPMENT.....175
APPENDIX 7: STATISTICAL ANALYSIS OF RESULTS.....178
APPENDIX 8: PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK 184

LIST OF FIGURES

Figure 1:1: Figure A shows the lateral view of the avian lung. 2
Figure 1:2: The Lung-air sac system of birds 4
Figure 1:3: The structure of the air capillary 6
Figure 1:4: Structure of the Air capillaries. 7
Figure 1:5: Microphotograph of vasculature of chick CAM (X40) 9
Figure 1:6: Arrangement of bronchi within the avian lungs and the direction of airflow 11
Figure 1:7: Scanning electron micrograph showing an air capillary (AC) surrounded by a network of blood capillaries (asterisk) in a chicken lung. 13
Figure 2:1: Growth factors in lung development and disease 18
Figure 2:2: Congenital holoprosencephaly and Anencephaly respectively 30
Figure 3:1 Stage 20 of development, showing the foregut tube 45
Figure 3:2: Figure 14A shows the primary bronchus 46
Figure 3:3: At stage 22 Embryo. A cross section through a stage 22 embryo 47
Figure 3:4: Cross section through the thoracic region of a stage 22 embryo 48
Figure 3:5: Stage 25 Embryo 49
Figure 3:6: This image shows a higher magnification 50
Figure 3:7: Cross section of a stage 29 Embryo. 51
Figure 3:8: Cross section of a stage 30 embryo. 52
Figure 3:9:showing stage 30 lung (A-D). 53
Figure 3:10: Section of a secondary bronchus (SB) of a Stage 31 embryo 54
Figure 3:11: Stage 33 Embryonic lung (LB). 55
Figure 3:12: Stage 33 Embryonic lung. 56
Figure 3:13: Stage 37 Lung. 57
Figure 3:14: Pulmonary arteries in a Stage 37 embryonic lung 58
Figure 3:15: Stage 3.13. Lung. 59
Figure 3:16: Section of a stage 39 lung. 60
Figure 3:17: Stage 40 embryonic lung 61
Figure 3:18: Positive control sections using pregnant mouse uterus. 64
Figure 3:19: Positive control section 65
Figure 3:20: Shows the thoracic segment of a Stage 22 embryo 66
Figure 3:21: Stage 22 Lung buds. 67
Figure 3:22: This is a longitudinal section of a Stage 22 Embryo. 68
Figure 3:23: e apical and basal aspects of the epithelium (Arrow heads). 69
Figure 3:24: Shows the thoracic segment of a stage 25 embryo. 70

Figure 3:25: Figure A shows the spinal cord 71
Figure 3:26: Showing Structures in a stage 27 embryo 72
Figure 3:27: Stage 27 embryonic lung bud. 73
Figure 3:28: Stage 29 lung showing the developing lung bud SB) 74
Figure 3:29: Shows a stage 29 lung bud 75
Figure 3:30: Shows the developing kidney tubules 76
Figure 3:31: Shows a H & E section of the developing Spinal cord in a stage 29 embryo ... 77
Figure 3:32: Spinal cord in a stage 29 embryo. 78
Figure 3:33: Stage 29 embryonic spinal cord 79
Figure 3:34: Stage 29 Spinal cord. 80
Figure 3:35: This is a section through the thoracic segment of an embryo at stage 31 81
Figure 3:36: Primary bronchus at Stage 31 showing VEGF expression 82
Figure 3:37: Stage 33 Lung. Developing parabronchi. 83
Figure 3:38: Stage 37 lung. 84
Figure 3:39: Parabronchi (PB) at stage 37 lung. 85
Figure 3:40: Stage 38 lung. Note numerous parabronchi (PB). 86
Figure 3:41: A stage 39 (day 13) showing a typical avian (parabronchial) lung. 87
Figure 3:42: Stage 40 Lung. 88
Figure 3:43: Negative section performed on a stage 40 embryonic lung 89
Figure 3:44: Stage 40 Lung with air capillaries 90
Figure 3:45: Stage 40 lung at X400 magnification 91
Figure 4:1: Figure A) is an illustration of how the eggs were treated. 104
Figure 4:2: This is an image of the CAM Capillary plexus of a 10-day old chick embryo ... 105
Figure 4:3: Angiogenesis in control chick CAM. 108
Figure 4:4: Angiogenesis in chick CAM treated with VEGF-A. 109
Figure 4:5: Angiogenesis inhibition as seen in chick CAMs treated with thalidomide 111
Figure 4:6: Absolute inhibition of angiogenesis in CAM treated with Efavirenz. 112
Figure 4:7: Bar graph representing the comparative efficacy of treatment drugs 113
Figure 4:8: Mean Trend of CAM vessel formation across Treatment Groups 115
Figure 4:9: Control embryo. Magnification: X40. 116
Figure 4:10: Dissected trunk of a Control embryo (Magnification: X40).. 117
Figure 4:11: VEGF-treated embryo. Magnification: X40.. 118
Figure 4:12: Dissection of the viable thalidomide-treated embryo. 119

LIST OF TABLES

Table 3:1: List of Antibodies used 43
Table 3:2: Scoring scheme for VEGF detection in lung tissue 62
Table 3:3: Localization of VEGF in the embryonic lung 63
Table 4:1: Summary of the effects of treatment 105
Table 4:2: Analysis of Results using ANOVA 106
Table 4:3: Fisher's exact test 107
Table 4:4: Comparison of the anti-Angiogenic effect of Efavirenz to Thalidomide. 114

NOMENCLATURE/ LIST OF ABBREVIATIONS AND SYMBOLS

AIDS: Acquired Immune Deficiency Syndrome

AC: Air cell

AMD: Age-related macular degeneration

bFGF: Basic Fibroblast growth factors (FGF 2)

BMP4: Bone morphogenetic protein 4

BSA: Bovine serum albumin

BSA/PBS: Bovine serum albumin/Phosphate buffered saline

CAM: Chorioallantoic membrane

CD4: cluster of differentiation 4 (T- cells)

CNS: central nervous system

CSF2: Colony stimulating factor 2

DAPI: DAPI: 4′, 6-diamidino-2-phenylindole (with little or no cytoplasmic labelling)

DNA: Deoxyribonucleic acid

EBP50: Ezrin-radixin-moesin (ERM)-binding phosphoprotein 50 (an anti-cancer drug)

EC: Endothelial cells

ECM: Extracellular matrix

EFV: Efavirenz

EGF: Epidermal growth factor

EPPB: Extrapulmonary primary bronchi

ERM: Ezrin-Radixin-Moesin, the 3 protein family members present in vertebrates (other species have only one ERM gene)

FGF: Fibroblast growth factors

FGF2: Fibroblast growth factor 2

FGF 7: Fibroblast growth factors 7

FGF10: Fibroblast growth factors 10

FGFR2β: Beta isoform of the type 2 FGFR

FGFR 1: Fibroblast growth factors Receptor 1

FGFR 2: Fibroblast growth factors Receptor 2

FGFR 3: Fibroblast growth factors Receptor 3

FGFR-4: Fibroblast growth factors Receptor 4

FGFR: Fibroblast growth factors Receptor

Flk-1: Designated VEGFR2

Flt-1: Designated VEGFR1

GM-CSF: Granulocyte-macrophage colony-stimulating factor

HAART: Highly active anti-retroviral therapy

H&E: Haematoxylin and Eosin

HGF: Hepatocyte growth factor

HIV: Human immunodeficiency virus

HIV-1: Human immunodeficiency virus-1

HPLC: High Performance Liquid Chromatography

HS: Heparan Sulphate

IAEA: International Atomic Energy Agency

IL-8: Interleukin 8

IgG: Immunoglobulin G

IPPB: Intrapulmonary primary bronchi

NNRTI: Non-nucleoside reverse-transcriptase inhibitor

NRP: Neuropilins (Transmembrane glycoproteins which act as receptors in neurons; enhancing axon function).

NRP-1: Neuropilins receptor, type I

NRP-2: Neuropilins receptor, type II

RT: Reverse transcriptase

IPC: Intrapulmonary chemoreceptors

IPS: Interparabronchial septum

IPPB: Intrapulmonary primary bronchi

KS: Kaposi sarcoma

NGF: Nerve growth factor

NNRTI: Non-nucleoside reverse-transcriptase inhibitor

$PaCO_2$: Partial pressure of carbon dioxide

PaO_2: Partial pressure of Oxygen

PB: Parabronchus/Parabronchi

PBS: Bovine serum albumin/Phosphate buffer

PCO2: Partial pressure of carbon dioxide

PO2: Partial pressure of Oxygen

PDGF: Platelet derived growth factor

PDGFA: Platelet derived growth factor A

PDGFB: Platelet derived growth factor B

PDGFC: Platelet derived growth factor C

PDGFR: Platelet derived growth factor receptor

PDGFRα: Platelet derived growth factor receptor alpha

PDGFRβ: Platelet derived growth factor receptor beta

PEPFAR: President's Emergency Plan for AIDS Relief's (USA)

pH: A numeric scale used to specify the acidity or basicity of an aqueous solution

PI: Protease Inhibitors

PO_2: Partial pressure of Oxygen

RBC: Red Blood Cell

RT: Reverse transcriptase

RNA: Ribonucleic acid

SB: Secondary Bronchi

SEM: Scanning electron microscopy

SMU: Sefako Makgatho Health Science University (in Ga-Rankuwa South Africa)

STAT: 7-Membered protein (transcription factors) family

STAT5A and STAT5B: Two related members of STAT family that mediate signals for abroad spectrum of cytokines involved in immune response

STAT3: member of STAT family, that causes zinc starvation and so, is antifungal TK: Tyrosine Kinase

TrKs: Threonine Kinases

TGF: Transforming growth factor

TGFß: Transforming growth factor beta

TGF-β1: transforming growth factor-beta-1

TGF-βRs I: Type I receptors for TGF-βI

TGF-βRsII: Type II receptors for TGF-βI

TNF: tumour necrosis factor

TNF- α: tumour necrosis factor alpha

VEGF: Vascular endothelial growth factor

VEGF-A: Vascular endothelial growth factor A

VEGF121: Vascular endothelial growth factor 121

VEGF165: Vascular endothelial growth factor 165

VEGF189: Vascular endothelial growth factor 189

VEGF206: Vascular endothelial growth factor 206

VEGFR: Vascular endothelial growth factor receptor

WHO: World Health Organization

CHAPTER ONE

1.0. THE AVIAN RESPIRATORY SYSTEM

1.1. Introduction

Birds evolved from reptiles and share some morphological characteristics in their respiratory system (Pough, 1989). However, there is a major difference in the respiratory system of birds when compared to that of reptiles and mammals; birds have a parabronchial lung, while reptiles have faveolar and the mammalian lung is bronchioalveolar (Duncker, 1978, Maina, 2002b, Maina, 2002a, Maina, 2003b). Of the three types, the avian lung- air sac respiratory system is the most efficient in function (Powell and Scheid, 1989). Avian lungs are small, flattened and dorsal in position (King and McLelland, 1984) with two openings, one for taking in oxygen-filled air and the other for expelling carbon dioxide-laden air (Ludders, 2001). The avian lung system is explained in some detail below:

King and McLelland (1984) described the avian respiratory system, the lung-air sac system as the most structurally complex and functionally efficient of all air-breathing vertebrates. Besides gas exchange, the avian respiratory system also plays an important role in thermoregulation (Powell *et al.,* 2000). Inspired air loops through the lungs, to ensure a continuous supply of oxygen (Powell *et al.,* 2000), allowing for the extraction of a greater concentration of oxygen from inhaled air (King and McLelland, 1984); and ensuring that intrapulmonary air remains largely 'fresh' with a high oxygen content. This respiratory design enables birds to obtain the amount of oxygen needed to power the extremely high energy demands of flight (Ludders, 2001). Complete air movement through the lung at any time requires two inspiratory and two expiratory cycles (King and McLelland, 1984). During the first inhalation, air flows through the trachea and bronchi into the posterior air sacs at which time the sternum moves forward and downward, while the vertebral ribs move cranially to expand

the thoraco-abdominal cavity and lower the pressure therein, so that air moves into the air sacs.

1.2. Structure of the Avian Respiratory System

1.2.1. The Lung

The rigidity of avian lungs prevents air capillary collapse (Maina and King, 1982) and allows for an intense subdivision of the parenchyma into very small terminal respiratory units, providing the avian lung with a much greater respiratory surface area than that of mammals (Maina, 2006). This anatomical modification thus compensates for the smallness of avian lungs (Maina, 1989) and endows it with a functional respiratory advantage over the mammalian lung.

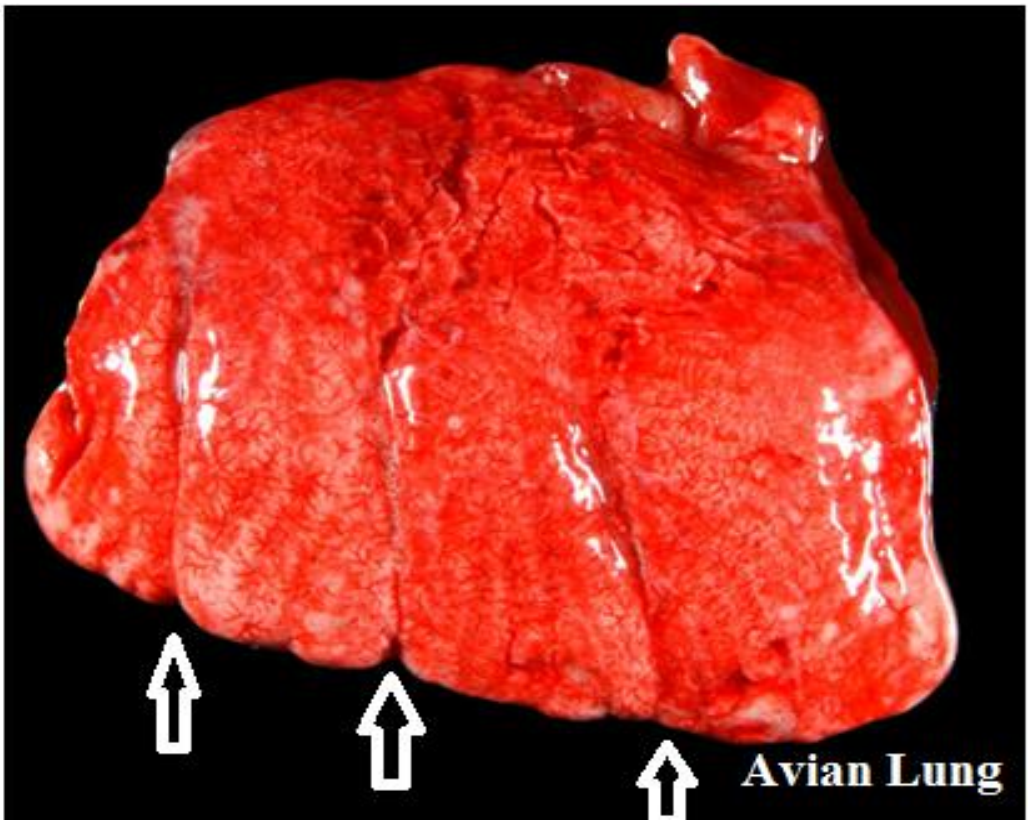

Figure 1.0:1: The lateral view of the avian lung. Note the costal sulci (arrow heads). Cornell University College of Veterinary Medicine (2012)

P-Primary bronchus, v-medioventral secondary bronchi, d-mediodorsal secondary bronchi, o-ostium into the abdominal air sac (Maina, 2002b). Legend lacks detail. Unlike the pliant mammalian lung, that of birds is structurally rigid and therefore isovolumic (Duncker, 1972). There is a directional relationship between the airways and their connection to the air sacs with the parabronchi running perpendicular to the secondary bronchi (Maina and Woodward, 2009).

1.2.2. Air Sacs

Air sacs are very thin-walled, transparent with poor blood supply (Powell *et al.,* 2000). They ventilate the lungs but do not take part in gaseous exchange (King and McLelland, 1989). Air sacs are functionally grouped into anterior (interclavicular, cervicals and anterior thoracics) and posterior air sacs (posterior thoracics and abdominals) (Ludders, 2001). Unlike reptiles and mammals that alternate inspiration with expiration, birds have two inspirations and two expirations per cycle. Inspired air loops through the lungs, to ensure a continuous supply of oxygen (Powell *et al.,* 2000).

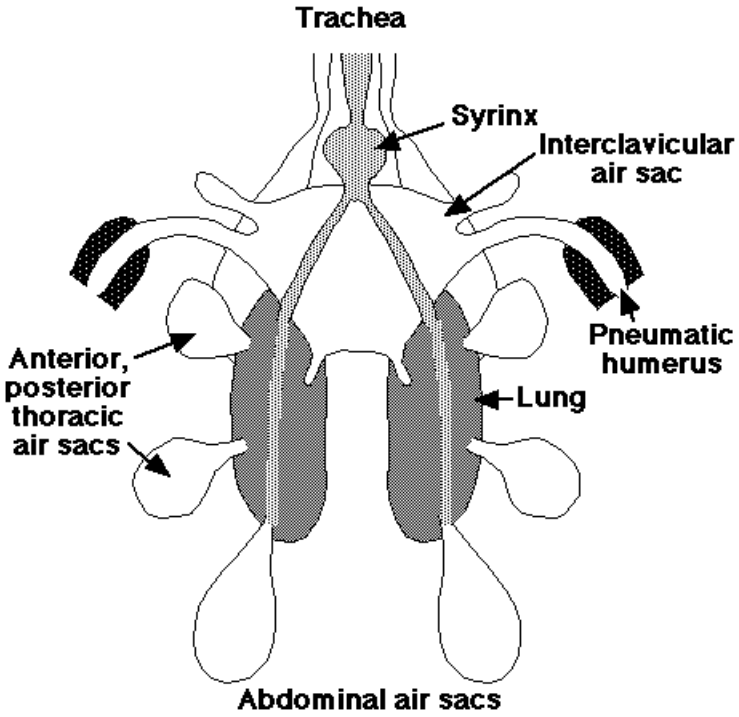

Figure 1.0:2: The Lung-air sac system of birds. The avian lung typically has nine air sacs. They act as "bellows" (Powell *et al.,* 2000) ventilating the lungs in a posterior-anterior (or caudo-cranial) direction (Maina *et al.,* 2003).

1.2.3. The Avian Bronchial System

The avian bronchial system consists of the trachea, the bronchi and its divisions and the parabronchi. Each of these structures is described below:

a) Trachea

The typical bird trachea is 2.7 times longer and 1.29 times wider than that of similarly-sized mammals (Ludders, 2001). Tracheal resistance to air flow is similar to that in mammals, but the tracheal dead space volume is about 4.5 times larger in birds. To compensate for the larger tracheal dead space, birds have a relatively larger tidal volume and a lower respiratory rate of approximately one-third that of mammals (Ludders, 2001). These two factors downplay the impact of the larger tracheal dead space volume on ventilation, so that the minute tracheal ventilation is only about 1.5 to 1.9 times that of mammals (Ludders, 2001).

b) Primary Bronchi

The trachea bifurcates into two extra pulmonary primary bronchi (EPPB) at the syrinx or 'voice box' which is unique to birds (Maina, 1989). In mammals, sound is produced in the larynx. Histologically, EPPBs are lined by pseudostratified epithelium which contain goblet cells, intraepithelial mucous alveoli and projecting ridges, bearing cilia (King and McLelland, 1989).

c) Secondary Bronchi

Each EPPB enters the hilum of the lung to become the intrapulmonary bronchi (IPPB) or mesobronchus each giving rise to four sets of second order or secondary bronchi (SB) named according to the region of the lung they supply (King and McLelland, 1989, Maina, 2005), namely the medioventral, mediodorsal, lateroventral and laterodorsal SB (King and McLelland, 1989). All SB arise from the caudal two-thirds of the IPPB at constricted necks of 1-2cm (King

and McLelland, 1989). The necks have the same histological appearance as the IPPB, but subsequently become lined by squamous epithelium, as they become SB. However, approximately 4mm from their origin, they give off numerous small tubes of similar calibre called parabronchi or tertiary bronchi (King and McLelland, 1984).

d) Parabronchi

Parabronchi are 0.5 - 2.0mm in diameter (depending on the size of the bird) and are connected at either ends by dorsobronchi and ventrobronchi. They can be several millimetres long (Maina, 1989).). They are lined by simple-squamous epithelium with a network of spiral bands of muscles (which is homologous to the helical bronchial muscles of mammalian lungs). In-between the muscles there are numerous pocket-like polygonal cavities of 100-200μm diameter, termed atria. Atria are lined by flat or cuboidal epithelium and open into the parabronchial lumen. PB are lined by osmophilic cells which produce osmophilic bodies which are believed to be the source of surfactant (King and McLelland, 1989). At the bottom of the atria, there are several funnel-shaped openings called infundibula which connect the atria to narrow tortuous tubes called air capillaries. Air capillaries anastomose freely, forming hundreds of tiny, branching, and anastomosing 'air capillaries' in the walls of the parabronchi (King and McLelland, 1989). These air capillaries are surrounded by a dense network of blood capillaries (Welty and Baptista, 1988).

e) Air Capillaries

Gas exchange occurs within the 'air capillaries' by diffusion, between the lungs and blood (BrownBrain *et al.,* 1997). Oxygen and carbon-dioxide are exchanged with cross-flowing blood capillaries in a process described as “counter-current exchange” (Maina, 1989). The functional efficiency of the avian lung is further enhanced by an ultra-thin blood-gas barrier, as the pneumocytes (especially the type II cells) and connective tissue elements are not located on the respiratory surface of the air capillaries (as in mammals), but are rather confined to the atria and infundibulae. There is therefore, separation or uncoupling of the gas exchanger (the

lung tissue) from the mechanical ventilators (the air sacs). This ensures a continuous, unidirectional ventilation of the lung (Maina, 2006).

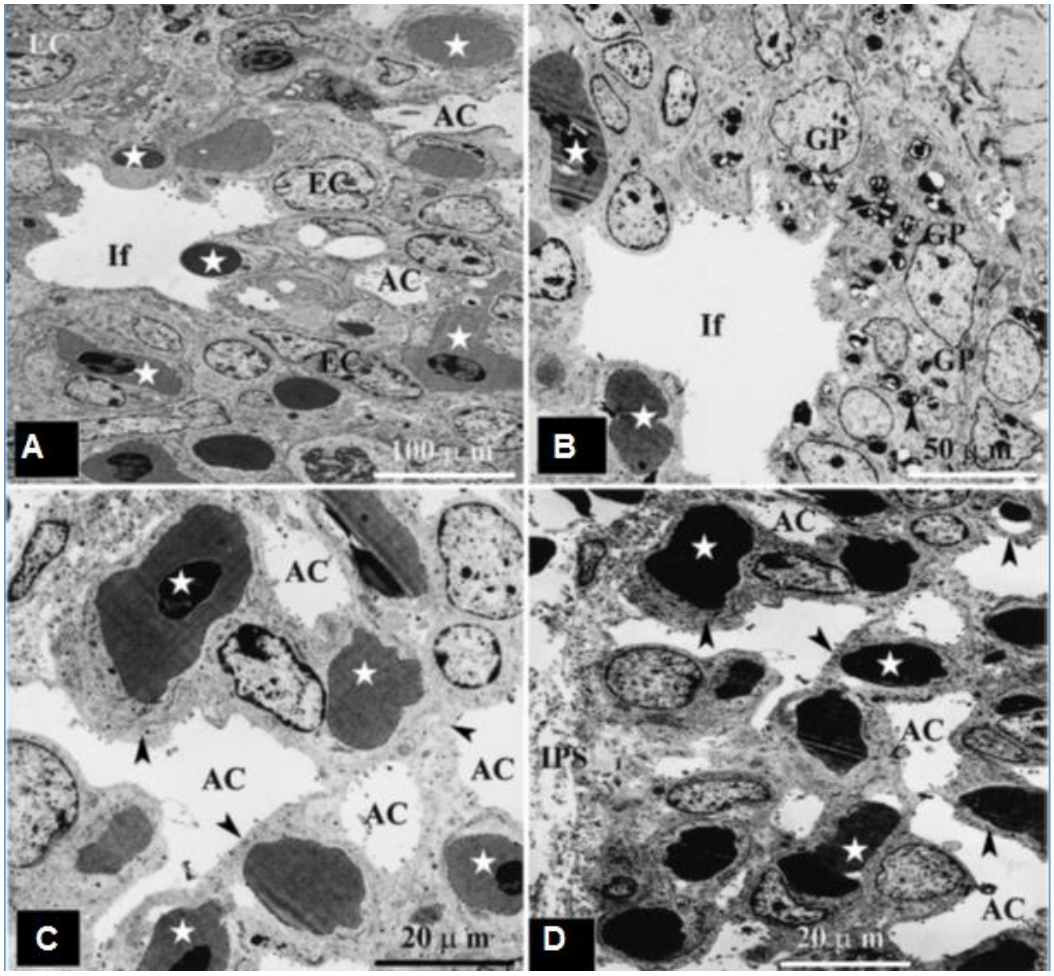

Figure 1.0:3: Photomicrograph, showing the structure of the air capillary In all panels the stars represent red blood cells. (A) A formative infundibulum (If) on day 16, giving rise to air capillaries (AC) and epithelial cells (EC). (B) An infundibulum (if), at day 17, with type II granular pneumocytes (GP) in the surrounding epithelium. (C) On day 18 of development Gas exchange tissue with blood capillaries containing red blood cells surrounded by developing air capillaries (AC). (D) Gas exchange tissue on day 20 with much thinner blood–gas barrier (arrow heads) (Maina, 2003b).

Furthermore, BrownDelp et al. (1997) used a 3-dimensional reconstruction to describe the gas-exchange region:

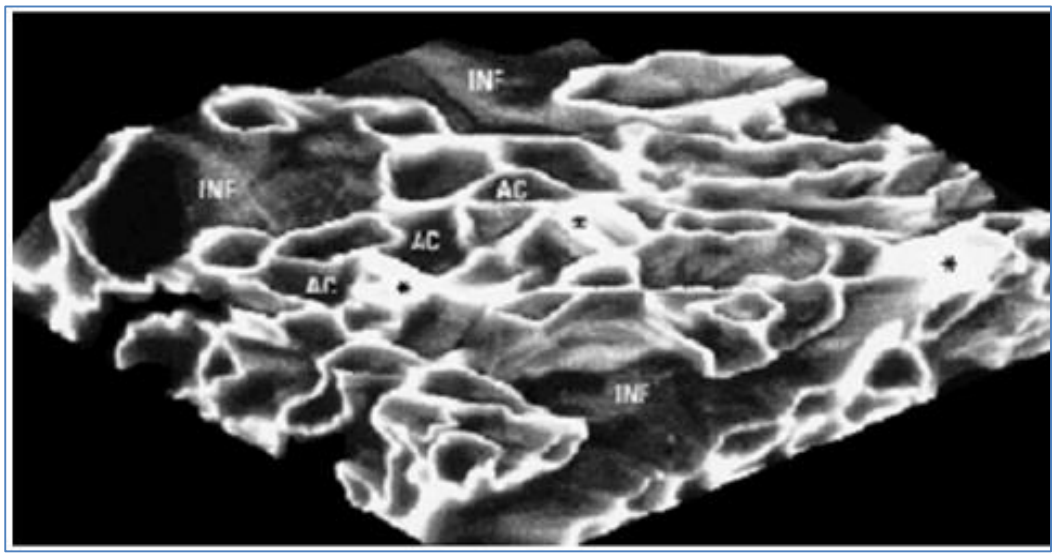

Figure 1.0:4: Photomicrograph showing a 3-dimensional reconstruction of the gas-exchange region–AC. Air capillaries are gas-exchange tissues (BrownBrain *et al.,* 1997).

A scanning electron microscopic studies both strong flying birds and flightless birds showed a structural support system for avian gas-exchange tissue that consists of a spider web-like system of trilaminar lipoproteinaceous substance that is secreted by the respiratory cells. They are found between blood capillaries and air capillaries, thereby anchoring and supporting the gas-exchange tissue (Klika *et al.,* 1997).

1.3. Flight

There are three main types of flight:

1. Buoyant flight
2. Aerodynamic or unpowered flight (as animals such as birds do)
3. Ballistic flight.

In a Buoyant flight system, an aerostat system gives an aircraft the same overall density as air. Ballistic flight refers to the use of accelerating projectiles in order to achieve a desired performance. With the exception of bats, birds are the only vertebrates that can fly (Tucker, 1972) and among all air-breathing vertebrates, the lung-air sac system of the avian respiratory apparatus is the most structurally complex and functionally efficient (Maina, 2000). Flight is a highly energetic form of locomotion. It is expensive in its absolute demands for energy when compared with other forms of locomotion, such as swimming, running or walking. However, active flight is a more cost-effective form of locomotion (Tucker, 1973) and birds use the interplay of aerodynamic and gravitational forces, to travel considerable distances on fixed wings. And of all animals, only birds, insects, and bats employ powered flight. And only birds can achieve sustained flight. It is documented that birds in flight have collided with airplanes; altitudes at which mammals would succumb to hypoxia (Sodhi, 2002, Manville II, 2005, Gard *et al.,* 2007).

1.4. Altitude and Adaptation

People who live in mountainous areas undergo adaptive processes such as polycythaemia to maintain their circulatory system. Poor adaptation causes heart failure from pulmonary hypertension (Sands *et al.,* 2011). Animals such as the flying squirrel exploit rising air to “fly” by gliding between trees. This is also called soaring. In birds, bats and all powered aircraft a source of propulsion is necessary for flight. Prolonged gliding requires enormous oxygen consumption (Baudinette and Schmidt-Nielsen, 1974) and birds can achieve remarkable energetic feats (King and McLelland, 1989). It has been calculated that the average energy expended by birds in avoiding predators, procuring food and reproduction is about 50 to 75% higher in a 380g bird than a rodent of the same body weight (King and McLelland, 1989). This is because flight involves virtually all body organ systems and in particular, the sensory, digestive, the cardiovascular, locomotory and pulmonary systems (Maina, 2005). With respect to its cost per unit distance covered however, powered flight is a very efficient cost-effective mode of transport (Tucker, 1973). Powered flight has been achieved in only two phyla,

(Chordata and Arthropoda) and among all chordates, in only three taxa namely–birds, (de Beer, 1954), bats (Yalden and Morris, 1975) and pterodactyls, (Bramwell, 1971). The respiratory system of birds is therefore unique in structure and function, being adapted to withstand the demands of flight (Tucker, 1973). Birds can fly at altitudes at which mammals would die from hypoxia (Maina, 2000).

1.5. Development of the Avian Lung

Tazawa and Rahn (1987) described three sequential developmental stages of respiration in avian embryos, namely, prenatal or embryonic, paranatal or hatchling and postnatal or post-hatching. Angiogenesis begins at the area vasculosa; it is at this area that blood island develops in early embryonic life. Gas exchange occurs by diffusion. The area vasculosa is the primordium of the chorioallantoic membrane (CAM) and the initial gas exchanger. The developing lung begins to replace the chorioallantois in the paranatal period and becomes the sole gas exchanger after hatching (BrownBrain *et al.,* 1997).

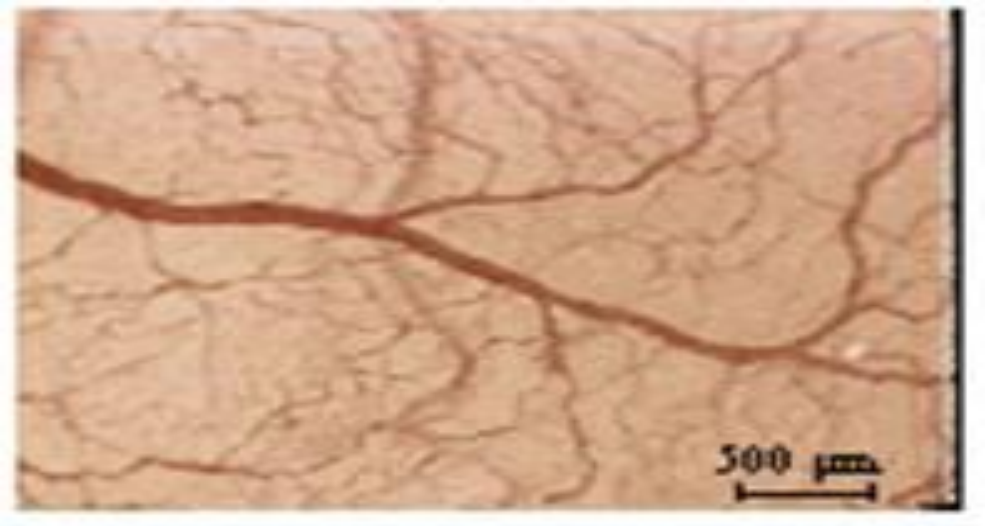

Figure 1.0:5: Microphotograph of vasculature of chick CAM (X40)

At the prenatal stage of avian development, gas exchange occurs by diffusion since the lung is not functional. The area vasculosa is therefore the initial gas exchanger.

http://www.ece.utexas.edu/~bevans/courses/ee381k/lectures/Tomography/milner/sld003.htm

As the embryonic development of the lung is of importance in this study, it is described here. Similar to mammalian lung development, developmental processes in the avian lung involves growth factors and the assembly of pulmonary vasculature, resulting in the complex architecture of the avian lung, with its exceptional respiratory efficiency (Maina, 2004). When compared to mammals, it is considered that the avian respiratory system seems to follow a more intricate process of development (Duncker, 1978), which may explain why of all vertebrates, and with the exception of bats, only birds can fly (Tucker, 1972).

1.5.1. The Lung Primordium

The pharyngeal endoderm gives rise to the lungs and the bronchial tree, whilst the mesenchyme forms the muscles, connective tissue, and the lymphatics that accompany the bronchial system (Bellairs and Osmond, 1998). It is known that mammalian lung development is regulated by distinct epithelial-mesenchymal interactions along the developing respiratory tract (Cardoso, 2001). The avian lungs develop from endodermal ridges that arise from the floor of the primitive pharynx on day 3.5 (Maina *et al.,* 2003) to day 4 (Bellairs and Osmond, 1998) post-incubation. This corresponds to Hamburger and Hamilton's stages 21-23 (Hamburger and Hamilton, 1951). According to Maina (2003b) the endodermal ridges fuse in the ventral midline on day 4 post-incubation (Hamburger and Hamilton's stage 24) to form a single lung bud that divides on day 5 (Hamburger and Hamilton's stage 27) into the left and right primordial lungs. The lung primordia elongate caudally and diverge to reach the dorso-lateral aspect of the embryonic coelom on day 6, post incubation (Hamburger and Hamilton's stage 29). On day 7 (stage 31), they rotate, attach onto the ribs and gradually start to slide into them. They become deeply inserted into the ribs by day 8 post incubation (Hamburger and Hamilton's stage 34). On day 9, the parabronchi (PR) begin to bud from the secondary bronchi (SB). Projecting into the adjacent mesenchyme, they greatly increase in length, number, diameter and commence to canalize on day 10 (stage 36). By day 13 (stage 39), the parabronchi anastomoses profusely and the luminal surface is lined by columnar epithelium.

The atria, infundibulae, and air capillaries develop from the luminal surface of the PB on days 15, 16 and 18 (stages 41, 42 and 44) respectively (Maina, 2003b).

1.5.2. Morphogenesis

According to Lü et al. (2004), mammalian lung bud morphogenesis involves major changes in cytoskeletal organization and fibroblast growth factor 10 (FGF10) and its receptor FGFr2b signalling is critical for bud morphogenesis. FGF 10 is expressed by the mesoderm during organogenesis, from where it diffuses to activate FGFr2b in the adjacent endoderm (Orr-Urtreger *et al.,* 1993). The intrapulmonary primary bronchus develops from an epithelial cell cord that runs in a craniocaudal direction, deep within the developing lung (Maina, 2003b). It gives rise to secondary bronchi along its proximo-distal extent, while parabronchi arise from and connect to the secondary bronchi (Maina, 2003b, Sakiyama *et al.,* 2003). Air sacs develop as “blister-like” diverticula from the cranial, ventral and caudal aspects of the developing lung. The abdominal air sac appears between days 5-7, the cervical between days 6-8 and the others (inter-clavicular, anterior thoracic and posterior thoracic air sacs), between days 7-12 of development (Maina, 2006). These correspond to Hamburger and Hamilton’s stages 27-38 (Hamburger and Hamilton, 1951).

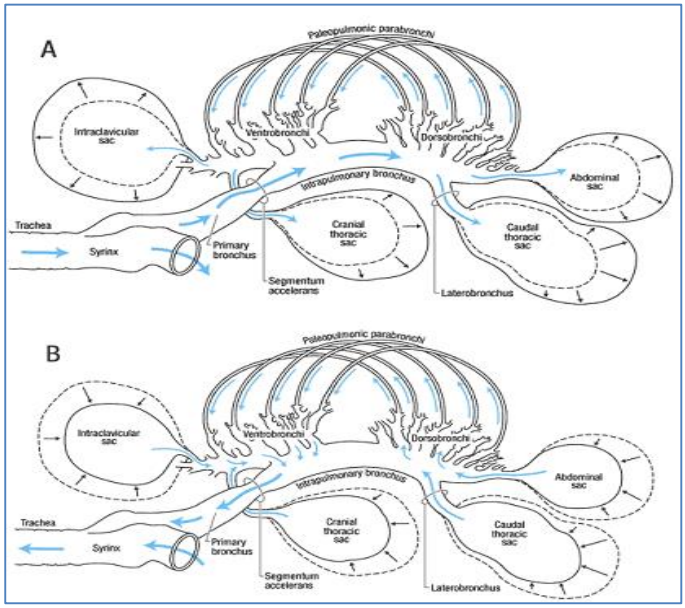

Figure 1.0:6: Arrangement of bronchi within the avian lungs and the direction of airflow

The direction of airflow is indicated by the blue arrows. Birds have two inspirations and two expirations per cycle. Figure A - Inspiration. Figure B – Expiration From the trachea, the extrapulmonary principal bronchus continues as the intrapulmonary principal bronchus (also known as the mesobronchus). Parabronchi connect the dorsobronchi and the ventrobronchi. Air flows from dorsobronchi to ventrobronchi. In the first inspiration, all air sacs expand as inhaled air enters the posterior air sacs and lungs and, at the same time, “older air” moves from the lungs into the anterior air sacs. In the second expiration, the air sacs diminish in volume as air moves from the posterior air sacs through the lungs and also, from the anterior air sacs into the trachea and out of the body (Ritchison, (2017)).

1.5.3. Pulmonary Angiogenesis

The pulmonary and bronchial vasculature develops from the splanchnic mesoderm, which forms around the foregut before the lung buds develop (Deruiter *et al.,* 1993, Bellairs and Osmond, 1998). According to Maina (2004), haematogenic and vasculogenic cells become ultrastructurally distinguishable on day 4 (stage 23) of development, with vasculogenesis beginning from day 7 (stage 31). This occurs in different areas of the developing lung. The blood vessels form from mesenchyme (angioblasts) and elongate by augmentation and alignment of the surrounding angioblasts (Maina, 2004). Angiogenesis occurs largely by sprouting and intussusception. Sprouting guides, the emerging vessels to form the primordial vascular plexus (which then surrounds and encloses the parabronchi). Intussusceptive angiogenesis begins on embryonic day 15 (E15) and is responsible for the rapid expansion of the capillary plexus (Makanya *et al.,* 2006). Mature vessel structure forms following remodelling of the primary capillary plexus (Darland and D’Amore, 1999, Conway *et al.,* 2001). Smooth muscle cells and pericytes surround the endothelial cell layer. According to Maina (2004), endothelial cells change into erythrocytes and drop into the lumen of the developing blood vessels and by day 10 the pulmonary vasculature is largely formed. Complete pulmonary circulation, however occurs only after the development of the blood capillaries on day 18 (refer to Figure 1.7).

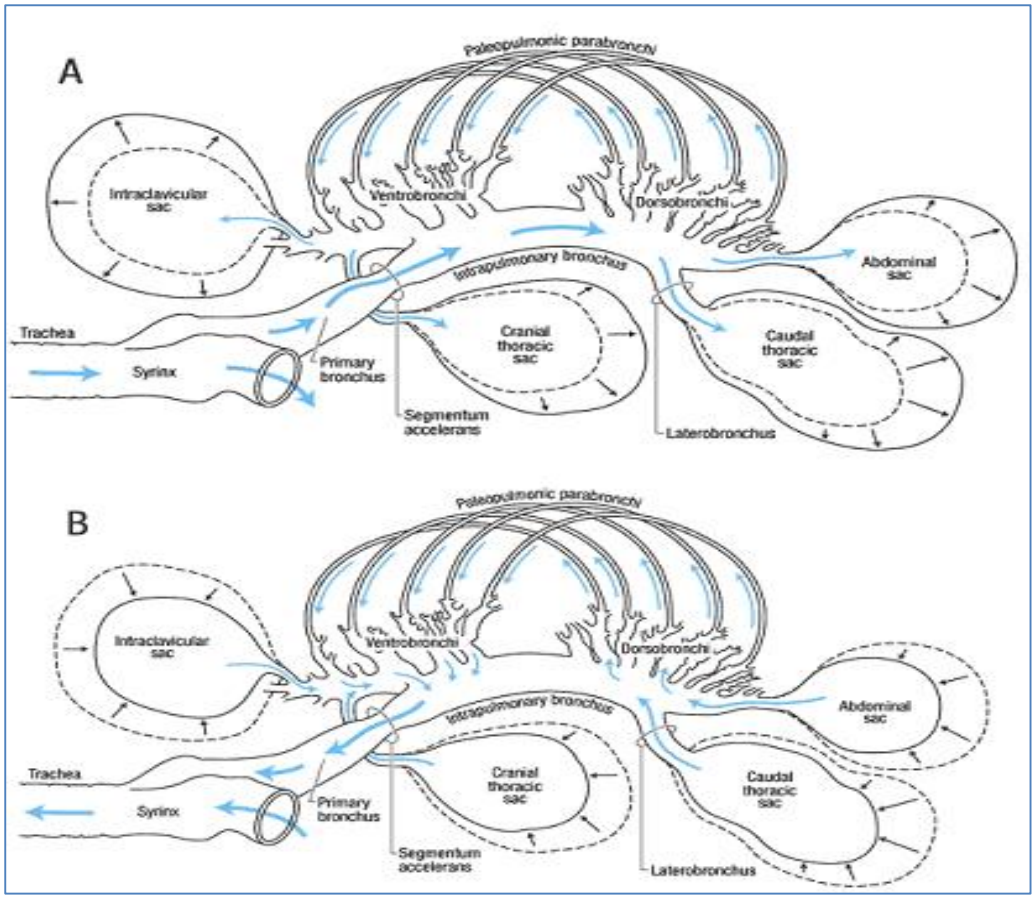

Figure 1.0:7: Scanning electron micrograph showing an air capillary (AC) surrounded by a network of blood capillaries (asterisk) in a chicken lung. The blood capillaries drain into a larger vein (V6) adjacent to an infundibulum (IF) (Makanya *et al.,* 2009).

1.6. Regulation of Respiration

Birds have central chemoreceptors that regulate ventilation in response to changes in arterial partial pressure of carbon dioxide (PCO_2) and hydrogen ion concentrations. They also have peripheral extrapulmonary chemoreceptors such as carotid bodies in their carotid arteries that are influenced by PCO_2. They increase their discharge rate as PCO_2 decreases, thereby increasing ventilation and decrease their rate of discharge in response to increase in PO_2 (Ludders, 2001). These are similar to those found in mammals but unlike mammals, birds have a unique group of peripheral receptors in the lung, the intrapulmonary chemoreceptors

(IPC) which are acutely sensitive to carbon dioxide, but insensitive to hypoxia. They regulate respiratory rate and volume on a breath-to-breath basis (Ludders, 2001).

CHAPTER TWO

2.0. MOLECULAR REGULATION OF LUNG DEVELOPMENT

2.1. Morphogenesis

Developmental similarities have been noted in the different species of vertebrates in that their embryos all show similarities in developmental pattern and present with the processes of fertilization, cleavage, blastulation/ gastrulation and formation of the three germ layers (Wolpert *et al.,* 1998). Similarities in vertebrate embryonic development were first described by the evolutionary biologist, Ernst Mayr (Mayr, 1970).

The term 'morphogenesis' is derived from the Greek word morphê for shape, and genesis, for creation. Morphogenesis describes the biological process of vertebrate development, or organogenesis, with the associated change in the shape of the organism or organs during formation. The process involves cell growth and cellular differentiation (Slack, 2013). Morphogenesis, or ontogeny, also refers to the development of various cell types within an organism (Thiery, 2003). It is guided by both molecular and cellular factors and is regulated by morphogens also known as transcription factor proteins or growth factors. Growth factors are characteristically, soluble and so diffuse through tissues, carrying signals that regulate cell differentiation along a concentration gradient. They act by binding to specific protein receptors.

Transcription factor proteins determine the fate of cells by interacting with their DNA and so, controlling cellular behaviours such as migration, adhesion and contractility (as seen during gastrulation) (Kouros-Mehr and Werb, 2006). Embryonic cells are restricted by cell-to-cell adhesion molecules such as cadherins that are found on many epithelial cells, without cadherins cells migrate. They are a class of type-1 transmembrane proteins. They bind cells together within tissues by forming adherens junctions. This process is dependent on calcium (Ca^{2+}) ions. Cadherins are therefore called "calcium-dependent adhesion" protein molecules (Hulpiau and Van Roy, 2009). The presence or absence of cell-to-cell adhesion molecules

therefore confers differential affinities on cells. Cadherins bind to other cadherins in a like-to-like manner: E-cadherin (found on many epithelial cells) binds preferentially to other E-cadherin molecules. Mesenchymal cells usually express other cadherin types such as N-cadherin. Morphogenesis occurs also as a result of changes in the cellular structure and cells interaction within tissues; thinning, elongation, folding or movement/ separation resulting from differential cell adhesion or cell differentiation such as epithelial-mesenchymal transition (Gilbert, 2000). Similarities have been noted in vertebrate development but divergence occurs early in morphogenesis) (Wolpert *et al.,* 1998). Many growths factors or signal Proteins regulate animal development. They act as morphogens and long-range inducers with grade defects. Growth factors are tempero-spatial in function and act to regulate morphogenesis along a concentration gradient (Rogers and Schier, 2011). Growth factors are also known as transcription factor because of their sequence-specific DNA-binding. Thus, they control the rate of transcription of genetic information from DNA to messenger RNA (Karin *et al.,* 1990). Although they are encoded by developmental control genes their concentration and morphogenetic functions can be up regulated or down-regulated. The morphogenetic properties of growth factors cause changes in the shape and structure of organs or the embryo and by morphogenetic movement cell, groups or sheets of cells are brought into new but necessary spatial relationships e.g. to bring about new phases of signalling and facilitate specific cell communication or induction. For example, the cells of each germ layer move to form epithelia that are arranged in the known order; outer most ectoderm, inner most endoderm and the mesoderm layer in between (Hardin and Walston, 2004).

2.2. Growth Factors Implicated in Lung Development

Early during the development of the mammalian respiratory system, an off-shoot of the foregut endoderm (called the laryngo-tracheal diverticulum) gives rise to the trachea and the lung. Through branching morphogenesis, and together with development of the pulmonary vascular system, the tree-like epithelial tubular diverticulum differentiates into the bronchial airways and

alveoli (Cardoso and Lü, 2006). Many growth factors and molecular pathways have been implicated in the developmental regulation of the lung (Ferrara, 2000, Gjorevski and Nelson, 2011) and lung development is spatially and temporally coordinated through the role of these growth factors (Maina, 2004). Cross-talk between epithelium and mesenchyme is necessary for any two tissues to successfully form one cohesive functioning unit as is found between the air-conducting system and pulmonary endothelium (Bellusci *et al.,* 1996). According to Desai and Cardoso (2001), the following factors have been found to be involved in mammalian development and disease causation:

1. Vascular endothelial growth factor (VEGF)
2. Fibroblast growth factors (FGF)
3. Epidermal growth factor (EGF)
4. Transforming growth factor beta (TGFß)
5. Bone morphogenetic protein 4 (BMP4)
6. Platelet derived growth factor (PDGF)
7. Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Growth factors are diffusible mitogen proteins that act to induce a variety of cellular activities usually within a short distance of where they are produced (Desai and Cardoso, 2001). Mammalian lung development is regulated by distinct epithelial-mesenchymal interactions of several growth factors along the developing respiratory tract. These growth factors have been implicated in both mammalian lung development and disease (Cardoso, 2001). The figure below shows different growth factors that have been implicated in the development of lung diseases (Desai and Cardoso, 2001):

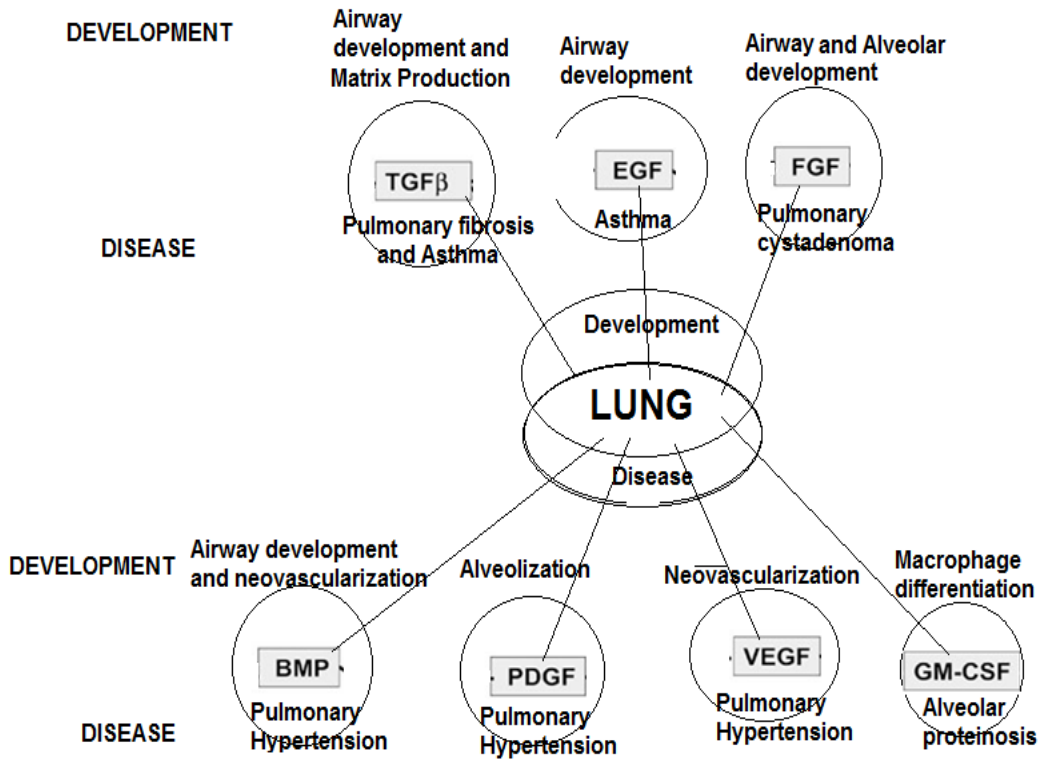

Figure 2.0:1: Growth factors that have been implicated in both lung development and disease. During development growth factors determine the branching patterns and size of airway (Warburton *et al.,* 2000) and they also regulate and mediate tissue injury and healing in the adult lung (Cardoso, 2001).

2.3. VEGF and Pulmonary Angiogenesis

The lung is a major site of VEGF gene expression, largely from type II alveolar epithelial cells (Kaner and Crystal, 2001). “The primary capillary plexus forms from the induced angioblasts by a poorly understood morphogenetic process influenced by FGF, vascular endothelial growth factor (VEGF), adhesion molecules, and the extracellular matrix” (Tallquist *et al.,* 1999, Poole *et al.,* 2001). VEGF is the key signal that leads to both developmental and pathological angiogenesis. Angioblasts, induced by FGF2 begin to express VEGFR2 (Flamme and Risau, 1992, Yamaguchi *et al.,* 1993, Eichmann *et al.,* 1996, Cox and Poole, 2000). It induces the cells to produce proteases which break through the basal lamina and allow cell migration into sites where angiogenesis is needed. The cells then proliferate to form new blood vessels (Goodsell, 2003). Vascular endothelial growth factor is a secreted angiogenic growth factor

which is highly specific for endothelial cells (Charnock-Jones *et al.,* 1994). According to Keyt et al. (1996), VEGF is a specific and potent mitogen for endothelial cells. It is known that VEGF is useful in the development of the mammalian pulmonary vascular system (Ferrara, 2000). It is produced by epithelial cells of the airway and deposited in the sub-endothelial matrix (Acarregui *et al.,* 1999). Its carboxyl-terminal domain is necessary for its potency. VEGF receptors are cell membrane receptor (tyrosine kinase) TKs and are characterized by the presence of seven immunoglobulin-like sequences in their extracellular domain. Two members of this receptor class, Flt-1 (designated VEGFR1) and Flk-1 (designated VEGFR2) are structurally similar and have been shown to represent high affinity receptors for vascular endothelial growth factors (Millauer *et al.,* 1993, Peters *et al.,* 1993). Charnock-Jones et al. (1994) demonstrated the presence of flt on non-endothelial cells in vivo and suggested a role for VEGF in the growth and differentiation of cytotrophoblast at implantation. VEGF receptor, the fms-like TK (flt), has been localized only to endothelial cells in vivo (Charnock-Jones *et al.,* 1994).

VEGF-A is said to be the founding member of the vascular permeability factor (VPF)/VEGF family of proteins and its role is critical in tumour angiogenesis (Dvorak, 2002). VEGF-A reprograms gene expression, enhances endothelial cell migration and division and also promotes endothelial cell survival, thereby inducing angiogenesis. FGFs and VEGF are major regulators of mammalian lung development (Hall *et al.,* 2002). They are both mediated by TK receptors and their activities complement each other (Hagedorn *et al.,* 2004) but their effects are opposed by serine-threonine kinase receptor signalling (e.g. TGFß family members) and by bone morphogenetic protein 4(BMP4) (Cardoso, 2001). It has been demonstrated that endogenous basic FGF activates the VEGF/VEGFR system, thereby indirectly inducing angiogenesis and influencing vascular development, both in vivo and in vitro (Tille *et al.,* 2001, Hagedorn *et al.,* 2004). FGFs mediate a multitude of physiological and pathological processes by activating the family of TK receptors (FGFRs) and each FGFR binds to a unique subset of FGFs. Binding specificity between FGFs and their receptors is essential for mammalian

development. Ligand- binding specificity is therefore essential in regulating FGF activity (Chelly *et al.,* 2001). FGFs and VEGF are known to regulate mammalian lung development (Hall *et al.,* 2002). FGF are a family of 23 polypeptides (Rapraeger, 2002). They possess a classical amino-terminal signal sequence and are secreted via the endoplasmic reticulum-Golgi secretory pathway. They activate TK receptors (FGFR) (Franch-Marro and Casanova, 2002). In the lung, FGFs regulate epithelio-mesenchymal interactions (Min *et al.,* 1998) and are useful in the development of organs that develop by branching and budding (Baird and Klagsbrun, 1991, Cardoso, 2001). Four FGF receptors (FGFR-1 to FGFR-4) have been demonstrated in post-natal mammalian lungs at varying levels (Weinstein *et al.,* 1998). Interaction with heparan sulphate (HS) stabilizes and so, limits the diffusion of FGFs into interstitial spaces. This suppresses FGF receptor signalling (Abraham *et al.,* 1986, Kreuger *et al.,* 2005). Moderate levels of basic FGF (FGF2) and VEGF are maintained during the sprouting phase of angiogenesis (Makanya *et al.,* 2007). VEGF and FGFs are mediated by TK receptors. Their activities therefore complement each other (Tille *et al.,* 2001, Hagedorn *et al.,* 2004). Moreover, VEGF A also induces lymphangiogenesis (Dvorak, 2002).

2.4. Role OF FGF7 and FGF10 in Pulmonary Morphogenesis

In the mammalian lung, morphogenesis is however largely attributed to FGF-10 and FGF-7 (Park *et al.,* 1998). FGF7 and FGF10 are both present in pulmonary mesenchymal cells during development. They are expressed in a distinct temporal manner (Cardoso, 2001). They activate (Yeh *et al.,* 2003) and bind with high affinity (Igarashi *et al.,* 1998) to the beta isoform of FGFR2 (FGFR2b). However, unlike FGF 7, FGF-10 also binds to FGFR1 (McKeehan *et al.,* 2009). Moderate levels of basic FGF and VEGF are maintained during the sprouting phase of angiogenesis (Makanya *et al.,* 2007). Distribution of FGF10 occurs in a spatial and temporal manner which is essential for patterning of lung epithelial tubules (Lü *et al.,* 2004). This is critical during both the initial stages of mammalian lung bud morphogenesis and subsequent formation of the bronchial tree (Sekine *et al.,* 1999). FGF 10 initiates lung bud formation and regulates lung bud morphogenesis. In the embryonic mouse lung, FGF10 regulates the

placement and expansion of the lung bud (Bellusci *et al.,* 1996) and FGFR2b signalling is critical for bud morphogenesis (Lü *et al.,* 2004). A study of lung epithelial explants undergoing FGF10-mediated budding in the `absence of other growth factors and mesenchyme showed that FGF 10 acts as a critical regulator of bud morphogenesis in the embryo (Lü *et al.,* 2004). Moreover, signalling by FGF10 and its receptor FGFR2b is also critical for other organs that develop by budding (for bud morphogenesis). Disruption of FGF10-FGFR2b signalling is therefore lethal at birth as a result of multiple organ defects (Lü *et al.,* 2004). FGF-7 is expressed early in lung development (Park *et al.,* 1998) at which time it is detectable throughout the mesenchyme surrounding the developing lung tubules (Finch *et al.,* 1989). It induces type II alveolar cell differentiation in the mammalian lung (Ulich *et al.,* 1994). In the presence of other soluble factors, FGF 7 induces trans-differentiation of the distal end of the trachea into lung (Shannon *et al.,* 1998). Cardoso (2001) demonstrated that development of the mammalian lung requires, in addition to FGF-7, the presence of factors such as endothelial growth factor (EGF), transforming growth factor alpha (TGF α) and hepatocyte growth factor (HGF). FGF 7 and FGF 10 have an evolutionary relationship (Emoto *et al.,* 1997) and have been implicated in the morphogenesis of the mammalian airway (Park *et al.,* 1998). They are expressed early in mesenchymal cells of the developing lung (Cardoso, 2001) and both activate the beta isoform of the type 2 FGFR (FGFR2β) (Yeh *et al.,* 2003). However, very little is known about their roles in avian lung development.

2.5. Epidermal Growth Factor (EGF)

EGF was first reported on by Cohen (1962). He found EGF as an accidental discovery during a study of nerve growth factor (NGF) in mice. Injecting purified extracts of submaxillary gland into new-born mice, caused other gross anatomical changes in nerve cells, in addition to that previously noted; precocious eruption of teeth and eyelids, diminished hair growth and poor growth/stuntedness in the mice (Cohen, 1962). Since then EGF has been found in many body tissues (Zeng and Harris, 2014) such as kidney (Fisher *et al.,* 1989, Harris, 1991), milk and breast during lactation (Dvorak, 2010, Nojiri *et al.,* 2012), in colostrum (Nojiri *et al.,* 2012) and

body fluids such as saliva and blood plasma (Carpenter and Cohen, 1979), maternal urine during pregnancy (Hofmann and Abramowicz, 1990), and amniotic fluid (Hofmann and Abramowicz, 1990). Human EGF has since then been cloned and sequenced (Bell *et al.,* 1986). Colostrum is known to contain many nutritional elements and immunoglobulins, which is clinically significant for the new-born e.g. for protection against infection (Nojiri *et al.,* 2012).

EGF is now known as a prototype member of a peptide family of growth factors that play an important role in epithelial cell differentiation, proliferation and migration (Zeng and Harris, 2014). EGF receptor (EGFR) is a tyrosine kinase activity receptor (Olayioye *et al.,* 2000) whose signalling has been implicated in cancer of the breast, where it is expressed at higher levels than in normal breast tissue (Yao *et al.,* 2012). EGFR is a point of convergence for many growth factors and so when over activated, disrupts the EGFR-dependent signaling networks, leading to cancers in many body organs and areas; including breast, lung, colon, bladder, kidney brain, pancreas, prostate and head and neck (Kolibaba and Druker, 1997). The anti-cancer drug, Ezrin-Radixin-Moesin (ERM)-binding phosphoprotein 50 (EBP50) suppresses EGF-induced proliferation by blocking EGFR signalling in breast cancer cells (Yao *et al.,* 2012). EBP50 is a protein normally found at the apical region of epithelial cells and acts as an adaptor in the modulation of epithelial cell proliferation (Voltz *et al.,* 2001). It is therefore required for the maintenance of active Ezrin-Radixin-Moesin (ERM) at the brush-border membranes of polarized epithelia (Morales *et al.,* 2004). Fouassier et al. (2009) studied EBP50 expression in normal human liver and in human cholangiopathies such as primary biliary cirrhosis, cystic fibrosis and primary sclerosing cholangitis and found that EBP50 is induced by oestrogen since it was found to be elevated in these liver pathologies, but its expression was down-regulated in ovariectomized rats (Fouassier *et al.,* 2009).

2.6. Transforming Growth Factor Beta (TGFß)

TGF-β signalling is of critical importance for normal vascular development and physiology and cellular responses to TGF- are elicited via specific transmembrane type I and type II

serine/threonine kinase receptors (Goumans *et al.,* 2003). An established vascular supply is an absolute requirement for the growth of normal and neoplastic tissues and transforming growth factor-beta-1 (TGF-β1) and its receptors (TGF-βRs I and II are useful not only in embryonic vascular assembly but also in the formation, maturation and maintenance of vessel wall integrity. TGF-β1 null mice present with fragile blood vessels (Pepper, 1997). The transforming growth factor beta also regulates orofacial development. In mammals, the mouth region of the face develops from the first pharyngeal arch (Greene and Pisano, 2005). According to Mukhopadhyay et al. (2006), among the many signalling molecules required for orofacial development, the TGFß superfamily have key roles to play since the genes encoding the different TGFß superfamily members are functionally associated with proteins that are expressed in the developing oro-facial tissues. Moreover, these growth factors also regulate tissue growth and morphogenesis in diverse species of organisms, along with cell apoptosis and formation of extracellular matrix (Moses and Serra, 1996) and TGFß has been implicated in vascular diseases such as acute macular degeneration and cancer. Moreover, TGF beta induces angiogenesis indirectly by enhancing cellular production of VEGF (Dolecki and Connolly, 1991). In order to investigate the role of epithelial-mesenchymal interactions during facial development, pieces of facial tissue were grafted to host chick wing buds and allowed to continue their development in isolation. It was observed that during chick facial development, buds of tissue outgrowth form and differentiates into the specified pattern, of cartilage and bone at a specific developmental period (Wedden, 1987). According to Pardali et al. (2010), through a complex and poorly understood process, TGFβ 1 signalling is able to switch in function, from being angiostatic to pro-angiogenic. This is because TGF- Signalling is complex in that it requires a balance between the two distinct TGF-β Receptor signalling pathways (Goumans *et al.,* 2003). Furthermore, it has been reported that TGFβ, along with several cytokines, have anti-inflammatory effects (Borish and Steinke, 2003).

2.7. Bone Morphogenetic Protein (BMP)

BMP were known as morphogens or moleculite but are now described as metabologens (substances that are able to induce and enhance metabolism and homeostasis). This is because BMPs are involved in fat formation and iron homeostasis (Reddi and Reddi, 2009). BMP is now known to be a subfamily of TGFß and is thus named because it was first characterized in demineralized bone matrix for its in vivo induction of ectopic bone formation (Rittenberg *et al.,* 2005). TGFßs and BMPs function together to regulate target gene expression; their activities balance out gene activities in the regulation of the transcription process of protein synthesis (Wotton and Massague, 2001, Harada *et al.,* 2003).

2.8. Platelet Derived Growth Factor (PDGF)

Like VEGF in humans, PDGF has been found in both developmental and pathological conditions. A study of Paediatric clear cell meningiomas (WHO grade II), which are aggressive but rare tumours, shows progressive excessive receptor expression for PDGF, VEGF and epidermal growth factor (EGF). Targeting these receptors therefore provides possible therapy for these tumours (Juratli *et al.,* 2015). PDGF is expressed in the developing and mature human kidneys and in Wilms' tumor (a cancer of the kidneys that usually affects newborns and the very young, also known as nephroblastoma) (Alpers *et al.,* 1995). Four members of the PDGF family have been identified; PDGFA, PDGFB, PDGFC and PDGFD (Li *et al.,* 2000). PDGFs are potent early developmental mitogens for cells of mesenchymal origin, such as haemoblasts, myoblasts (smooth muscle cells), fibroblasts and glial cells but is also required for tissue maturation such as cell induction, differentiation and patterning formation, thereby being necessary for morphogenesis) (Betsholtz *et al.,* 2001). PDGF was first discovered during a study of factors that stimulate arterial smooth muscle cell proliferation and that it drives cellular responses and promotes the deposition of extracellular matrix (ECM) and tissue remodelling (Ross *et al.,* 1974). Genetic studies on developing mice have demonstrated two

forms of PDGF; PDGFA, PDGFB and their receptors (PDGFRα and PDGFR β respectively) (Gnessi *et al.,* 2000).

While PDGFA and PDGFRα (Platelet derived growth factor receptor alpha) are required for normal embryogenesis, PDGFB and PDGFRβ (Platelet derived growth factor receptor beta) are useful in the development of vascular support cells during angiogenesis (Fruttiger *et al.,* 1999, Karlsson *et al.,* 2000). Moreover, PDGF is implicated in organogenesis (Karlsson *et al.,* 2000) such as in the development of the nervous system and neural crest function. PDGF plays a role in the development of neural and vascular support cells such as the mammalian myelin-forming oligodendrocytes in the central nervous system (CNS) (Leveen *et al.,* 1994). PDGFA is expressed by neurons and astrocytes in the developing spinal cord (Aase *et al.,* 2002). Studies on cellular responses to PDGFs show that they are implicated in mesenchymal function; proliferation, differentiation, and migration during development and also play a role in adult organisms such as Drosophila, Xenopus, zebrafish, Caenorhabditis elegans and mouse in which a PDGF/VEGF-like signalling networks had been identified (Hoch and Soriano, 2003).

2.9. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

GM-CSF, also known as colony stimulating factor 2 (CSF2), is a glycoprotein cytokine produced by cells of the immune system, endothelial cells and fibroblasts (Francisco-Cruz *et al.,* 2014). Cytokines act in an endocrine, autocrine and paracrine manner (Tato and Cua, 2008b), as immunostimulatory agents and may provide interventions against cancer (Vacchelli *et al.,* 2013). The immune system of an individual seems to play an important role in oncogenesis and the spread of cancer (Vacchelli *et al.,* 2013). Though tumour cells limit immunity, it has been recognized that the body's immune system retains some capacity to fight cancers such as prostate cancer. Moreover, in development, GM-CSF is produced by cells of the genital tract and acts as an embryonic cytokine (embryokine) (Hansen *et al.,* 2014) which regulates (facilitates) the development of the immune system. GM-CSF stimulates stem

cells to produce granulocytes (neutrophils, eosinophils, and basophils) and monocytes (Francisco-Cruz *et al.,* 2014), as part of the immune or inflammatory response (Voehringer, 2012) to threats such as infection. GM-CSF activates the production of two related proteins STAT5A and STAT5B (Voehringer, 2012). These proteins are part of the 7-membered STAT family, but they are encoded by separate genes (Grimley *et al.,* 1999). Moreover, GM-CSF activates macrophages to act through STAT3. STAT3 is antifungal since it causes zinc starvation and so, lethality to the pathogen (Vignesh *et al.,* 2013). DNA recombinant GM-CSF was first used in 1987, to treat accidental cesium irradiation in Goiania, Brazil (International Atomic Energy Agency, 1989). Molgramostim and Sargramostim are two forms of GM-CSF of yeast origin that have been used to stimulate cancer-specific immune responses. They are marketed as protein therapy (Vacchelli *et al.,* 2013).

2.10. Some Growth Factors That Regulation Animal Development

Many genes have been implicated in limb bud development e.g. retinoic acid and sonic hedgehog gene (SHH) (Riddle *et al.,* 1993), Hoxa complex-20 and 21 (Charité *et al.,* 1994) and fibroblasts growth factors (FGFs) -14-17 and BMP-23 (Francis *et al.,* 1994). In the chick embryo, retinoic acid is needed for initiation of limb bud outgrowth (Stratford *et al.,* 1996).

Limb buds are formed through interactions between mesoderm and ectoderm. Proximo-distal patterning of the limb bud is regulated by “Apical Ectodermal Ridge (AER), formed from limb bud ectoderm through induction by the underlying limb bud mesoderm. AER facilitate cell proliferation. Mesoderm provides cell signals for dorsal and ventral patterning. The fore limbs are notable in the lateral plate mesoderm, adjacent to 16-20th somites within 50 hours of incubation and cartilaginous wing models form by day 10. The hind limbs form later at about 72 hours of incubation and they form as buds jutting out from the flanks (Choi *et al.,* 2013). On the other hand, dorsal ectoderm regulates the dorso-ventral polarity and signals from the ectoderm control dorso-ventral positioning of distal limb structures. Anterior-posterior (A-P) limb alignment is regulated by the Zone of Polarizing Activity (ZPA). Wolpert theory suggests

that the ZPA produces diffusible morphogen whose concentration determines A-P position (Choi *et al.,* 2013). The same is the case with sonic hedgehog (SHH) with Lewis Wolpert's French flag model; SHH diffuses to form a concentration gradient thereby exerting different effects on embryonic cells (Marigo *et al.,* 1995).

Two growth factor signalling centres have been identified in the definition of the anterior-posterior axis of the avian embryo; one in the primitive node and one in the anterior visceral endoderm. In contrast to amphibians, the relatively large yolks of avian and reptilian embryos impede the organizer in that it is not able to move from anterior to posterior poles in order to lay down the dorsal axis around the vegetal hemisphere. Embryonic axis therefore begins and ends at the same side of the egg (Arendt and Nübler-Jung, 1997, Arendt and Nübler-Jung, 1999). Chick egg is large, very yolky and telolecithal. Telolecithal or macrolecithal describes uneven distribution of yolk in the cytoplasm of eggs, as is found in birds, reptiles, and fish. The yolk is concentrated at one pole of the egg, separate from the developing embryo. The chick blastodisc has two developmental areas which have been described: a light area called the area pellucida and its surrounding, a dark ring called the area opaca (Wolpert *et al.,* 1998).

2.10.1. Hox genes

Hox genes are also called homeobox genes or homeotic genes (called thus in that they regulate anatomical structures). They were first found in drosophila but are also found in vertebrate central nervous systems, including humans. Hox Genes specify limb bud position (Davis *et al.,* 1995). Hox and parahox genes regulate segmentation (formation of repeated segments such as the vertebral column or division of an organism into repeated segments) in plants and animals. Hox gene is expressed in limb buds along proximal-distal axis (Izpisua-Belmonte *et al.,* 1991), with 3′ gene expressed first (most proximally) and 5′ genes expressed last (most distally) and targeted disruption of Hox-D11and Hoxa-11 led to loss of the radius and ulna in mouse forelimbs (Davis *et al.,* 1995). Moreover, ectopic limbs have resulted from manipulation of Hox (Hox b9, c9, d9) gene expression patterns in the lateral plate mesoderm (Cohn *et al.,* 1997). Hox genes pattern the anterior-posterior axis and help to specify positions

along the axis; its expression occurs along the dorsal axis in the neural tube, neural crest, paraxial mesoderm and surface ectoderm, extending from the anterior boundary of the hindbrain through the tail. For example, expression of different Hox genes specify different vertebrae type: In mice: seven cervical (neck), 14 thoracic (attached to the rib), 6 lumbar or abdominal vertebrae, 4 sacral but variable number in the coccygeal or tail region. This is so important that Hox gene knockout or changes in Retinoic Acid gradients (Hox genes are sensitive to levels) lead to segment-specific malformations and alteration in the vertebra identity e.g. lack of distinguishing vertebral features.

2.10.2. Transforming growth factors (TGF)

Balemans and Van Hul (2002) reported on a cocktail of modulators during vertebrate development, among which are the transforming growth factor (TGF) super-family. TGFs have important functions during embryonic development, particularly in pattern formation and tissue specification, exerting a wide range of biological effects on several tissue types in both vertebrates and invertebrates. Moreover, in adult tissues, TGFs enhance tissue repair, and are involved in processes such as wound healing, bone repair and remodelling.

2.10.3. Retinoic acid

Retinoids are known teratogens. Retinoic acid (RA) is a metabolite of retinol (vitamin A) that mediates the functions of vitamin A required for growth and development of organisms, ranging from fish to humans. It is an important morphogen for the development of many body systems (Tickle *et al.,* 1982) and has been found in the developing chick limb bud, excess of which induces limb duplications (Stratford *et al.,* 1996). RA is required for specification of embryonic anterior-posterior axis and for limb bud initiation, in zebrafish, chick and mouse embryos (O'Neil *et al.,* 2001). A posteriorizes embryos and mimics ZPA activity in limb buds (Tickle *et al.,* 1982, Niederreither and Dollé, 2008). Stratford et al. (1996) demonstrated that stage 20 and 21 limb bud synthesizes didehydroretinoic acid (ddRA), with the posterior half of the limb bud synthesizing ddRA at a higher rate than the anterior half. Mammals have numerous receptors for retinoic acid: retinoic acid receptor (RAR)-alpha, RAR-beta, and RAR-

gamma, each with splice variants, and RXR (retinoid X receptor), as well as a cellular retinoic acid binding protein (CRABP). RARs and RXR all belong to the type II nuclear hormone receptor family. Retinoic acid diffuses across plasma membrane into the cell and into the nucleus to bind to the RAR/RXR heterodimeric receptor, binding specifically to DNA (Niederreither and Dollé, 2008). Implants of beads coated with RA produces mirror-image duplications like ZPA grafts. RA is normally present in limb buds at 25 nM concentration and a concentration of 20 nM RA applied exogenously will cause duplication of digit. The posterior aspect of the embryo has a higher concentration of RA; up to 50 nM (Niederreither and Dollé, 2008). Stratford et al. (1996) administered the RA antagonist, disulphiram, to the ectoderm over the prospective limb bud of embryos before limb bud outgrowth (stages 12–18) and found no limb developed in the majority of cases. However, treatment with RA at later stages of chick limb development showed no morphological change (Helms and Wetzel, 1996). Treatment with disulphiram suppressed in addition to RA activity, sonic hedgehog (SHH) expression. In the absence of ligand (RA), RAR/RXR heterodimers bind transcriptional repressors. It is uncertain if RA is really the morphogen produced by the ZPA or if it induces formation of ZPA. However, implants of RA beads result in formation of ZPA, not around the bead, but distal to the bead, and only 16-24 hours after implantation (Niederreither and Dollé, 2008).

2.10.4. Sonic hedgehog

The sonic hedgehog gene (SHH) is well studied. It was first discovered in Drosophila melanogaster (fruit-fly) by Christiane Nüsslein-Volhard and Eric Wieschaus, as published in 1980 (Nüsslein-Volhard and Wieschaus, 1980). It is one of three proteins in the family called hedgehog found in mammals (the other two being desert hedgehog (DHH) and Indian hedgehog (IHH). SHH regulates vertebrate organogenesis such as brain development and growth of digits on the limbs but is also useful control of adult stem cells having been implicated in the development of some cancers (Marigo *et al.,* 1995). Smith-Lemli-Opitz Syndrome (SLOS) which is a rare hereditary autosomal recessive disorder is caused by deficiency of the enzyme, 7-dehydrocholesterol reductase, resulting in impaired cholesterol synthesis.

Reduced sonic hedgehog (SHH) signaling pathway has been associated with possible pathogenesis of a link between SLOS and brain tumours. A recent study by Aslan et al. (2017) reported for the first time, revealed coexistence of SLOS with intracranial undifferentiated malignant neuroglial tumor, at WHO grade 4, in a 10-year old child.

Figure 2.0:2: Congenital holoprosencephaly and Anencephaly respectively

Wada et al. (2005) suggested a novel role for SHH signalling in neural crest cell migration at the midline and their differentiation into cartilage. This finding throws light on why interference with SHH signalling results in failure of both skeletal fusions and incidence of cleft palate, as is found in holoprosencephalic human embryos. Normally, forebrain formation precedes development of the face, in the fifth and sixth weeks of human development.

2.10.5. WNT Gene

The WNT gene family are structurally related genes that regulate signalling glycoproteins that are 350–400 amino acids (Cadigan and Nusse, 1997). They are proteins involved with developmental processes such as regulation of cell fate, cell proliferation, cell migration, pattern formation (e.g. body axis patterning) and are therefore particularly important in bone,

heart and muscle development. WNT signalling pathways use either nearby cell-cell communication (paracrine) or same-cell communication (autocrine) and are conserved in animal species, from fruit flies to humans (Nusse, 2005). WNT genes were first discovered due to their role in carcinogenesis and have induced breast cancer in mice. WNT10A is over expressed in the cell lines of Burkitt's lymphoma, promyelocytic leukemia and WNT 6 gene in colorectal cancer cell lines (Kirikoshi *et al.,* 2001). Moreover, studies on mutations led to the finding that WNT genes are useful in embryonic development and tissue regeneration in adult bone marrow and skin (Goessling *et al.,* 2009). WNT signalling has also been implicated in type II diabetes. WNT-proteins act through the Frizzled family receptor, which passes the biological signal to the protein dishevelled inside the cell (Logan and Nusse, 2004).

2.10.6. Signal Transducer and Activator of Transcription (STAT)

STAT stands for Signal Transducer and Activator of Transcription. Aaronson and Horvath (2002) described the JAK-STAT signalling pathway as a cascade consisting of a cell surface receptor called Janus kinase (JAK) and two Signal Transducer and Activator of Transcription (STAT) proteins. The JAK are protein tyrosine kinases (TKs) mediated by cytokines (Rane and Reddy, 1994). Various ligands bind to the surface receptors to activate the Janus kinase (JAK), increasing their kinase activity. Three members of the family (JAK1, JAK2, and JAK3) have been described (Brooks *et al.,* 1994, Brooks *et al.,* 2014). The JAK-STAT pathway transfers extracellular chemical signals to the nucleus, thereby inducing DNA transcription and expression of genes that regulate cell proliferation, cell differentiation, immunity, apoptosis and oncogenesis (Aaronson and Horvath, 2002). STAT is a 7-Membered protein (transcription factors) family which mediate cytokine activity (Aaronson and Horvath, 2002) such as Granulocyte Colony Stimulating Factor (GCSF). STAT transmit signals for both cellular proliferation and differentiation. JAK3 was identified by RT-PCR of rat mesangial cells (Takahashi and Shirasaw, 1994) and is phosphorylated by receptor-associated JAK, thereby forming homo or heterodimers which translocate into the nucleus where it activates transcription.

Activation of JAK3 pathway has been associated with granulocytic differentiation of myeloid precursor cells (Rane *et al.,* 2002). It is now known that STAT3 becomes activated after phosphorylation of tyrosine 705 in response to ligands such as epidermal growth factor (EGF), Interleukin (IL-) 5, IL-6 and interferons (Takahashi and Shirasaw, 1994). In humans, STAT3 is encoded by the STAT3 gene, mutation of which has been associated with recurrent infections and hyper immunoglobulin E syndrome. Affected people present with auto-immune diseases in many organs resulting in diabetes and thyroid disorders (Milner *et al.,* 2014). In addition, bone disorders and tooth development have also been reported (Levy and Loomis, 2007). STAT5A and STAT5B, which are related proteins in the family, mediate signals for a broader spectrum of cytokines that are also involved in the immune response. Functional disorder of the JAK-STAT pathway results in immune deficiency syndromes and cancers (Akira *et al.,* 1994).

2.10.7. Testis-determining factor (TDF)

Testis-determining factor (TDF), also known as Sex determining region Y (SRY) is a DNA-binding protein that is encoded by the SRY gene. SRY gene is the mammalian Y-linked testis-determining gene (Wallis *et al.,* 2008) that initiates sex determination in humans (Berta *et al.,* 1990, Wallis *et al.,* 2008). TDF, also known as SOX2, belongs to the SOX (SRY-like box) gene family that has the ability to upregulate other transcription factors, e.g. SOX9 during chondrocyte differentiation (Kashimada and Koopman, 2010) and in the regulation of Anti-Müllerian hormone (AMH) activity in humans. AMH is encoded by the AMH gene and although its receptor is expressed in both male and female fetuses, the expression of this hormone is limited to male sertoli cells (Rey *et al.,* 2003); suppressing the development of the paramesonephric ducts (Müllerian ducts) in male embryos (Behringer *et al.,* 1994). Furthermore, SRY gene regulates self-renewal, or pluripotency of undifferentiated embryonic stem cells, and enhances maintenance of embryonic and neural stem cells (Chuang *et al.,* 2015). SRY also encodes a protein with 79 amino acid motif, called the High Mobility Group box (HMG box), a protein domain which is involved in DNA binding (Štros *et al.,* 2007), and

particularly causing the sequence-specific DNA binding in SRY and other known transcription factors (Giese *et al.,* 1992).

2.10.8. SOX

The Sox gene family are expressed during mouse development (Wright *et al.,* 1993) and in a number of other species, both vertebrate and invertebrate (Niwa *et al.,* 2009, Johansson and Simonsson, 2010). Takahashi and Yamanaka (2006), discovered that Sox2 in conjunction with other growth factors, caused induction of pluripotent stem cells in mouse embryonic and adult fibroblast cultures. Since then, it has been found that SOX2 genes improve dental cell proliferation, migration and adhesion by regulating gene expression about cell cycle, migration and adhesion, a novel strategy that has been applied in tissue engineering to develop seed cells with strong proliferation, migration and adhesion (Liu *et al.,* 2015). It has been documented by Masui et al. (2007) that the primary role of SOX2 in embryonic stem cells is to regulate the expression of another growth factor called Oct4 (octamer-binding transcription factor 4). Oct4, which is also known as POU5F1 or POU domain, class 5, transcription factor one is a protein which, in humans, is encoded by the POU5F1 gene. SOX2 regulates Oct4 expression in a way that they both perpetuate their own expression whenever they are expressed concurrently (Rizzino, 2009). Anomaly in SOX2 expression has been found in squamous cell carcinoma of the oesophagus and high SOX2 expression has been associated with spread of early oral SCC into the lymph nodes (Chuang *et al.,* 2015).

2.10.9. Guanine nucleotide-binding proteins (G-proteins)

G proteins are a protein family that belong to the larger group of enzymes called GTPases. They are also called guanine nucleotide-binding proteins from their ability to bind to, and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP). G proteins act by binding to G protein-coupled receptors (GPCRs) that span the cell membrane, activating the intracellular GPCR domain which, in turn, activates the particular G protein (Qin *et al.,* 2011). They therefore act as intracellular molecular switches in the transmitting of signals across the cell membrane. When bound to GTP, they are switched 'on', and, when they are bound to

GDP, they are 'off'. There are two classes of G proteins: monomeric small GTPases and heterotrimeric G protein complexes that are made up of alpha (α), beta (β) and gamma (γ) subunits (Hurowitz *et al.,* 2000).

2.10.10. Platelet-derived growth factors (PDGFs)

PDGFs are further discussed in the next chapter. Recent advances in genetic manipulation show cellular responses to platelet-derived growth factors (PDGFs) during animal development. PDGF enhance mesenchymal proliferation, cell migration, differentiation during development and in the adult animal. The genomic sequence led to the identification of new PDGF and PDGF receptors in mice, Drosophila, C. elegans, zebrafish and Xenopus. PDGFRs are TK receptors (Hoch and Soriano, 2003).

2.10.11. Chicken Gonadal and Embryonic Development – MHM gene

MHM gene is a chicken Z chromosome locus that is expressed in females but is methylated and silenced in males. It is useful in sex determination and gonadal development. Using different variants of mis-expression of the sense and antisense strands, the role of chicken Z chromosome-linked locus MHM has been found to be an integral part of a chicken's normal embryonic and gonadal development. While MHM is methylated and transcriptionally silent in male cells, in female sex cells, it is hypo-methylated and transcribed into a long non-coding RNA (Roeszler *et al.,* 2012).

2.10.12. Insulin-like growth factor-I (IGF-I)

Insulin-like growth factor-I (IGF-I) is a neural marker that regulates neurogenesis. IGF-I promotes adult neurogenesis by regulating activation of quiescent neural stem cells (NSCs), influencing not only the number and differentiation of the NSCs but also their positioning and migration of nerve cells (Nieto-Estévez *et al.,* 2016). It has been found that IGF-I administration causes pleiotropic or multiple effects on cellular processes. The degree of finding depends on the concentration used, the developmental stage of the organism and the type of body cell (Takata *et al.,* 2016). Also, IGF-I expression has been reported in the liver at E19 and E20

and a higher concentration at an even earlier developmental stage (E15 to E18) was found in the developing muscle, peaking on E17, after which IGF-I levels decline (Liu *et al.,* 2016). A study of both in vitro and in vivo models showed that IGFI enhances the maintenance and proliferation of NSCs progenitors and neuron integration in synaptic circuits (Nieto-Estévez *et al.,* 2016).

2.11. Experimental Embryology

Chick and mouse embryos have been used as models for the study of early development of vertebrates (Behringer *et al.,* 1994, Rashidi and Sottile, 2009). According to Gilbert (2000) the major developmental stages in mice include:

1. Rotational cleavage (with formation of the blastocyst),
2. Implantation,
3. Formation of embryonic disc,
4. Development of extra embryonic tissues,
5. Formation of the germ layers and gastrulation (with pattern formation, such as seen in the patterning of the body axes and degeneration of the primitive streak.).

In mouse embryo, gastrulation begins on day 6 and lasts for 1-2 days after fertilization. It begins with formation of the primitive streak. It is at this time that the future posterior pole is known. Mesoderm formed from cells of the primitive streak move through the streak and spread cranially and laterally between the ectoderm and the visceral endoderm. The definitive endoderm hypoblast forms later from cells that migrate through mesoderm. These cells also give rise to the primitive gut. Neurulation occurs in the late embryo (8-12 days after fertilization) at which time the gut endoderm canalizes, and the embryo undergoes a complex conformational change and become enclosed in amniotic membrane within amniotic fluid. Gastrulation is completed by day 9 after fertilization (Wolpert *et al.,* 1998). Avian development (unlike in mice and rats) can be directly observed if a small window is cut in the egg shell.

Moreover, the total developmental period is only 20-21 days (Hill, 2018). Gilbert (2000) described the major features that are seen in early chick development:

1. Discoidal cleavage with formation of a three¬-layered blastodisc
2. Formation of the primitive streak
3. Gastrulation
4. Regression of the primitive streak
5. Formation of embryonic axis.

Nevertheless, chick embryos are not that accessible since they are usually embedded in egg yolk. Moreover, cleavage pattern is influenced by the presence and location of yolk and cleavage consists of many processes that are fundamental to avian development e.g. zygotic gene activation (ZGA) and increases the thickness of the blastoderm as its cell-layer increases. During the period of chick development, the number of cells increases from 1 to about 2000 (Park *et al.,* 2006). This may be the reason why not much is known about chick embryogenesis, particularly about the stages involved in cleavage.

The relationship between stages of development and days of incubation at 39 ^{0}cC was described by Hamburger and Hamilton (1951). This, and the early stage timing (Carnegie) are often adopted in experimental research; besides being easy for surgical manipulation, the chick embryo serves as a good surrogate for human embryos since its organs form in ways that are very similar to those of mammals (Gilbert, 2000).

CHAPTER THREE

3.0. EXPRESSION PATTERN OF VEGF IN THE DEVELOPING AVIAN LUNG (*GALLUS GALLUS* VARIANT *DOMESTICUS*)

The molecular regulation of Lung development has been discussed in the preceding chapter.

In this chapter, *endogenous VEGF expression in the developing avian lung is studied in order to ascertain the onset of VEGF expression. In the second work (chapter Four), having noted the onset of VEGF expression in the lung, fertile eggs will be incubated until the onset of VEGF expression, and treated with the respective drugs (n=6) and angiogenesis in their CAM will be observed and analysed. The aim is to evaluate the test drug, Efavirenz, a first line ARD used in both HAART and combination therapy for type 1 HIV infection. The anti-angiogenic (and so, anti-cancer) properties of Efavirenz will be compared to the known anti-angiogenic/anti-cancer agent (thalidomide as the negative control drug). And the angiogenic drug, VEGF will serve as the positive control drug). While Control 1 group is the solvent control (for they will be treated with the diluent alone), Control 2 subjects will have uninterrupted, and so, normal embryonic development.*

3.1. **Ethics:** This work was done under at the University of the Witwatersrand Medical School (School of Anatomical Sciences and School of Clinical Medicine) under ethics clearance No. 2008/7/1 (see Appendix section),. The University subscribes to international ethical guidelines for research, in accordance with the NIH "Principles of Laboratory Animal Care" (NIH publication, 1985). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the institution.

3.2. **Problem statement:** The general structure and phylogenetic variations of the avian lung have been described (e.g. Duncker, 1971, 1972; Maina, 1982, 1993, 2000; Maina and King, 1982; Maina and Nathaniel, 2001) but lung development in birds has received limited scientific investigation. Makanya et al. (2007) described the development of the avian lung as “a complex and little-studied process” when compared to the mammalian lung with its wealth of information. In other words, there is a lack of data on avian lung morphogenesis (e.g. Maina, 2003a, 2003b, 2005, 2006; Maina *et al.,* 2003; Makanya, *et al.,* 2007). Furthermore, the commonest cause of death in poultry farms results from respiratory diseases. One can therefore say that avian respiratory research will contribute to knowledge, and lead to techniques for improving, treating and maintaining poultry farming (Jacob *et al.,* 2013).

3.3. Justification: The avian lung is anatomically different and functionally more efficient than the mammalian lung. Many researchers agree that there is a general lack of data on the molecular aspects of its development (Maina, 2003a, Maina, 2003b, Maina *et al.,* 2003, Maina, 2005, Maina, 2006, Makanya *et al.,* 2007).

3.4. **Aim:** Thus the aim of this work is to characterize the pattern of endogenous VEGF expression at stages 19-40 of the avian lung (the domestic fowl, *Gallus gallus* variant *domesticus*).

3.5. Objectives

1. To ascertain the age at which lung buds form from the foregut progenitor
2. To study the growth and branching pattern of the airway.
3. To ascertain the onset of VEGF expression, using routine immunofluorescent technique in order -

4. To determine the embryonic stage at which anti-angiogenic drugs would be most effective (in the inhibition of pulmonary angiogenesis.

3.6. Materials-

- Fertile eggs
- Incubator
- Dissecting microscope
- Microscope camera
- Magnifying glass
- Egg bowls
- Wooden wax dish
- Dissecting kit
- A Fine hand saw (for cutting observation windows into egg shells)
- Chick Ringer
- Reagents
- Pencil or marking pen
- Study Materials e.g. H & H staging manual

3.7. Methodology

3.7.1. Care of the Eggs

Freshly laid fertile eggs of the New Hampshire breed of chicken (*Gallus gallus* variant *domesticus*) and of the same clutches were used. Embryos were harvested and tissues were processed in the School of Anatomical sciences. All the eggs used for this work were obtained through the Central Animal Unit of the University of the Witwatersrand based within the Faculty of Health Sciences. They were incubated in thermostatically regulated incubators. Humidity was provided through a sponge placed in a bowl of water. The eggs were manually turned through 180^0 twice daily. And as the eggs matured, they were harvested at the appropriate stages (as estimated from the days of incubation). All reagents were prepared fresh and sterilized.

3.7.2. Light microscopy of H and E sections

These were first done to ascertain the extent of lung tissues within thoracic segments obtained from avian embryos at stage 19-40. The presence or absence of the following structural parameters was noted:

1. Growth and branching pattern of the lung buds
2. Formation of definitive lungs
3. Formation of the air sacs; the type(s) formed (e.g. cervical, interclavicular, thoracic or abdominal) and their sizes
4. Formation of blood vessels
5. Formation of the bronchial system:
    - Extra-pulmonary primary bronchi (EPPB)
    - Intra-pulmonary primary bronchi IPPB
    - Secondary bronchi (SB)
    - Parabronchi (PB)

3.7.3. Standard immunofluorescent technique

By standard immunofluorescent technique, expression of VEGF in lung sections was assessed in both the mesenchymal and endodermal cell compartments. Immunofluorescence staining was done on different days at both the Department of Anatomy and Internal medicine, but fluorescence microscopy was carried out in the Department of Internal Medicine. The Immunofluorescence scoring sheets were obtained from the School of Anatomical sciences of the University of the Witwatersrand. Antibodies were secured as indicated e.g. from Santa Cruz Biotechnology. South Africa.

3.7.4. Experimental Groups

Lung buds at stages 19-40 of development (Hamburger and Hamilton, 1951) were studied. Lung bud appears on day 3.5 (Maina *et al.,* 2003) which corresponds to Hamilton and Hamburger's stage 21. In order to cover all aspects of the lung development, the study started with younger embryos (at stage 19). The embryos were staged and grouped as follows:

- Group 1. Stages 19-21 (n=6)
- Group 2. Stages 22-24 (n=6)
- Group 3. Stages 25-27 (n=6)
- Group 4. Stages 28-30 (n=6)
- Group 5. Stages 31-33 (n=6)
- Group 6. Stages 34-36 (n=6)
- Group 7. Stages 37-40 (n=6)

3.8. Tissue Processing

42 eggs were used in the first aspect of this work (H & E sections and Immunohistochemistry) while 30 eggs were used for the second aspect of the work (angiogenesis). The embryos were processed as follows:

The shells were cracked, and the embryos were harvested and immersed in warm chick ringer (see Appendix section) at 37^{o}C. The embryos were then staged using Hamburger and Hamilton's staging for chick embryos (Hamburger and Hamilton, 1951, Hamburger and Hamilton, 1992).

3.8.1. Fixation

The developing lung buds were dissected out, fixed overnight by immersion in 4% Para formaldehyde. Embryos at the early stages (stages 19- 29) were fixed whole and the thoracic segments were excised, processed and sectioned for the study. Tissue Processing

The tissues were fixed in 4% paraformaldehyde overnight (24 hours). Because of this, the 1st station (fixative) of the processor was skipped (since the tissues were presented after fixation). The tissues were individually placed in two-piece cassettes with 0.67 mm pores, 45-degree writing surface and a secure latch closure. The cassettes were then placed in the tissue basket which automatically is rotated through the reagent stations: Dehydration and Clearing in graded concentrations of alcohols (70%, 95% absolute alcohol), passed through two changes of chloroform and cleared in 2 changes of xylene. After clearing, the tissue basket is immersed in melted paraffin wax) ready for blocking.

3.8.2. Paraffin sections

Paraffin sections of 5µm thickness were obtained using a rotary microtome (Leica 1400, Leica Instruments GmbH, Germany). For optimal sectioning, the blade was changed after every 30-50 sections. Paraffin sections were floated on a pre-heated water bath at 40 ^{o}C and left for a few seconds to straighten out and then with a gentle sweep action, the floating sections were picked up on clean frosted pre-labelled glass slides. With care not to trap bubbles, the slides were placed at an angle on a hot plate pre-set at 60 ^{o}C for about one hour. Dried slides were stored in slide boxes.

3.8.3. Haematoxylin and Eosin Staining

Every 10th section was stained with H & E. This was to ascertain the extent of the developing lung within the thoracic segment processed. Only sections with lung tissue were immunolocalized for VEGF. Staining protocol for Haematoxylin and Eosin (H & E) was as follows:

Tissue sections were incubated at 60^{o}C overnight and deparaffinized in two changes of 100% Xylene for 10 minutes each. They were then rehydrated in graded series of alcohol (100%, 95%, and 70%) each, for 1 minute, brought to distilled water, stained with haematoxylin for 5 minutes and rinsed in running tap water for 5 minutes. Sections were differentiated by dipping three times in 0.3% acid alcohol, rinsed by dipping in running tap water, stained with eosin for 1 min, again rinsed by dipping in running tap water, dehydrated in graded series of alcohol (70, 95 and 100%)

1 min each and cleared in Xylene. They were then mounted on glass slides in Entellen and allowed to dry at room temperature.

3.9. Standard Immunofluorescent Technique

3.9.1. **Antigen Unmasking**: The sections adjacent to H and E sections that had lung tissues were stained for VEGF. Sections were placed in a Coplin jar filled with citrate buffer with pH of 6.0 and heated in a standard microwave oven at medium heat (720W) for 5 min, top up buffer was added when necessary and it was heated for another 5 min. The sections were allowed to cool down for 20 minutes and rinsed in PBS (pH 7.6) in the presence of a magnetic stirrer for 5 minutes (Maina *et al.,* 2003). (For preparation of PBS, see Appendix section; chapter 8).

3.9.2. **Cell Permeabilisation**: This was done to facilitate tissue staining and to prevent nonspecific staining. Sections were blocked by non-immune serum for 25 min at room temperature and placed in 0.1% Triton-X100 in 0.1% Bovine Serum Albumin/Phosphate Buffered Saline (BSA/PBS) for 10 min. After this they were rinsed in 3 changes of 0.1% BSA/PBS for 5 min each, in the presence of a magnetic stirrer.

3.9.3. Antibodies

Table 2.0:1: List of Antibodies used

Antibody	**Description**	**Dilutions**
Primary Antibody (Santa Cruz)	Polyclonal rabbit anti-VEGF antibodies	1: 100
Secondary Antibody (Santa Cruz)	Anti-phosphotyrosine (4G10) – (Alexa Fluor® 568 (Goat Anti-Rabbit IgG; (Molecular Probes).	1: 300
Nuclear stain (DAPI	DAPI (Boehringer Mannheim)	1:10000

Fluorescence Microscopy & Imaging	Sections were then viewed on an Olympus IX71 inverted fluorescent microscope and the Images captured using Olympus Analysis Software.	

Primary antibody: Antibody concentration was predetermined through antibody optimization according to the manufacturer's protocol. Rabbit VEGF primary antibodies (VEGF, A-20: sc 152, Santa Cruz Biotechnology, Inc. USA, sc-2012, diluted with 0.1%BSA/PBS, 1:100 µl); according to the recommended dilution range (1:100-1:400). The slides were then incubated in a moist chamber overnight in the fridge at 40 C.

Secondary antibody: The next morning, the sections were washed in three changes of 0.1% BSA/PBS for 5 min. each and 50µl of the secondary antibodies (Anti-phosphotyrosine (4G10) – (Alexa Fluor 568) diluted in PBS was added to the sections and incubated for 1 hour at room temperature after which the sections were washed in three changes of 0.1% BSA/PBS for 5 min. each in foil covered glass containers and then rinsed in PBS. The nuclear stain (DAPI, dilution of 1: 100) was added to the sections for 10 min and rinsed off in PBS. The sections were mounted with cover slips using an aqueous mounting medium and images captured using a fluorescence confocal microscope.

3.9.4. Controls

Negative controls: Sections were incubated with primary antibody but without secondary antibody was replaced with 100µl of 0.1% BSA/PBS.

Positive controls: Positive controls were performed on histological sections of pregnant mouse uterus (5µm thickness) (Mowa *et al.,* 2004, Nguyen *et al.,* 2012).

3.10. Results

3.10.1. Results: H and E Sections

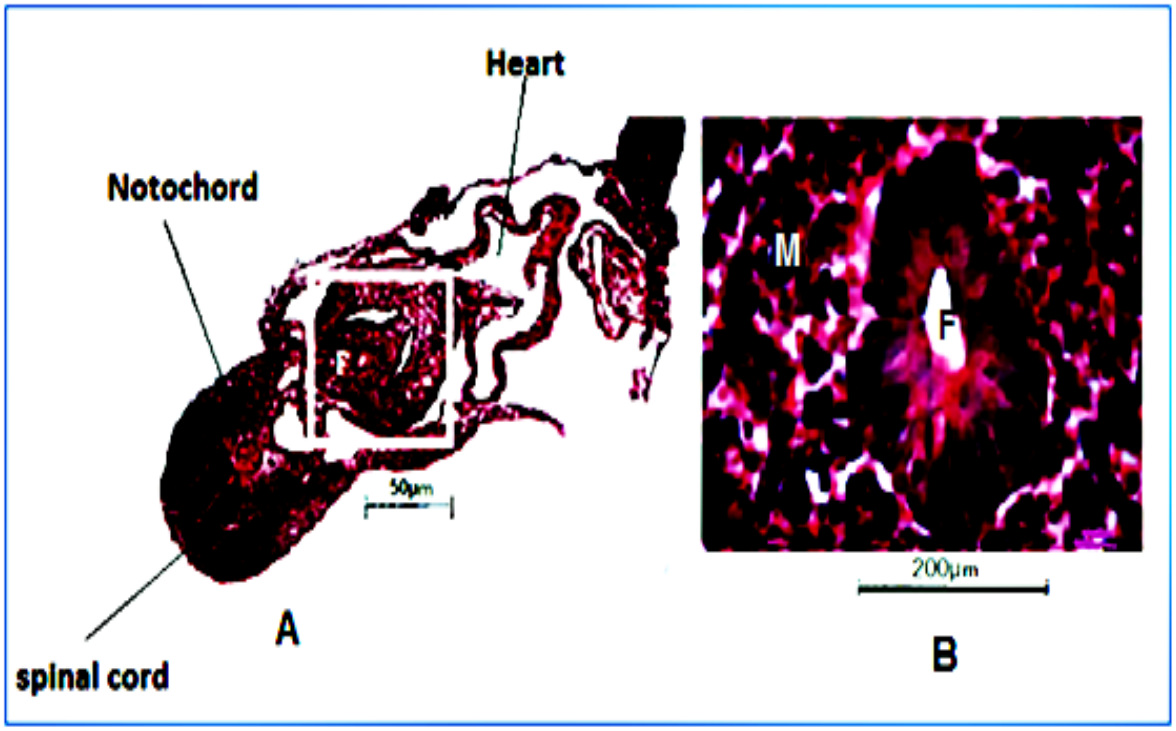

Figure 3.1. Stage 20 of lung development, showing the foregut tube. **Figure A is a cross section through the thoracic region of a stage 20 embryo. X50. The primitive foregut tube (F) is seen just dorsal to the developing heart but ventral to the notochord and spinal cord. Figure B shows the highlighted area in figure A. X200. It shows the foregut epithelial tube lying in surrounding mesenchyme (M).**

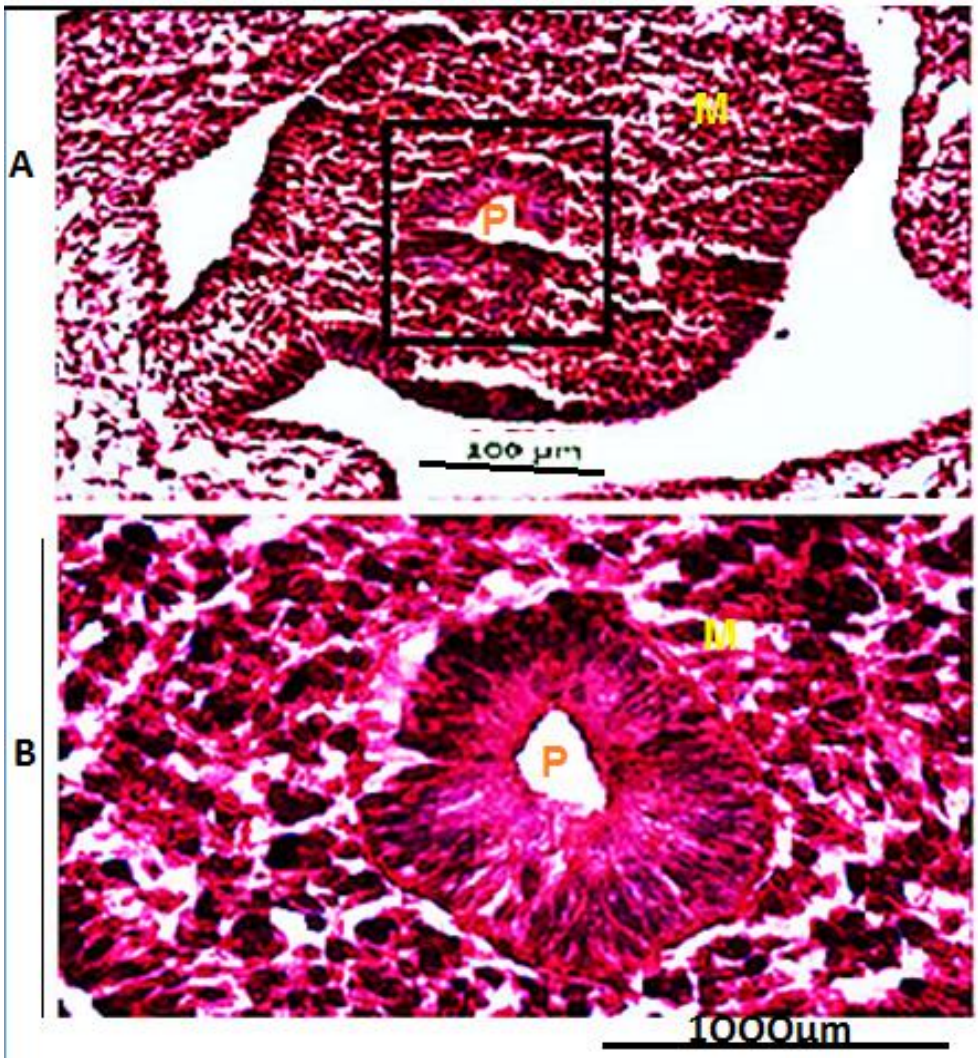

Figure 3.2. This shows the primary bronchus (P) within the lung bud mesenchyme (M). Figure 14B shows an epithelial primary bronchus (P) surrounded by lung bud mesenchyme (M) at a higher magnification (X1000).

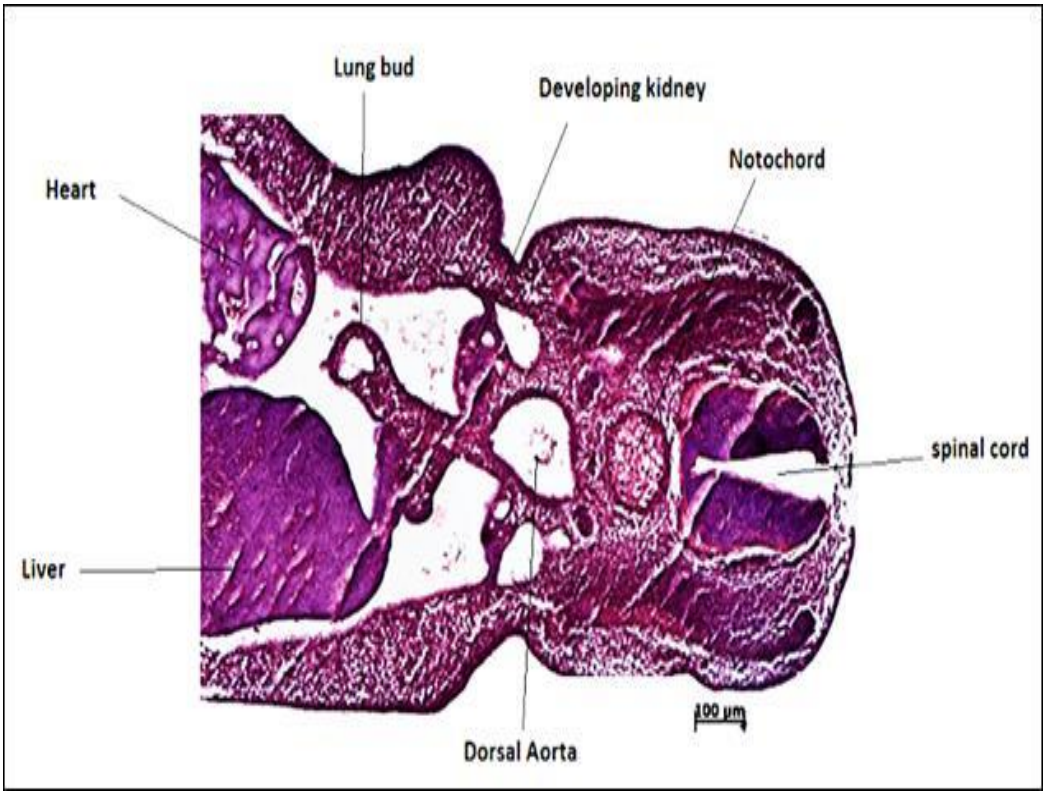

Figure 3.3. At stage 22 Embryo. A cross section through a stage 22 embryo (X100 magnification). At stage 22 both left and right lung buds are distinctly formed and lie just ventral to the developing kidney.

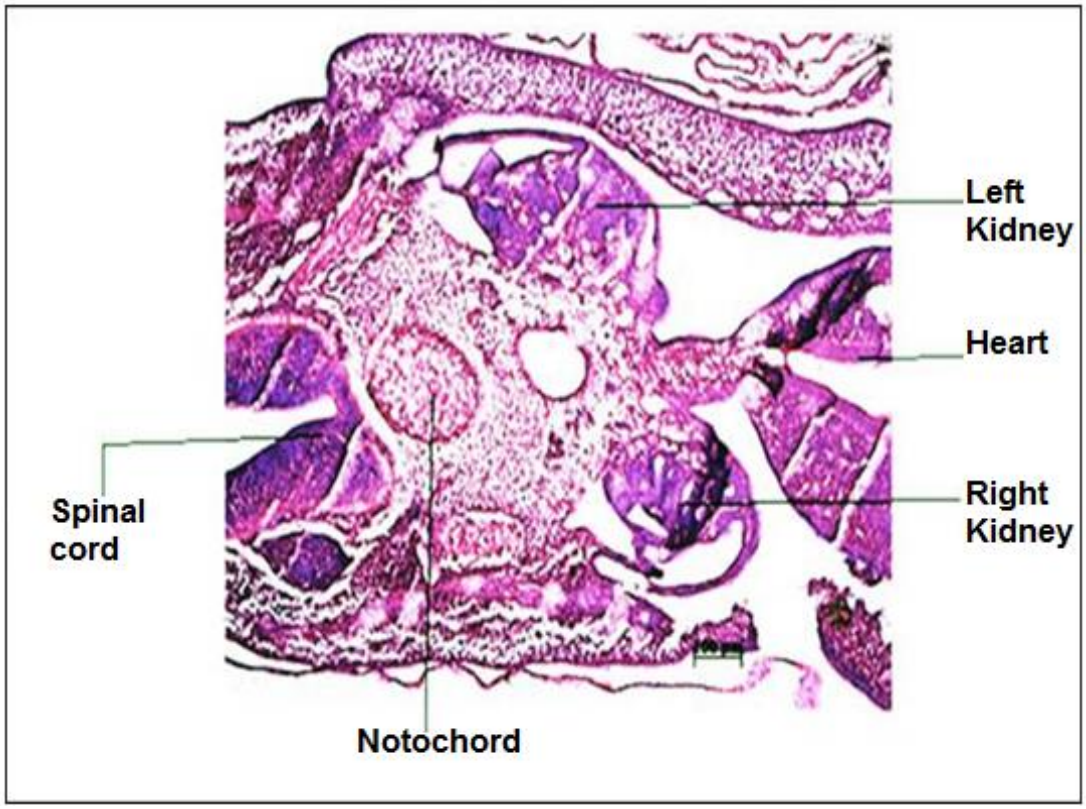

Figure 3.4. Cross section through the thoracic region of a stage 22 embryo at X100 magnification) showing the developing heart, kidneys, notochord and associated spinal cord

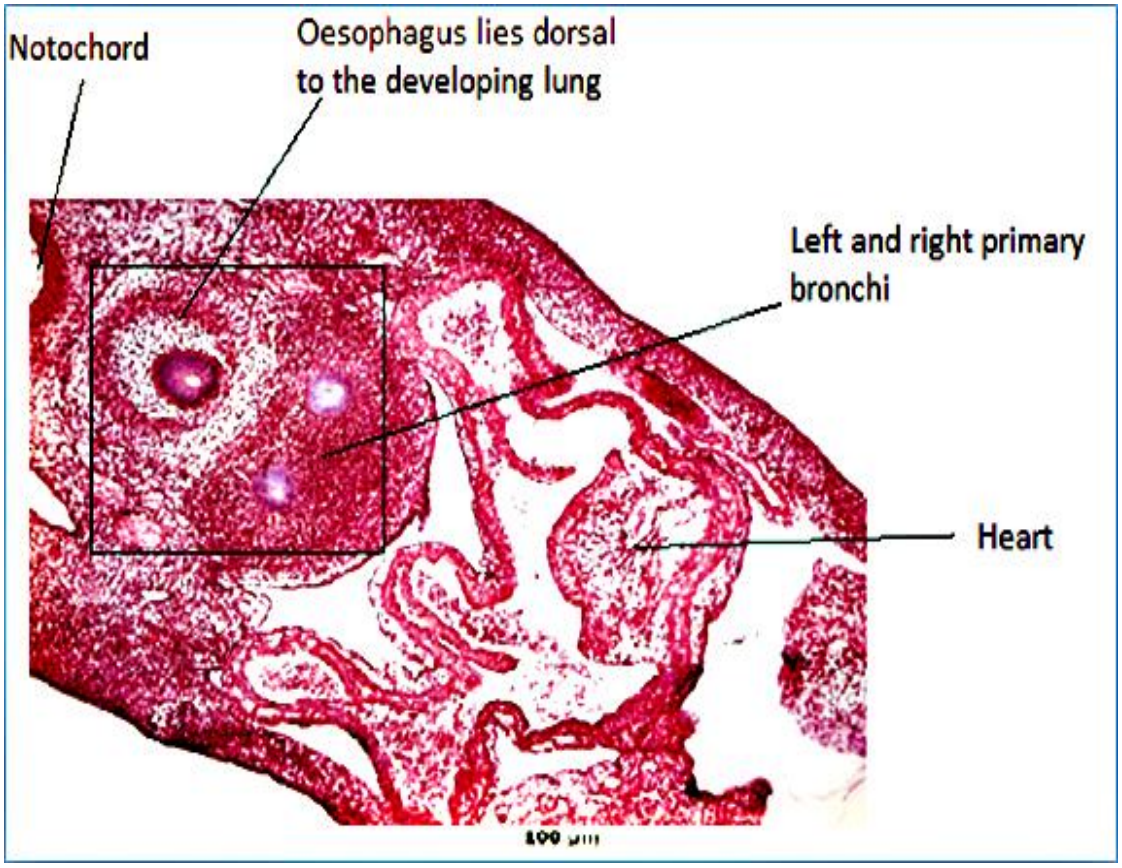

Figure 3.5. Stage 25 Embryo

A section of the stage 25 embryo showing the trachea bifurcation into the two primary or principal bronchus and lying just ventral to the oesophagus: Longitudinal section of a Stage 25 embryo (X100) showing the centrally placed oesophagus (E) dorsal to the two primary bronchi. The highlighted region in the image above is shown below:

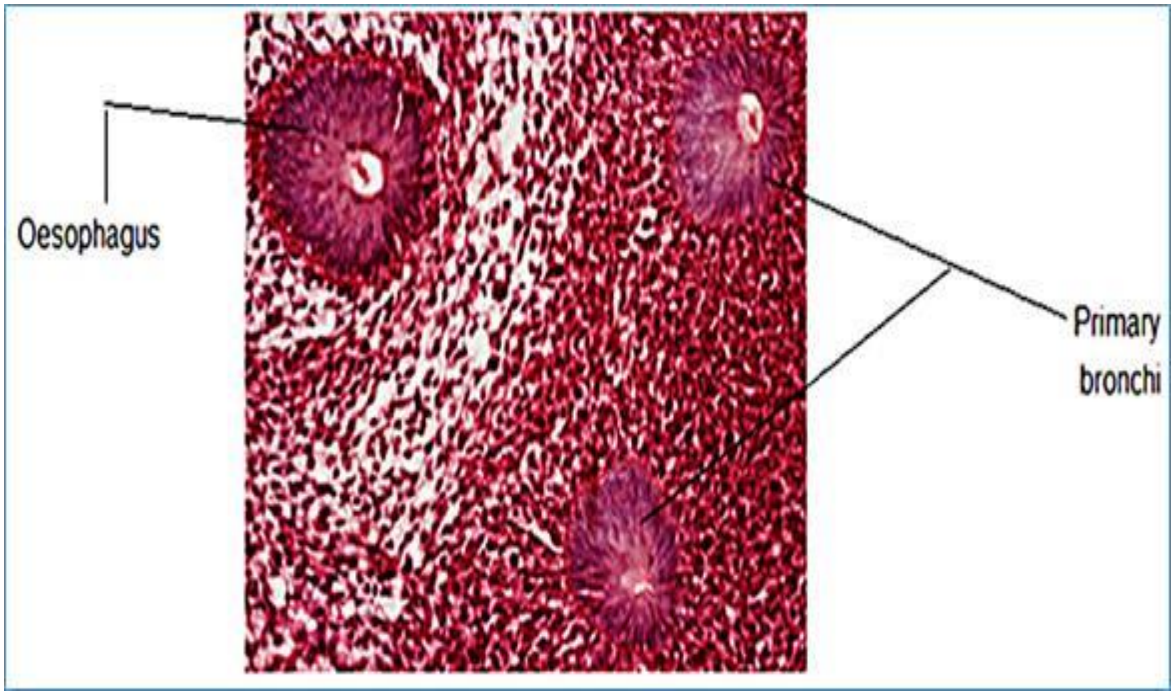

Figure 3.6. This image shows a higher magnification (X400) of the selected area in 3.6 above

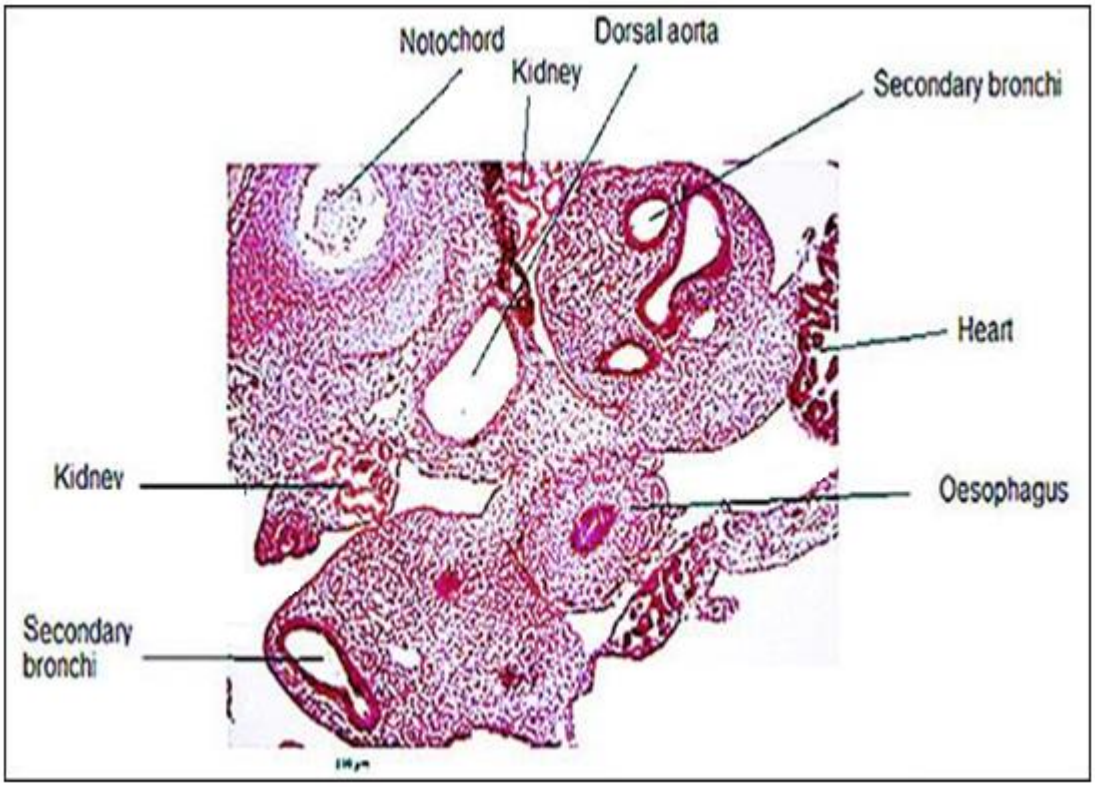

Figure 3.7. Cross section of a stage 29 Embryo. The lung bud is well developed. Secondary bronchi can be seen in the lung buds.

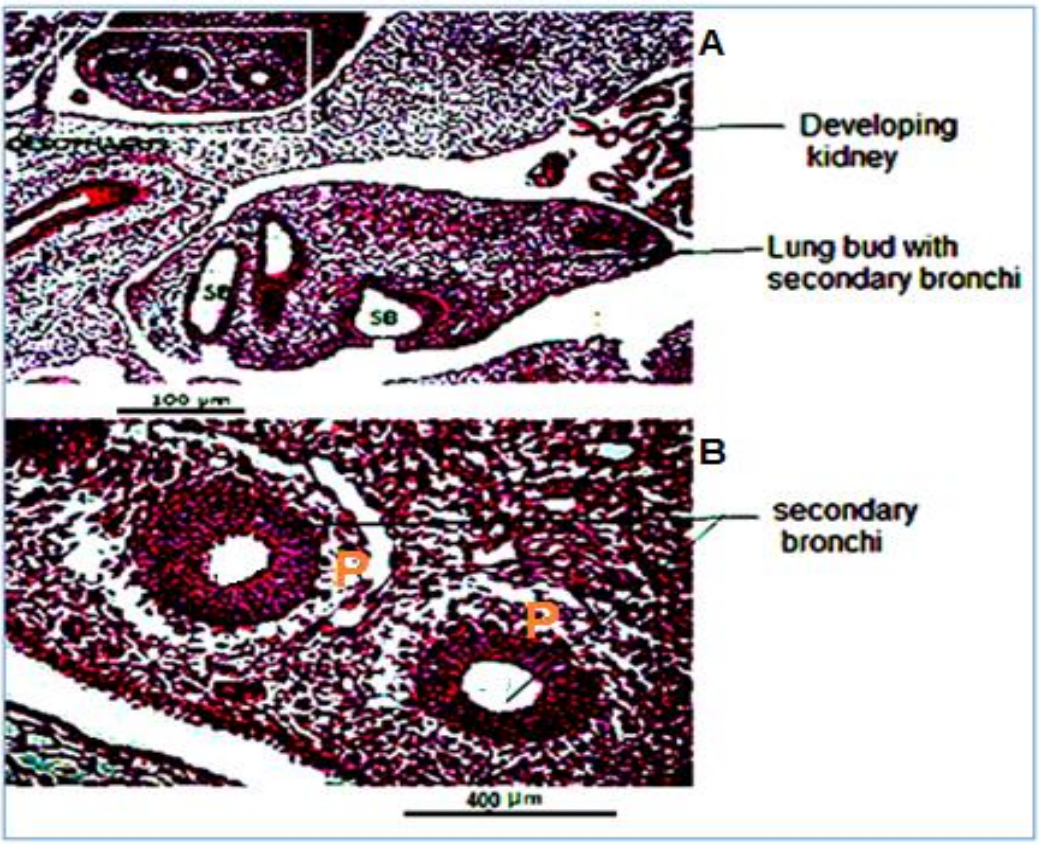

Figure 3.8. Cross section of a stage 30 embryo. Figure A shows the lung buds lying just ventral to the kidneys.

Figure A shows the lung buds lying just ventral to the kidneys. In the right lung, the secondary bronchi (SB) are arranged in a proximo-distal extent. When viewed closer (in figure B) there is peri-bronchial epithelial tissue reaction (P) with formation of bronchial musculature.

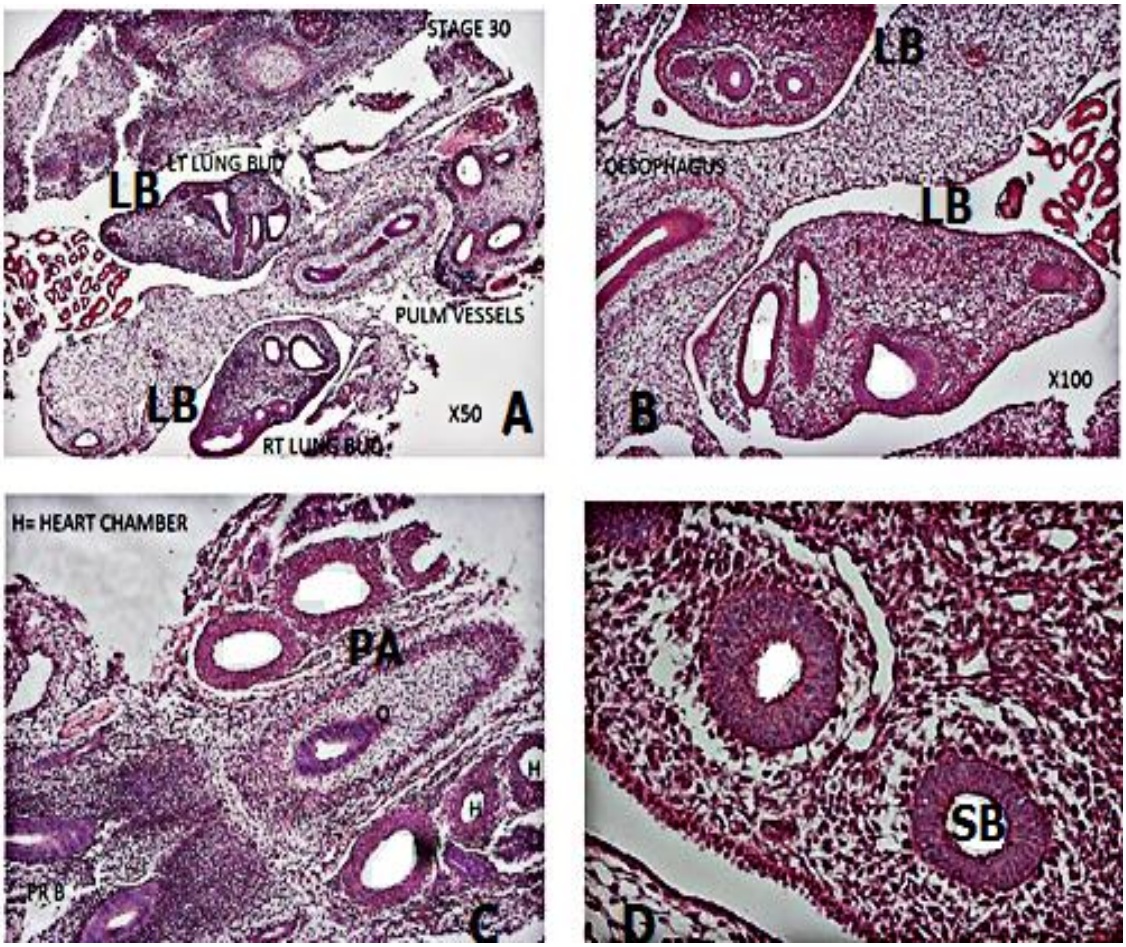

Figure 3.9. Stage 30 lung (A-D). **Image A) and B) show two well-formed lung buds (LB) present at this time. Pulmonary arteries (PA) are seen in image C). Image D shows presence of secondary bronchi (SB) at a higher magnification (than in B)**

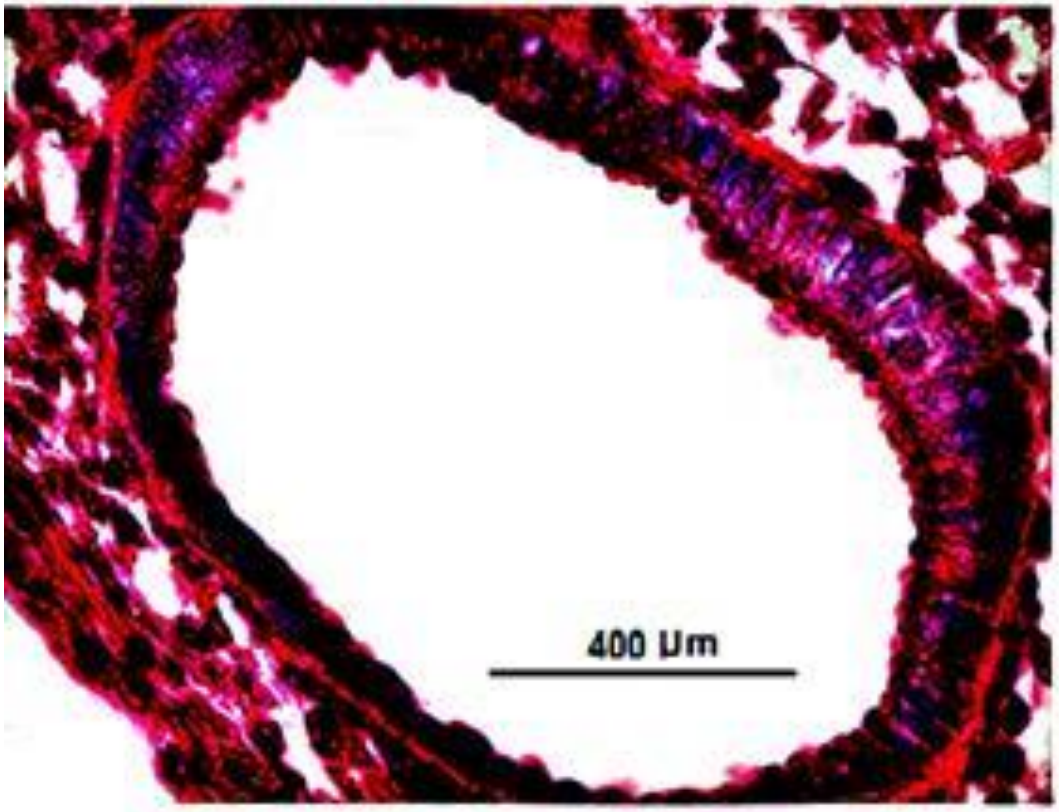

Figure 3.10. Section of a secondary bronchus (SB) of a Stage 31 embryo at a higher magnification (X400). Matured SB are lined by squamous epithelium.

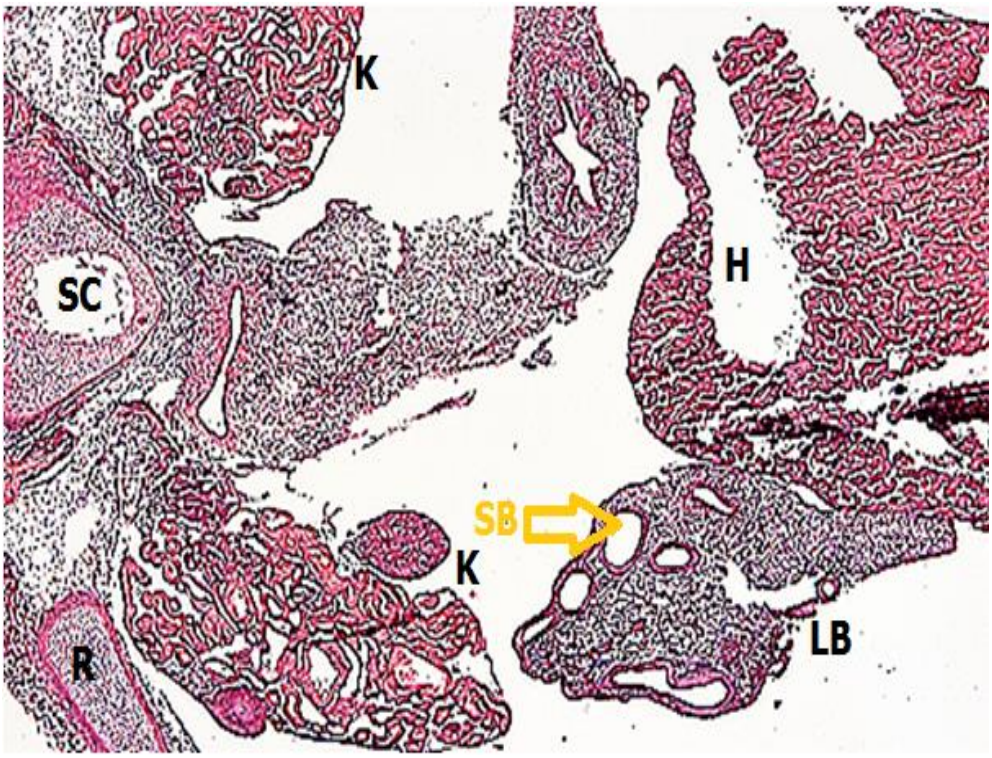

Figure 3.11. Stage 33 Embryonic lung (LB). H-Heart, K-Kidney, O-Oesophagus, SC-Spinal Canal, R-Ribs, SB-Secondary Bronchi

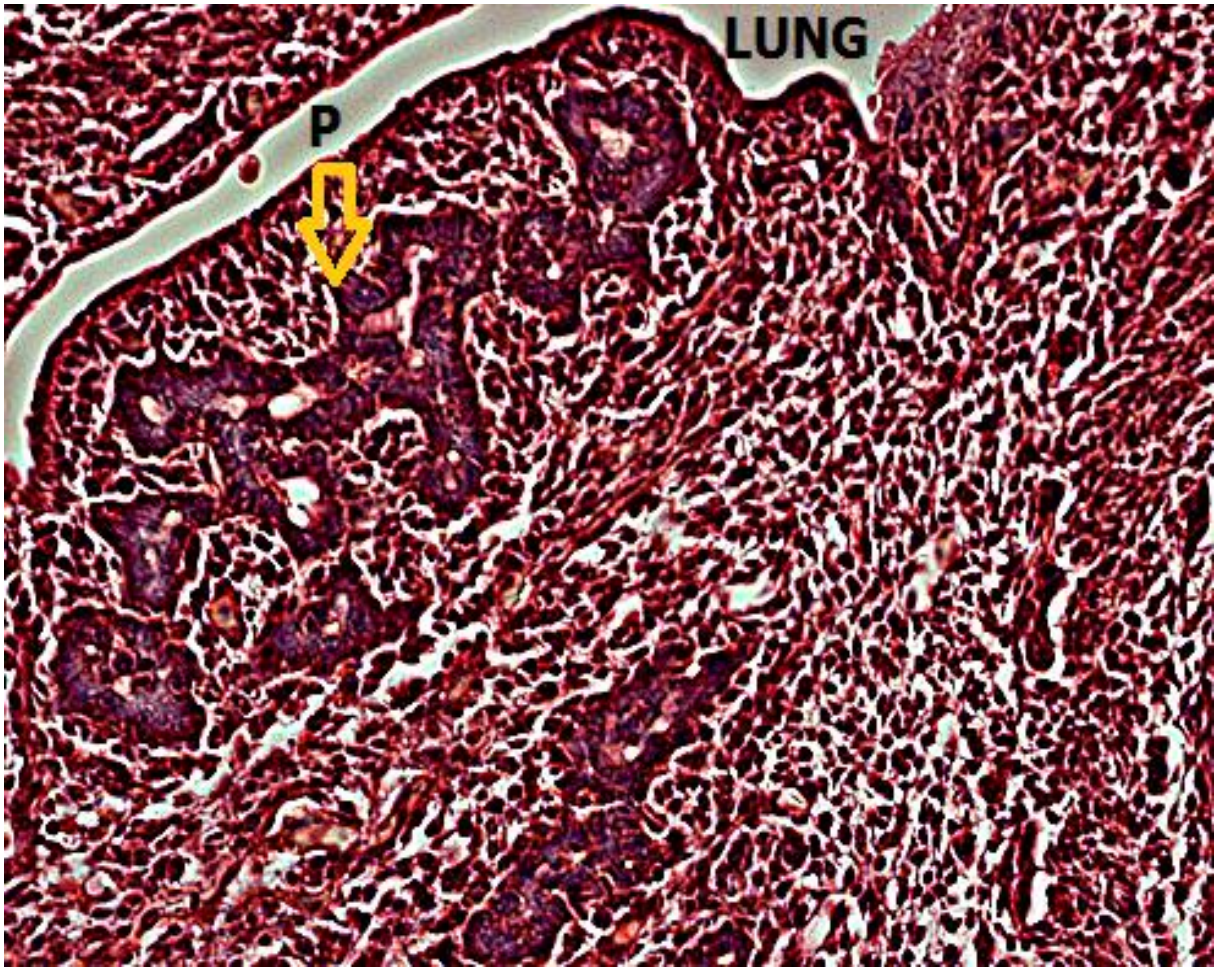

Figure 3.12. Stage 33 Embryonic lung. At this stage the parabronchi (arrow head) are cord-like and not yet fully canalized

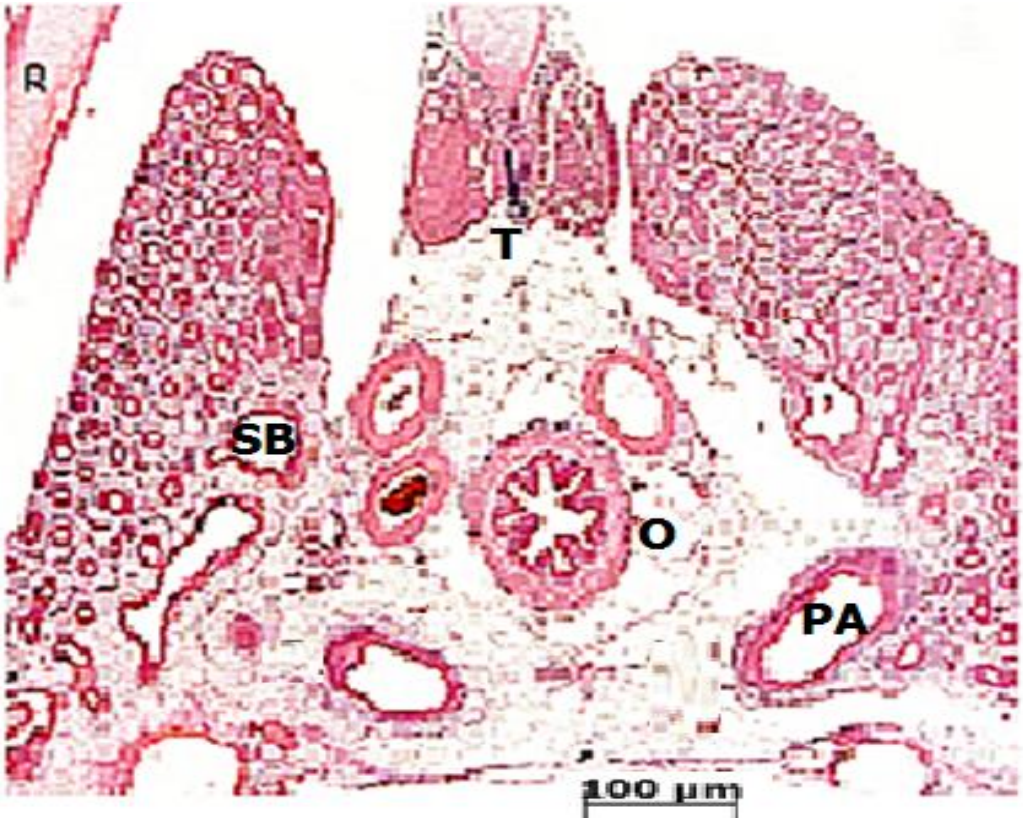

Figure 3.13. Stage 37 Lung. T-Trachea. O-Oesophagus. PA-Pulmonary arteries. R-Rib and SB-Secondary Bronchi

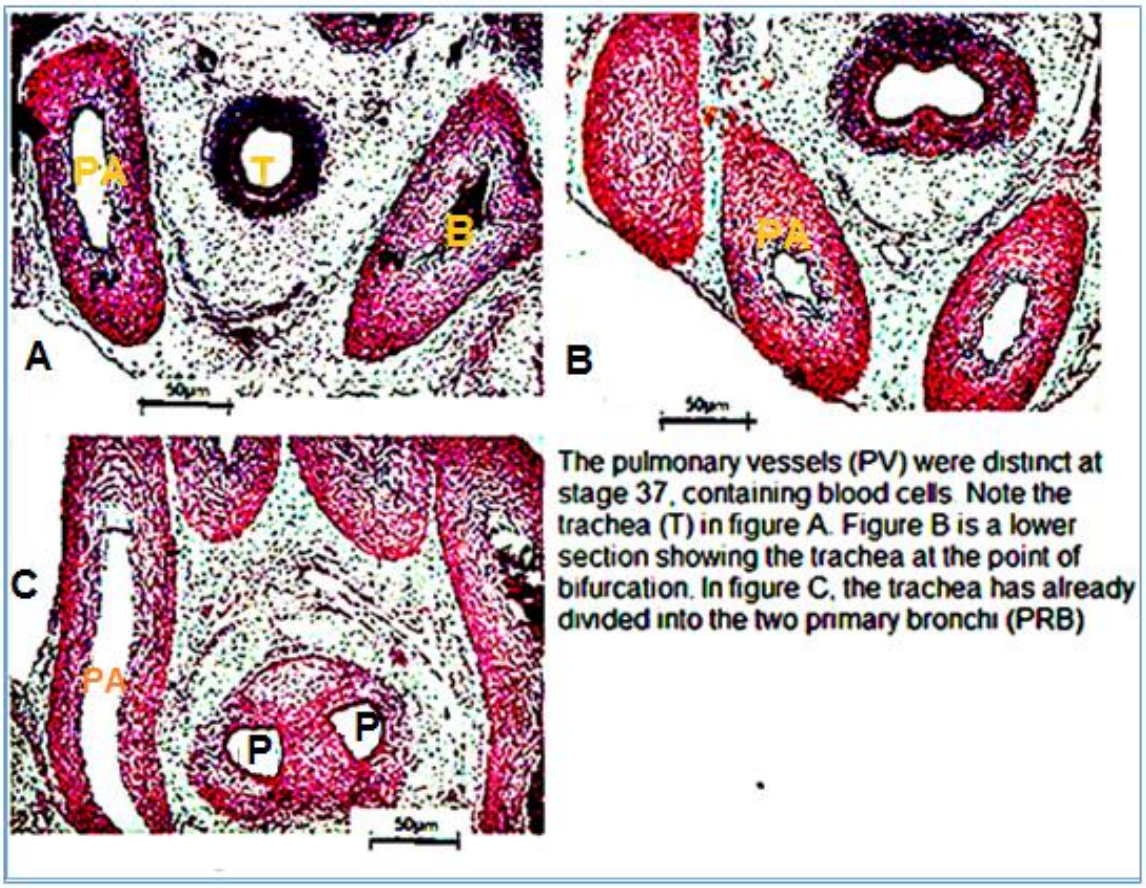

Figure 3.14. Pulmonary arteries in a Stage 37 embryonic lung. **Note the pulmonary vessels arteries (PA), dividing trachea (T) in figure A. Figure B shows the trachea at the point of bifurcation. The 2 pulmonary primary bronchi (P) are already formed in figure C, which is a lower section of the airway. Note presence of blood cells in the PA (B) e.g. in Figure A.**

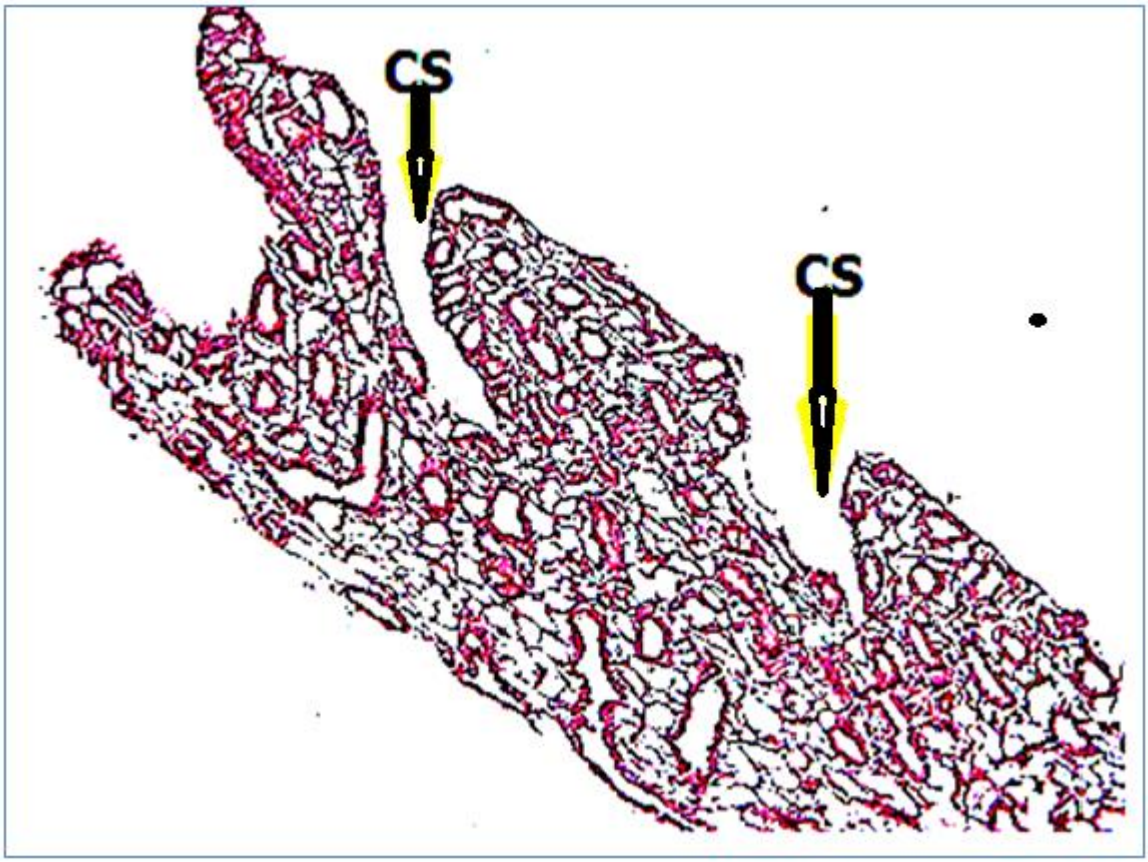

Figure 3.15. Stage 13 Lung. Note the costal sulci (CS) form because the avian lung is rigid, non-expansive and embedded into the ribs.

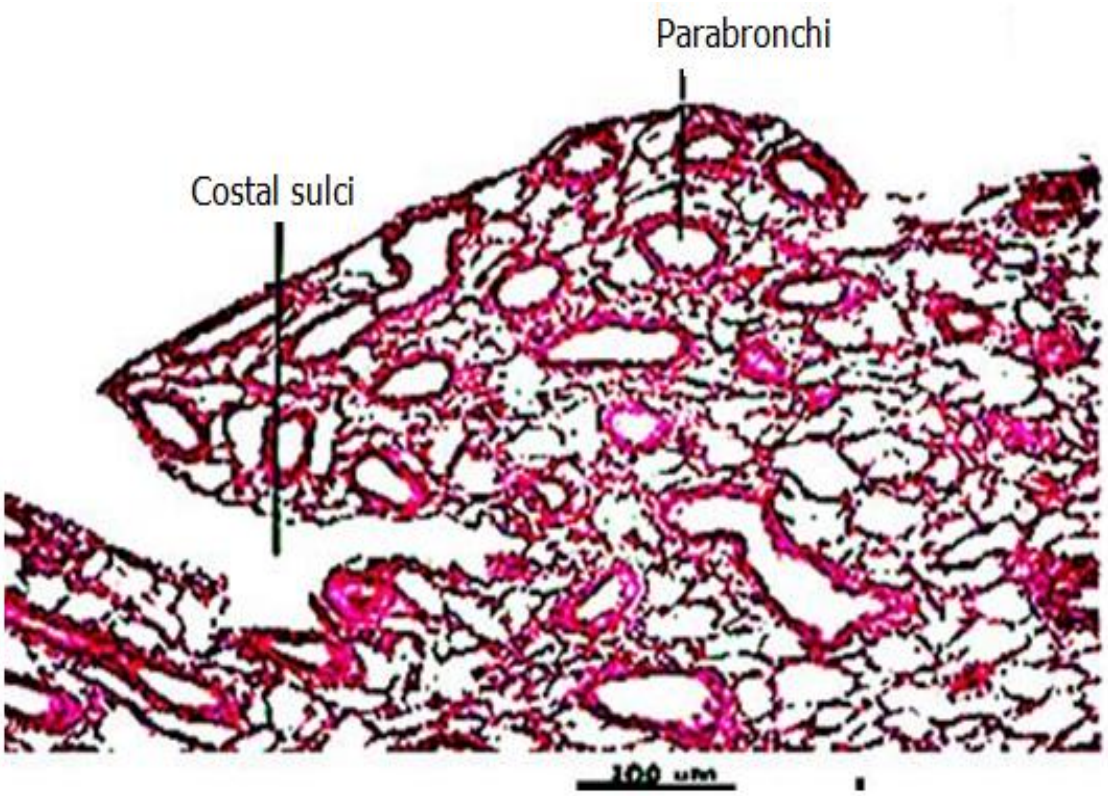

Figure 3.16. Section of a stage 39 lung. **It shows a parabronchial lung with costal sulci, as is typical of avian lungs since the lungs are wedged in between ribs.**

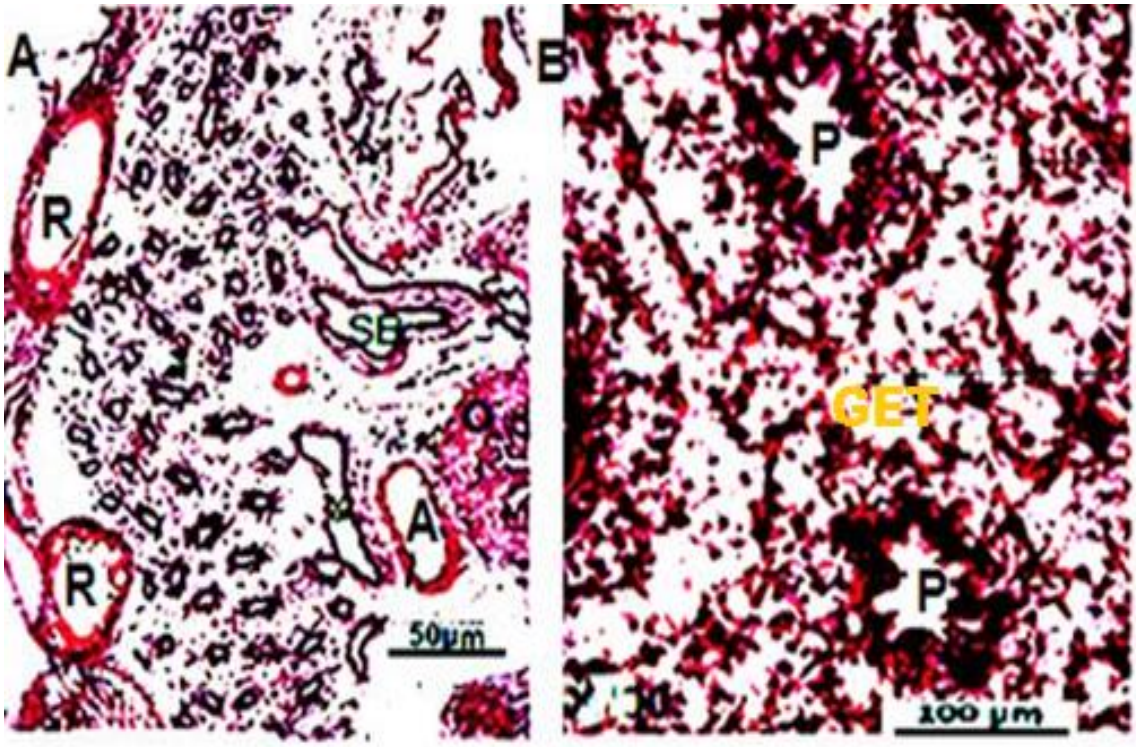

Figure 3.17. Stage 40 embryonic lung. In Figure A), R-rib, SB-secondary bronchi, A-pulmonary artery. In Figure B, P- Parabronchi and in between P is the interparabronchial or gas-exchange tissue, GET).

3.10.2. Results VEGF Staining

Tissue sections adjacent to the H & E sections were immunolocalized for VEGF (Red): Alexa Fluor 568): Nuclear stain, DAPI- Blue). In the H & E sections, no VEGF expression seen at stage 19 of avian development.

a) Scoring of VEGF Staining

b) After consultation with the Statistician (Prof Schoeman) at the Medunsa campus of the University of Limpopo (now called Sefako Makgatho Health Sciences University (SMU), VEGF detection in lung tissues at the different stages of development was scored as shown in the table below:.

Table 2.0:2: Scoring scheme for VEGF detection in lung tissue

0	No VEGF detected
+	VEGF mildly detected
++	VEGF moderately detected
+++	VEGF markedly/heavily detected

Table 3.2 shows how the tissue sections were scored for VEGF.

Table 2.0:3: Localization of VEGF in the embryonic lung

Developmental Group	Developmental Stage	Score	VEGF Detection in the Lung
1	19	0	Negative
2	22	+	Mild
3	27	+	Mild
4	29	++	Moderate
5	31	++	Moderate
6	33	++	Moderate
7	37	+++	Marked/Heavy
8	40	+++	Marked/Heavy

VEGF was not detected at embryonic stage 19 of avian development but was found from stage 22. VEGF levels then increased with lung maturation and was at its highest at stage 40. As will be seen below, VEGF expression was also noted in surrounding structures such as the developing heart, kidneys and spinal cord as will be seen in the images captured. At stage 19, the lung bud had not formed but the lung progenitor (foregut tube) was seen.

a) Control tissues

Pregnant mouse uterus was used as positive control tissues because of the typical endometrial angiogenesis associated with development.

b) Positive control

Below shows a uterine blood vessel (V) with marked VEGF expression. Nuclei stain blue with DAPI.

3.10.3. Results: Immunofluorescence

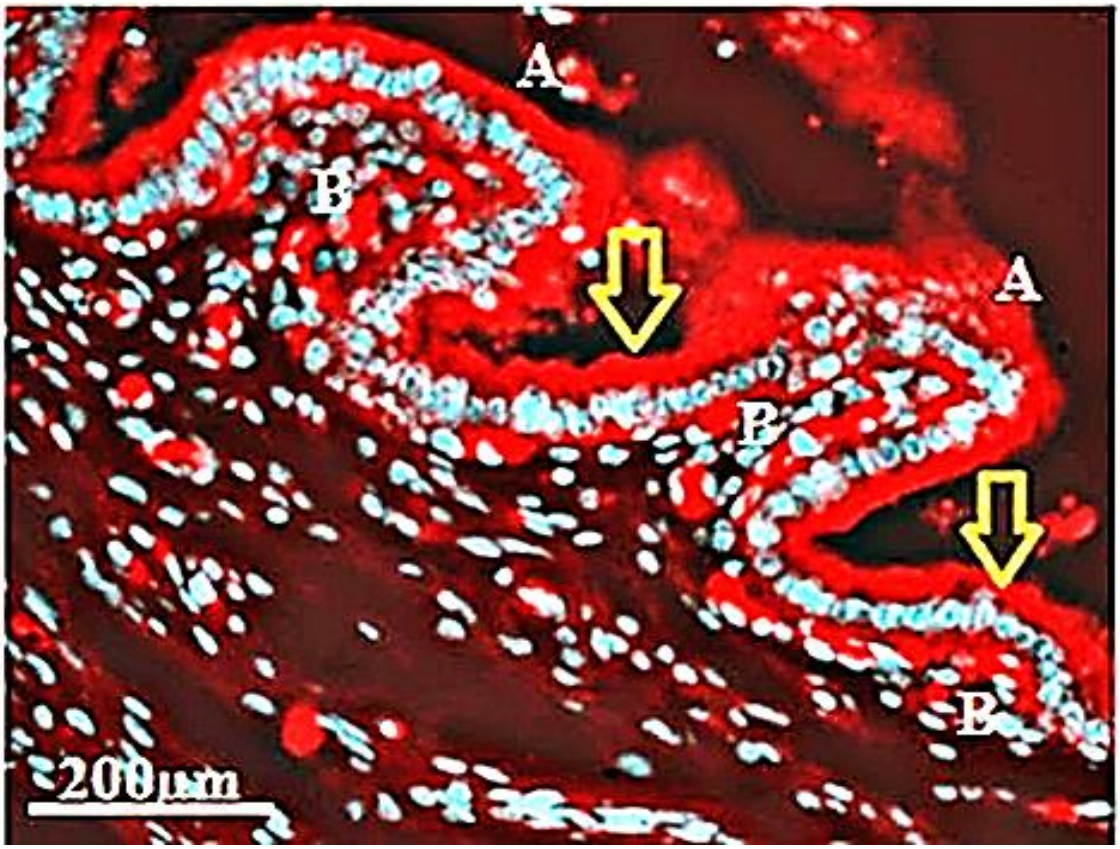

Figure 3.18. Positive control sections using pregnant mouse uterus stained for VEGF. Endometrial epithelium (arrow heads) shows VEGF expression in both the apical (A) and basal (B) borders.

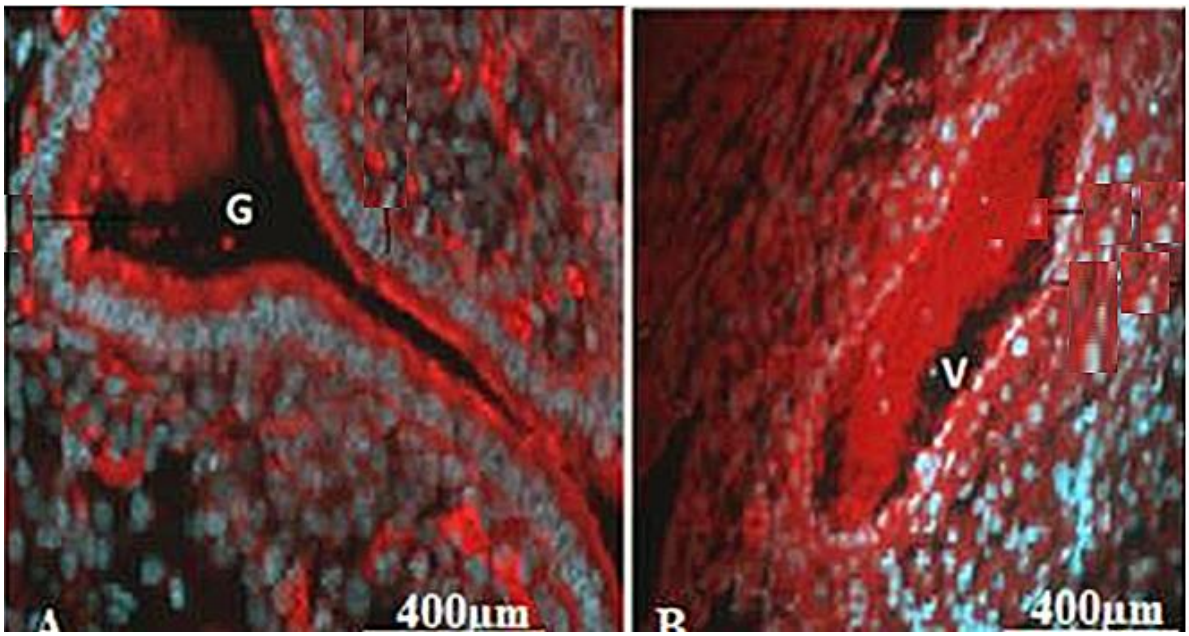

Figure 3.19. Positive control section. **Pregnant mouse uterine tissue was used as positive control. Image A) shows an endometrial gland (G) expressing VEGF (red) in its epithelium. Image B) shows an endometrial blood vessel (V) with blood clot in its lumen. Note that both vascular and endometrial tissues show diffuse VEGF expression.**

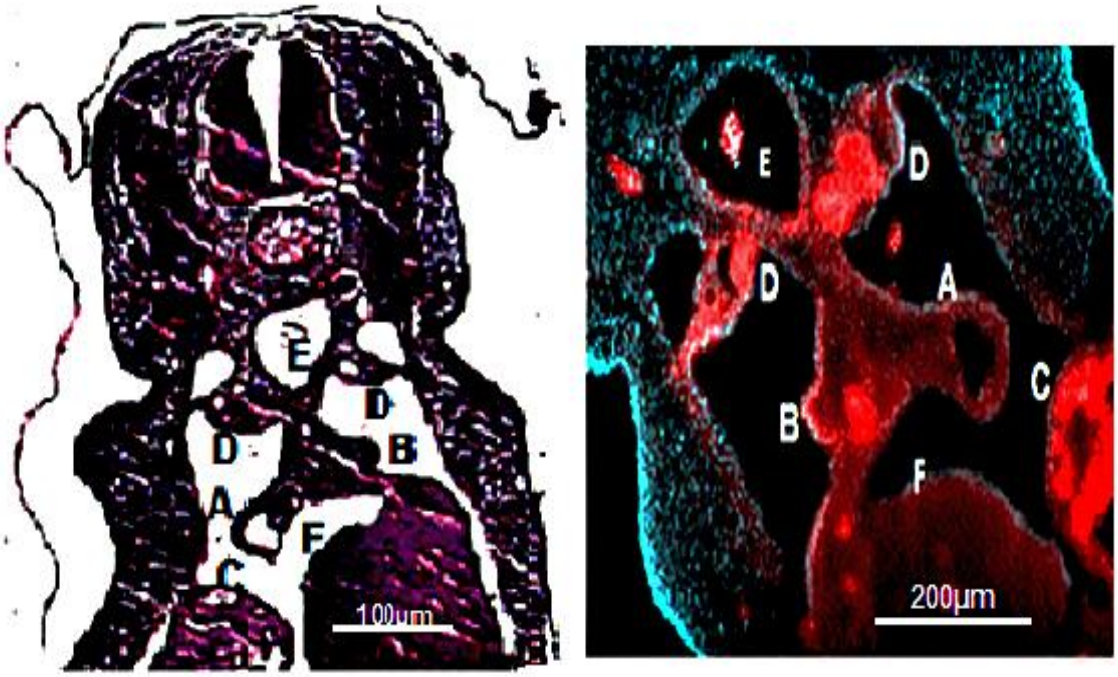

Figure 3.20. Shows the thoracic segment of a Stage 22 embryo. The earliest VEGF activity was seen at this stage. Red: Alexa Fluor 568, Blue: Nuclear stain, DAPI. (Blue): H & E. Figure A. shows H & E staining of a stage 22 avian embryo showing the developing left and right lung buds (A, B), heart (C), kidneys (D) and the dorsal aorta (E). Figure B shows VEGF expression (red) in several organs at this early stage (A- left lung bud, B- right lung bud, C- heart, D-kidneys, E-Notochord, F- Liver).

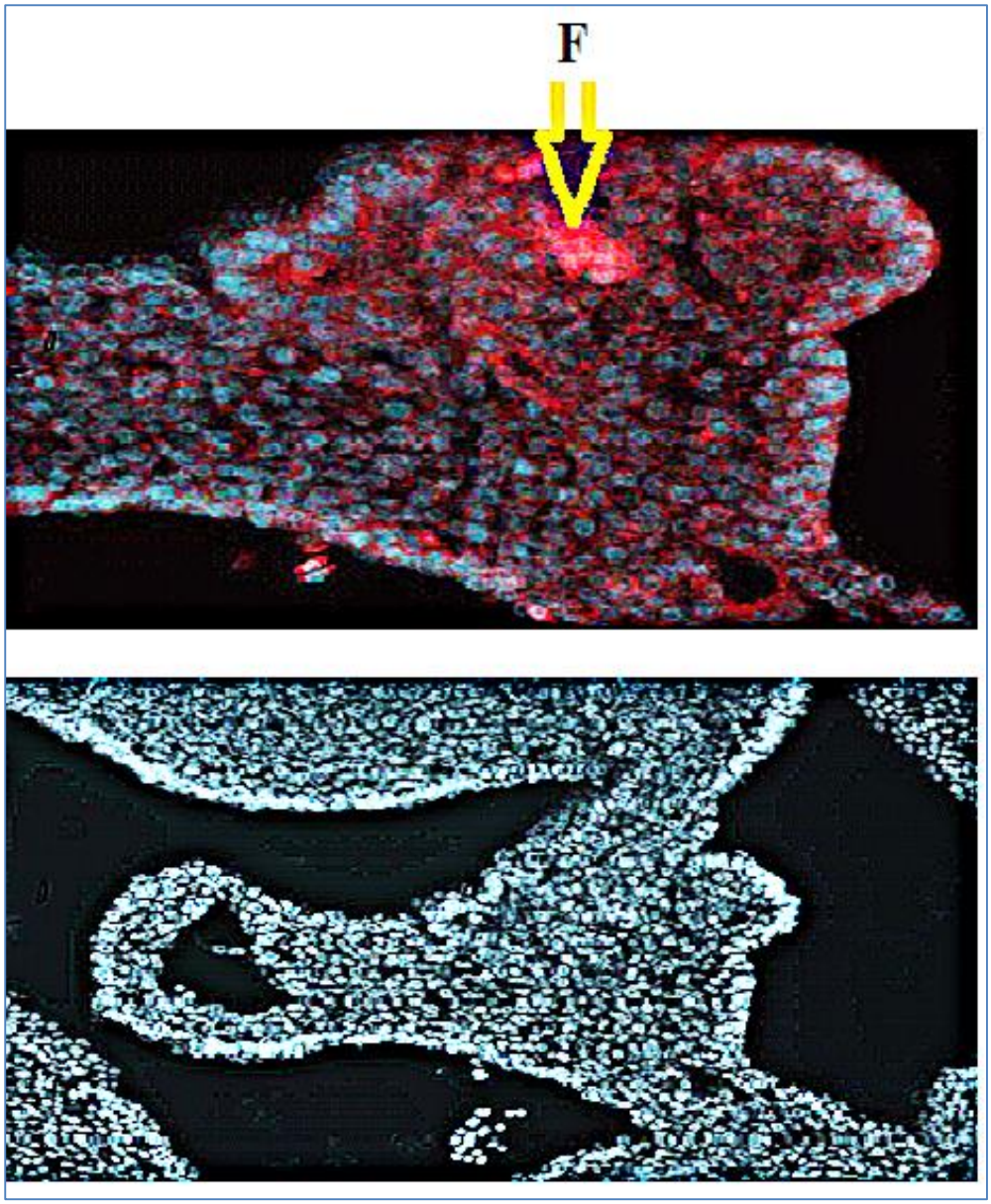

Figure 3.21. Stage 22 Lung buds. Figure A) shows VEGF activity in the immature lung bud. Note the VEGF foci (F). Figure B) is a negative control section of the lung bud at developmental stage 22.

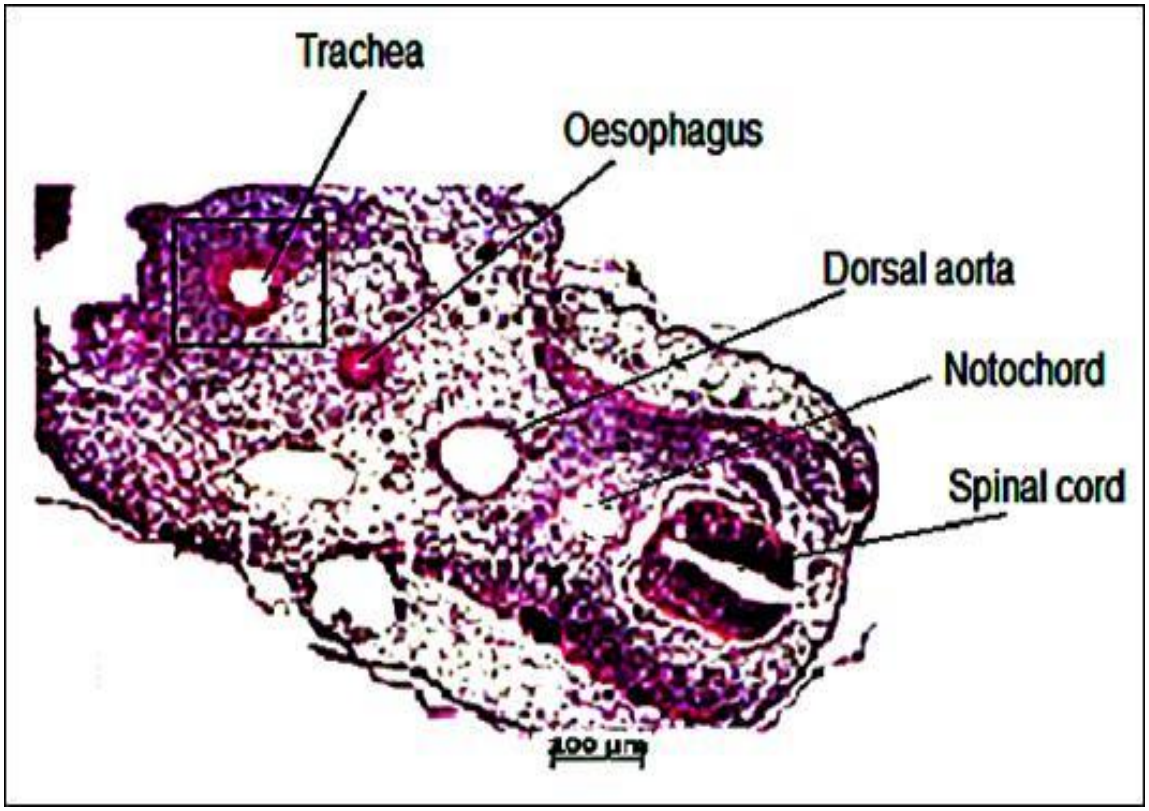

Figure 3.22. This is a longitudinal section of a Stage 22 Embryo. Note the oesophagus (O) lies just dorsal to the trachea (T).

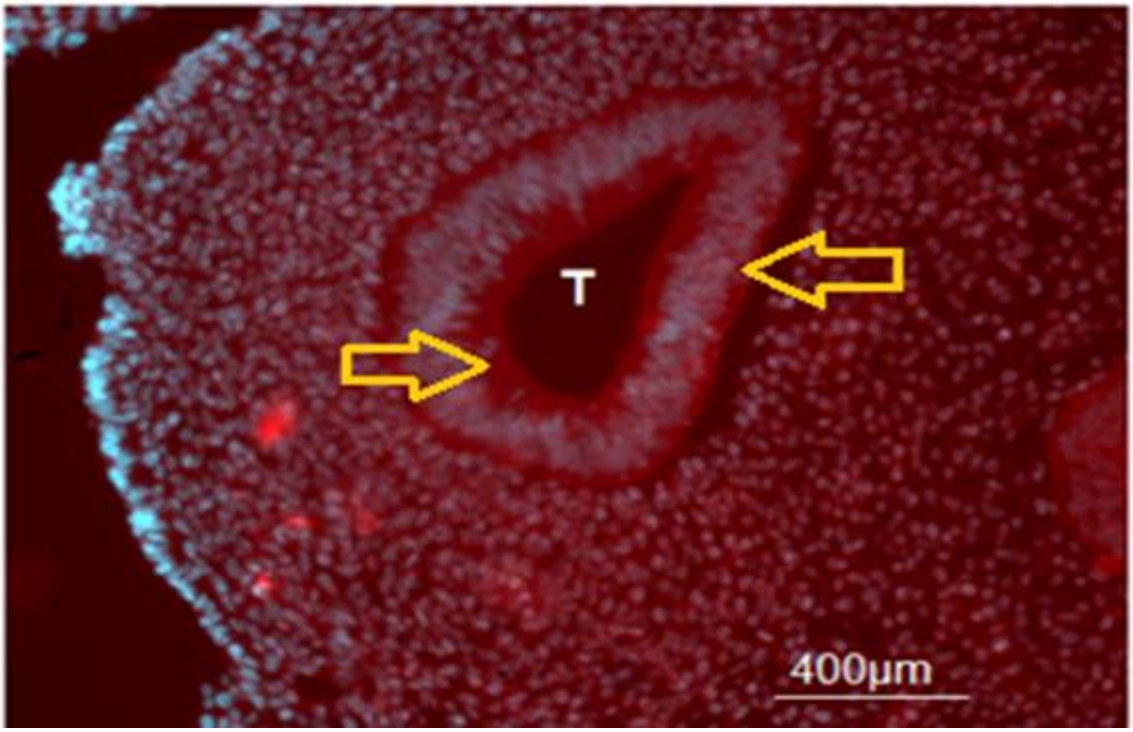

Figure 3.23. This shows the highlighted structure (trachea, T) in figure 20. The trachea (T) expressed VEGF in both the apical and basal aspects of the epithelium (Arrow heads).

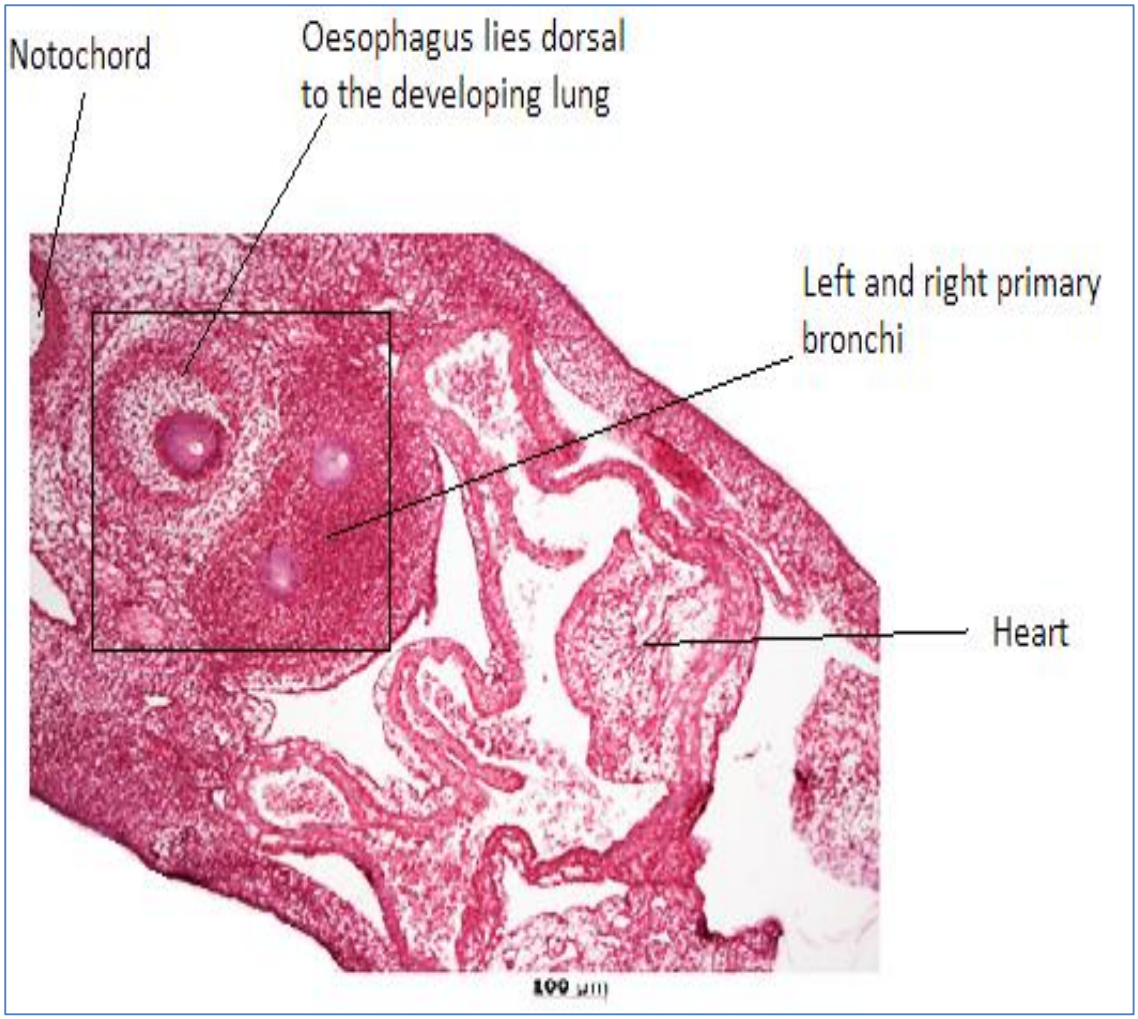

Figure 3.24. Shows the thoracic segment of a stage 25 embryo. The highlighted section shows the two primary bronchi ventral to the oesophagus. The developing heart is seen ventral to the bronchi.

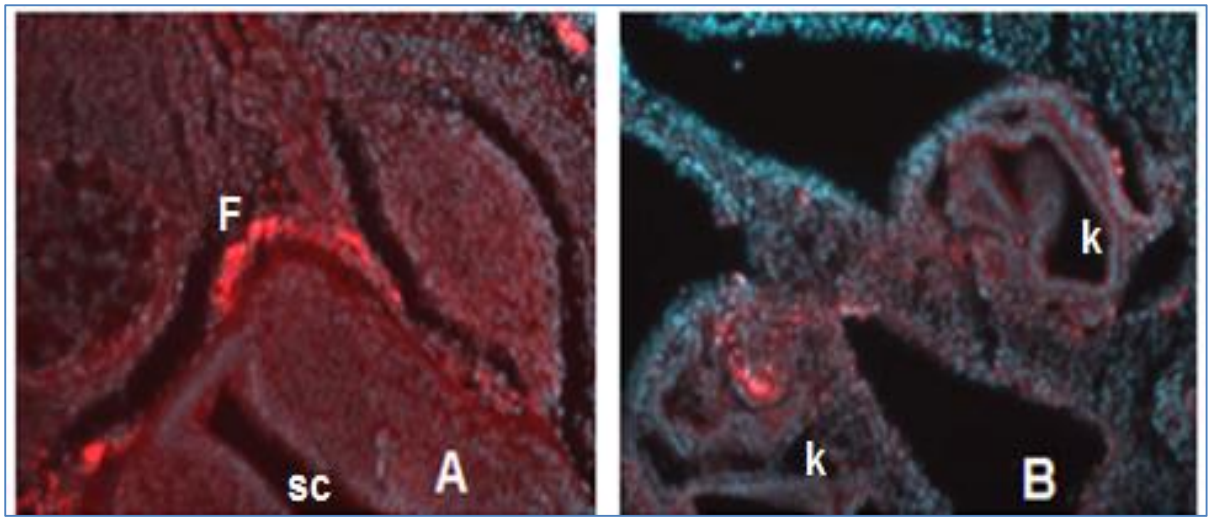

Figure 3.25. Figure A shows the spinal cord (sc) and B, the developing kidneys (K), both at stage 25 of development. Fluorescing cells (F) in A around the spinal canal (SC) suggest perineural angiogenesis. Original magnification for A & B-x400

Figure 3.26. Showing Structures in a stage 27 embryo; lung bud (highlighted), Heart (H), spinal cord (SC), liver (L), dorsal aorta (DA) and kidneys (K).

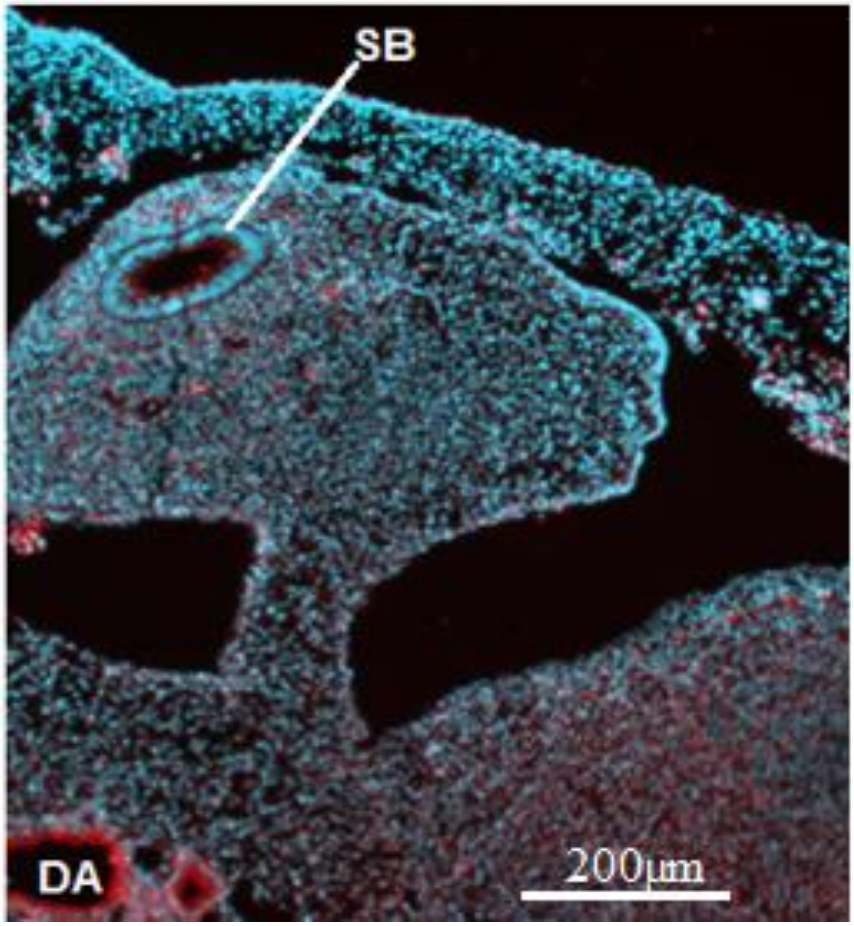

Figure 3.27. Stage 27 embryonic lung bud. The developing lung bud also lies just ventral to the dorsal aorta (DA) which expresses VEGF more markedly. Note the presence of one secondary bronchus (SB) at this stage.

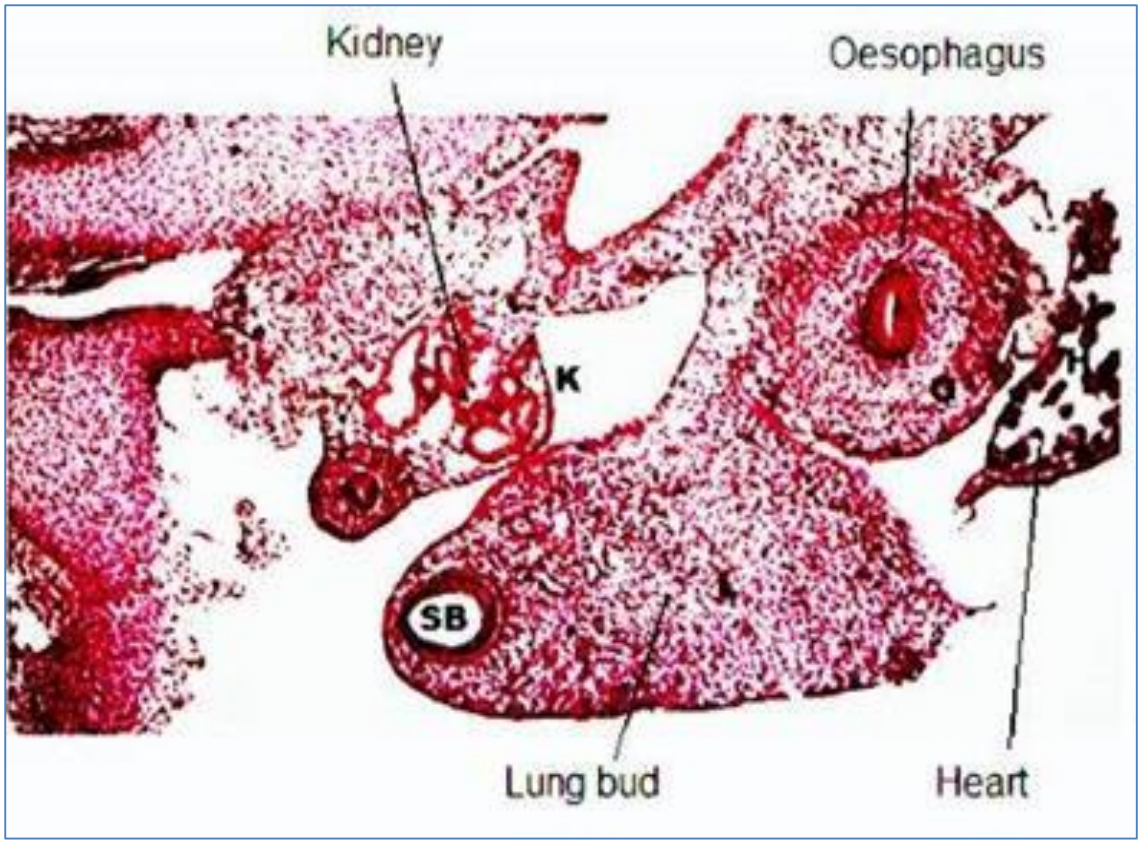

Figure 3.28. Stage 29 lung showing the developing lung bud lying just ventral to the oesophagus. Note the secondary bronchi (SB).

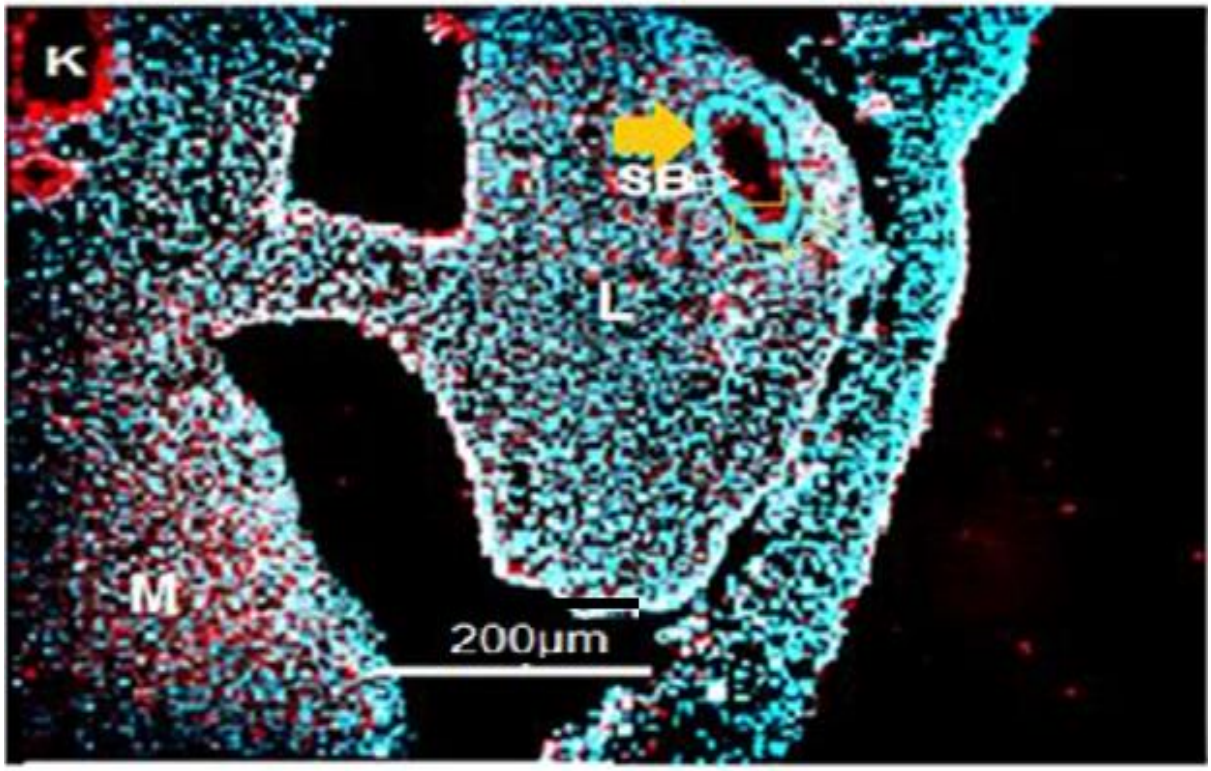

Figure 3.29. Shows a stage 29 lung bud. Note the VEGF activity (seen as dots of redness) in the developing lung parenchyma, its surrounding mesenchyme tissues and the secondary bronchi, SB (arrow head).SB-secondary bronchi.

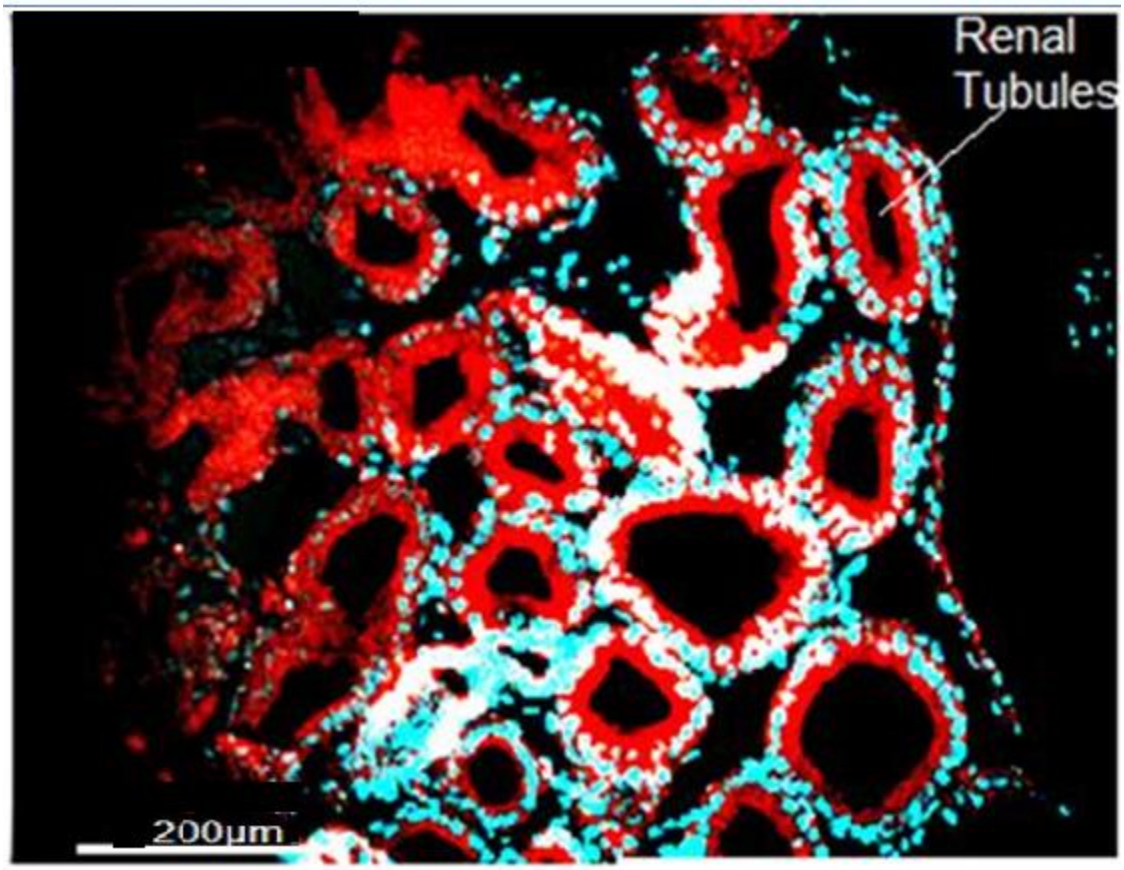

Figure 3.30. Shows the developing kidney tubules as the most fluorescing organ in the stage 29 embryo. The renal tubular epithelium had distinct VEGF expression

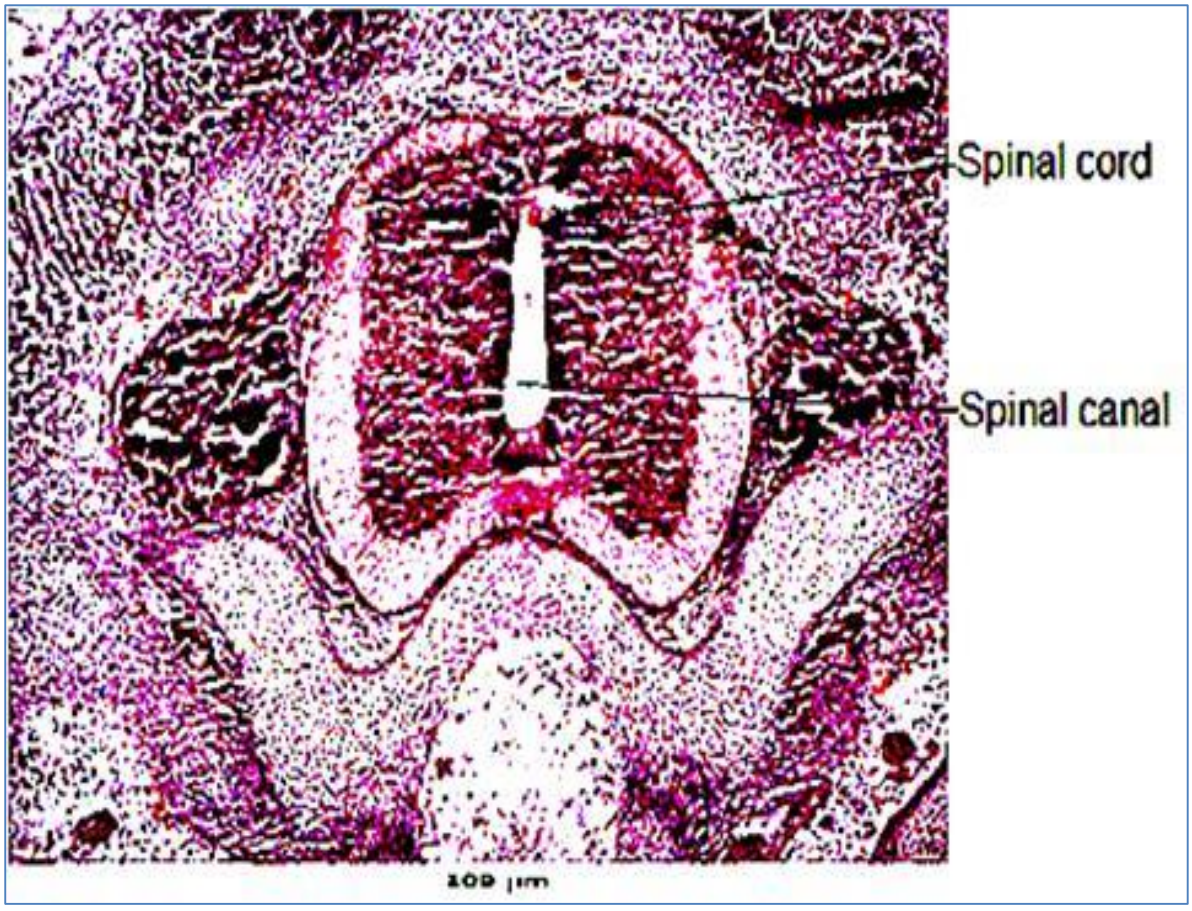

Figure 3.31. Shows H & E section of the developing Spinal cord in a stage 29 embryo

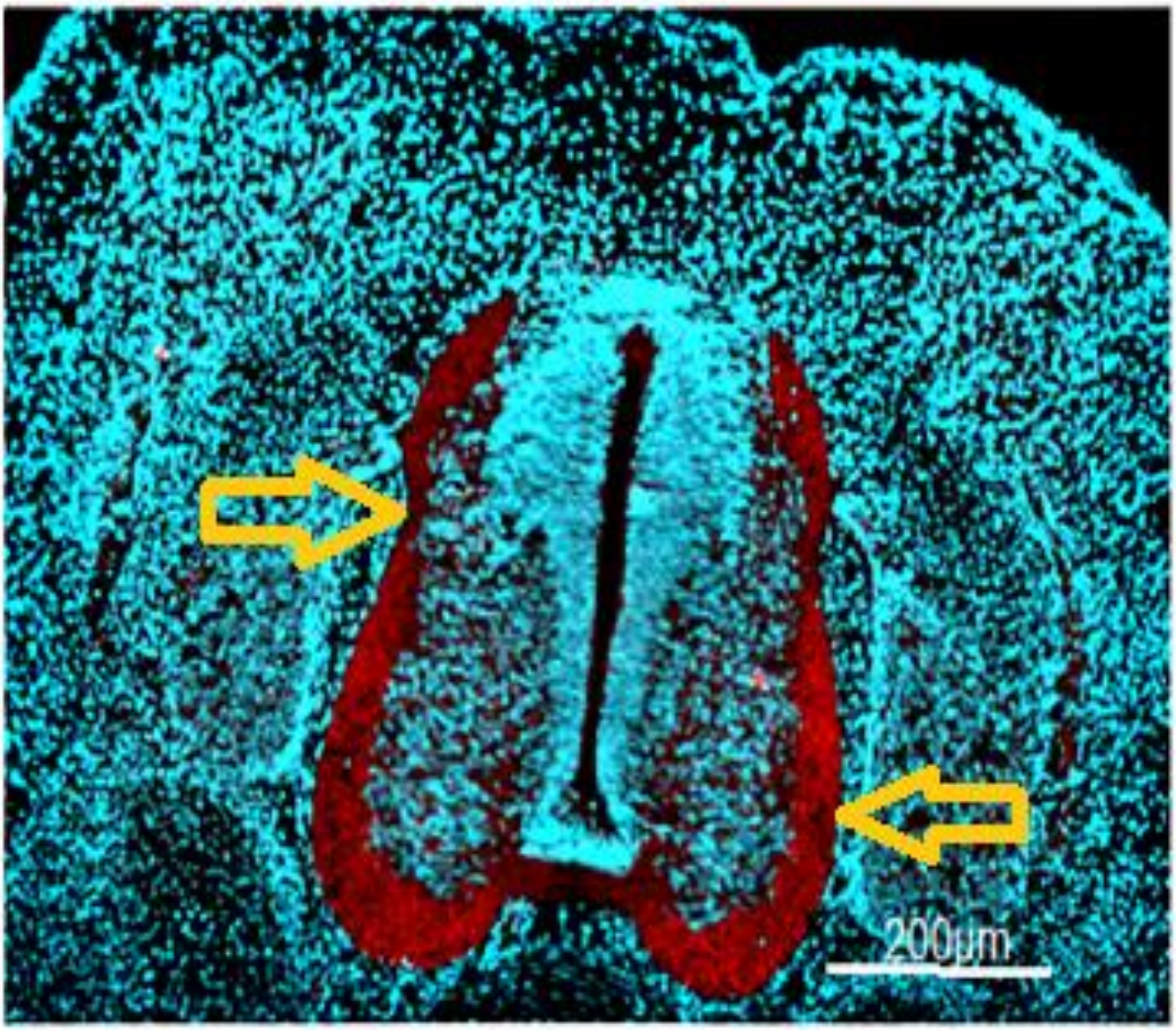

Figure 3.32. Spinal cord in a stage 29 embryo. There is a distinct perineural VEGF expression in the perineural tissues, suggesting perineural angiogenesis (arrow head)

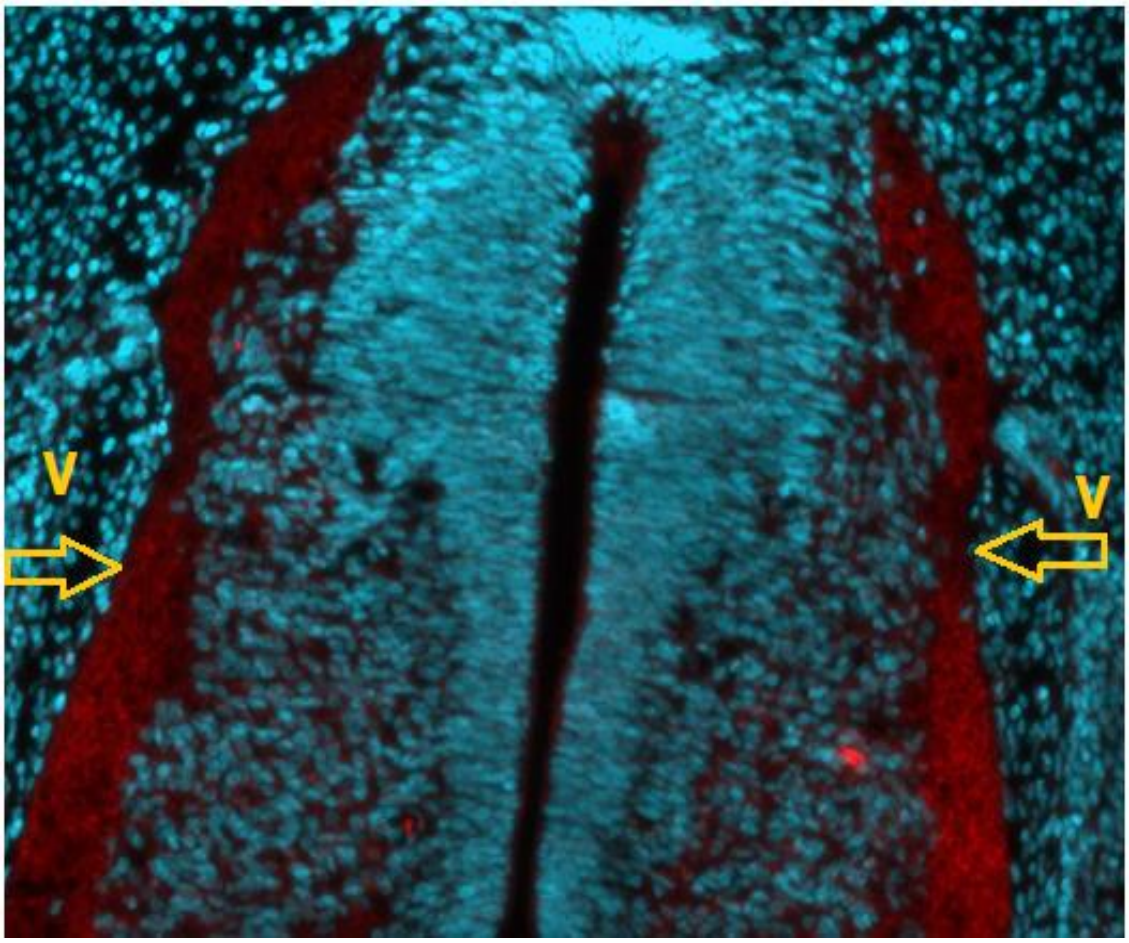

Figure 3.33. Stage 29 embryonic spinal cord at X400 magnification. Note the diffuse angiogenic activity around the spinal canal (arrow head V).

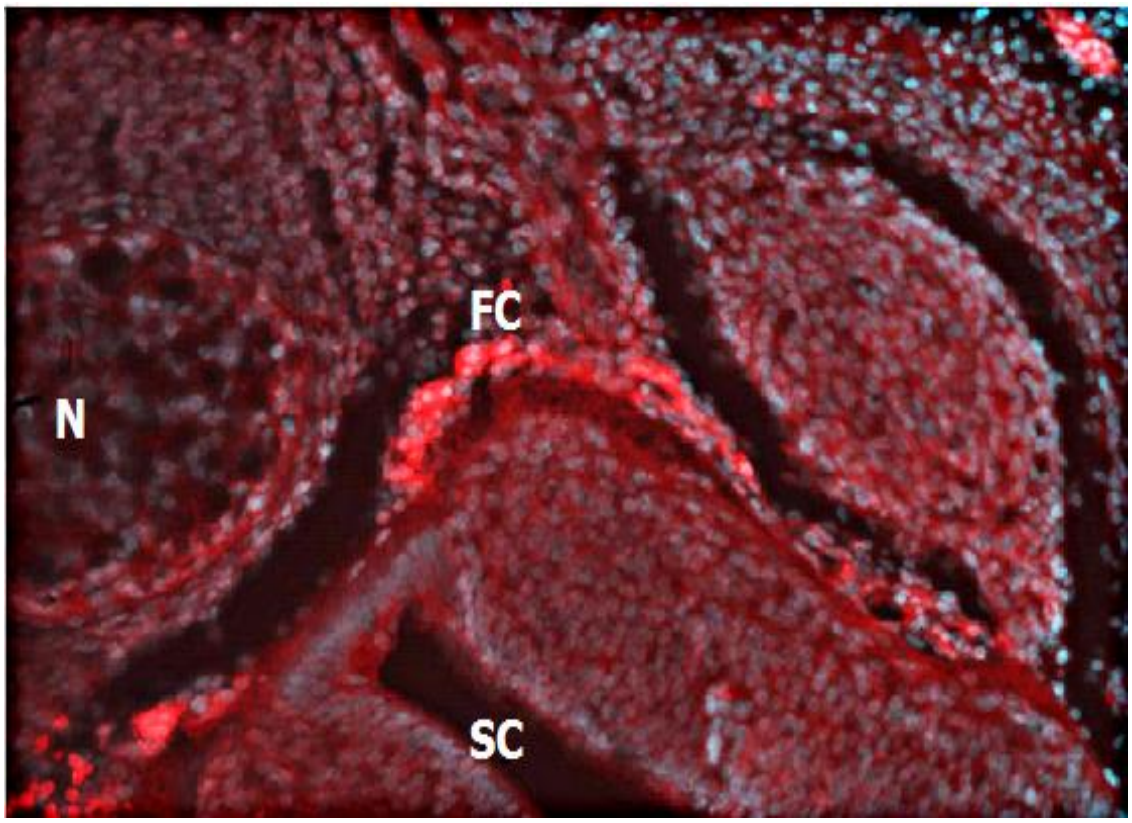

Figure 3.34. Stage 29 Spinal cord. Fluorescing cells (FC) around the developing spinal cord suggest on-going perineural angiogenesis. SC-Spinal canal X400

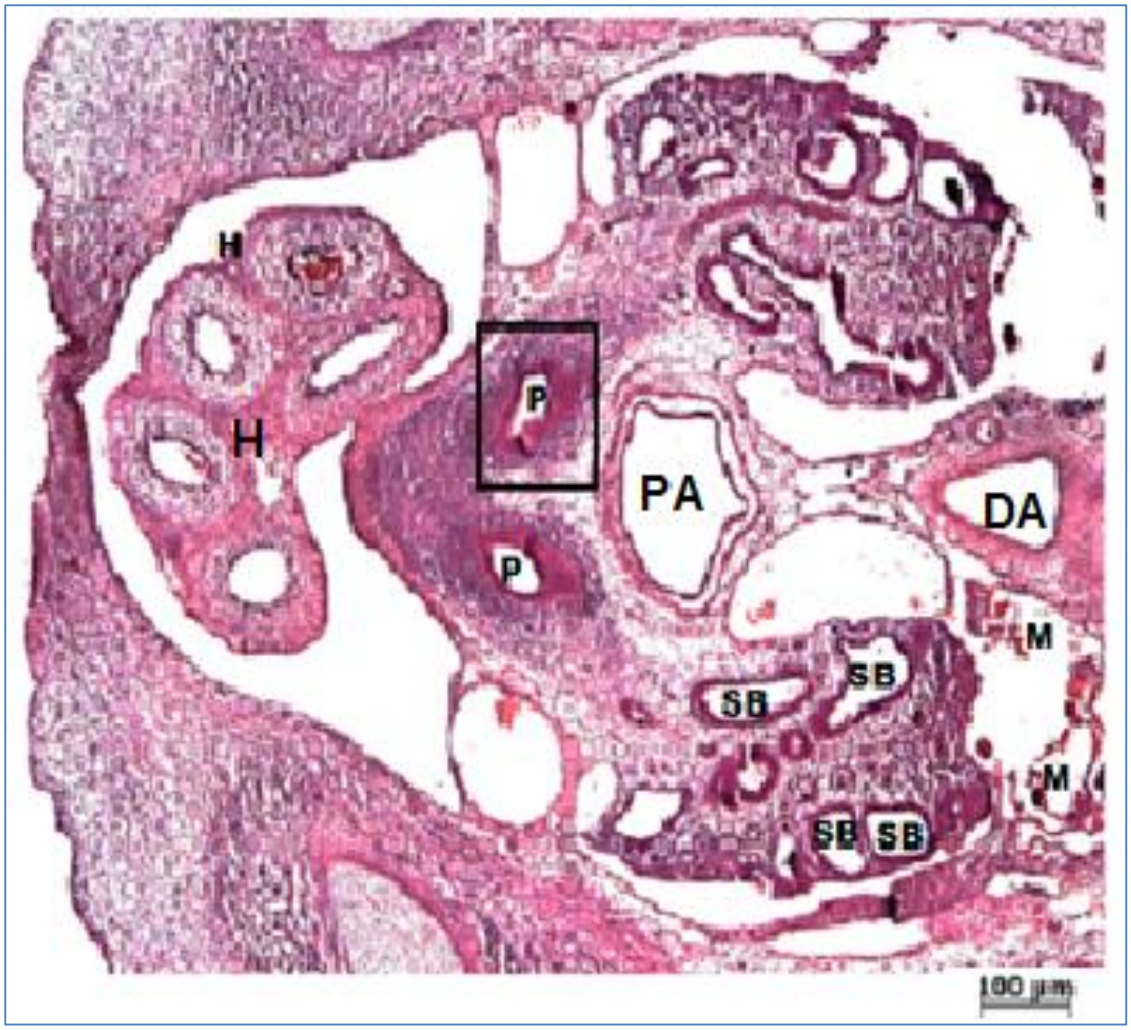

Figure 3.35. This is a section through the thoracic segment of an embryo at stage 31, showing from ventral (L) to dorsal: heart chambers (H), 2 primary bronchi (P), both lungs showing secondary bronchi (SB) at the lateral and medial bothers of the lungs, and kidney (mesonephric) tubules (M). PA- pulmonary artery, DA-Dorsal aorta

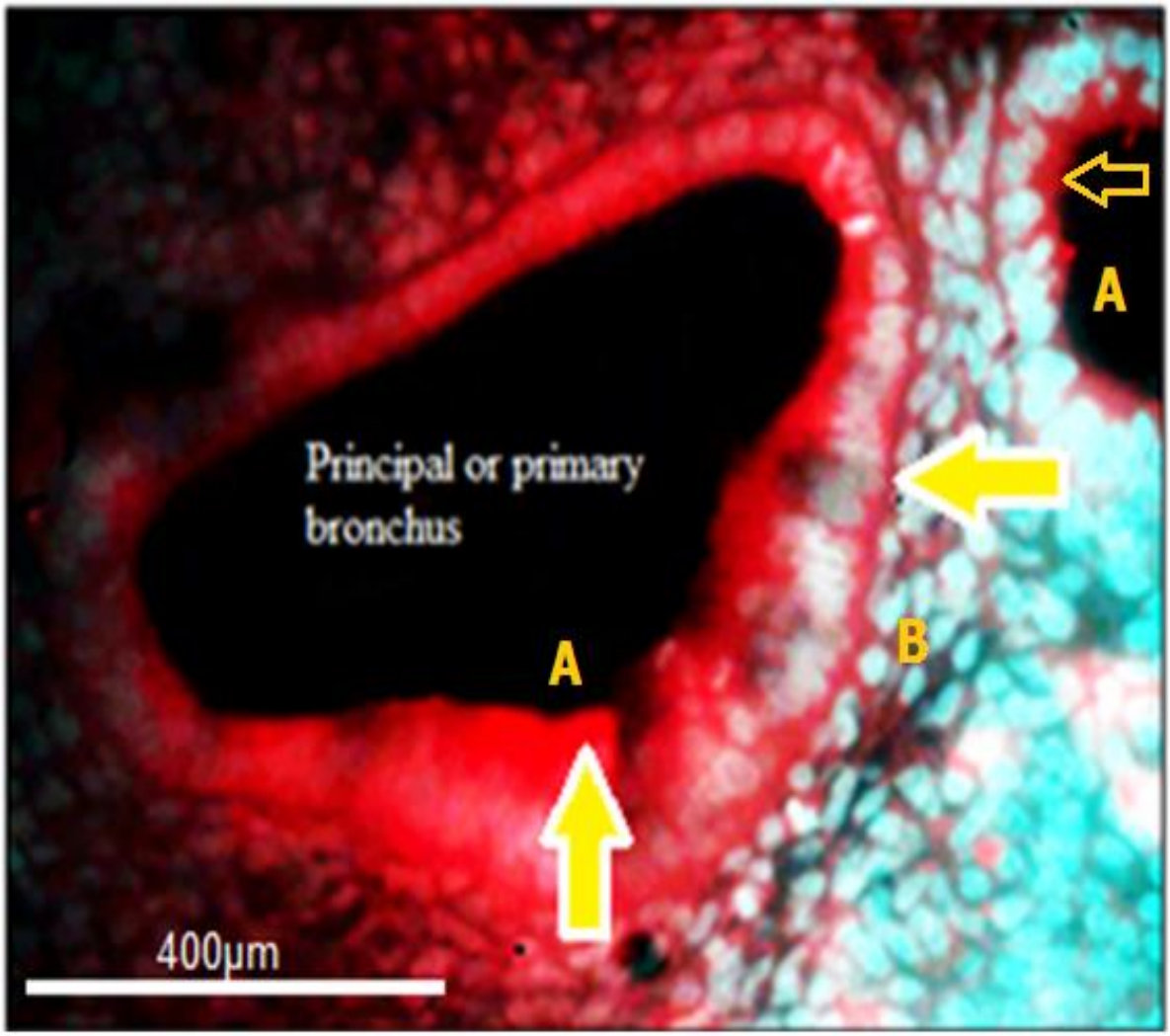

Figure 3.36. Primary bronchus at Stage 31 showing VEGF expression at both the apical A) and basal B) borders of the epithelial cells (arrow heads)

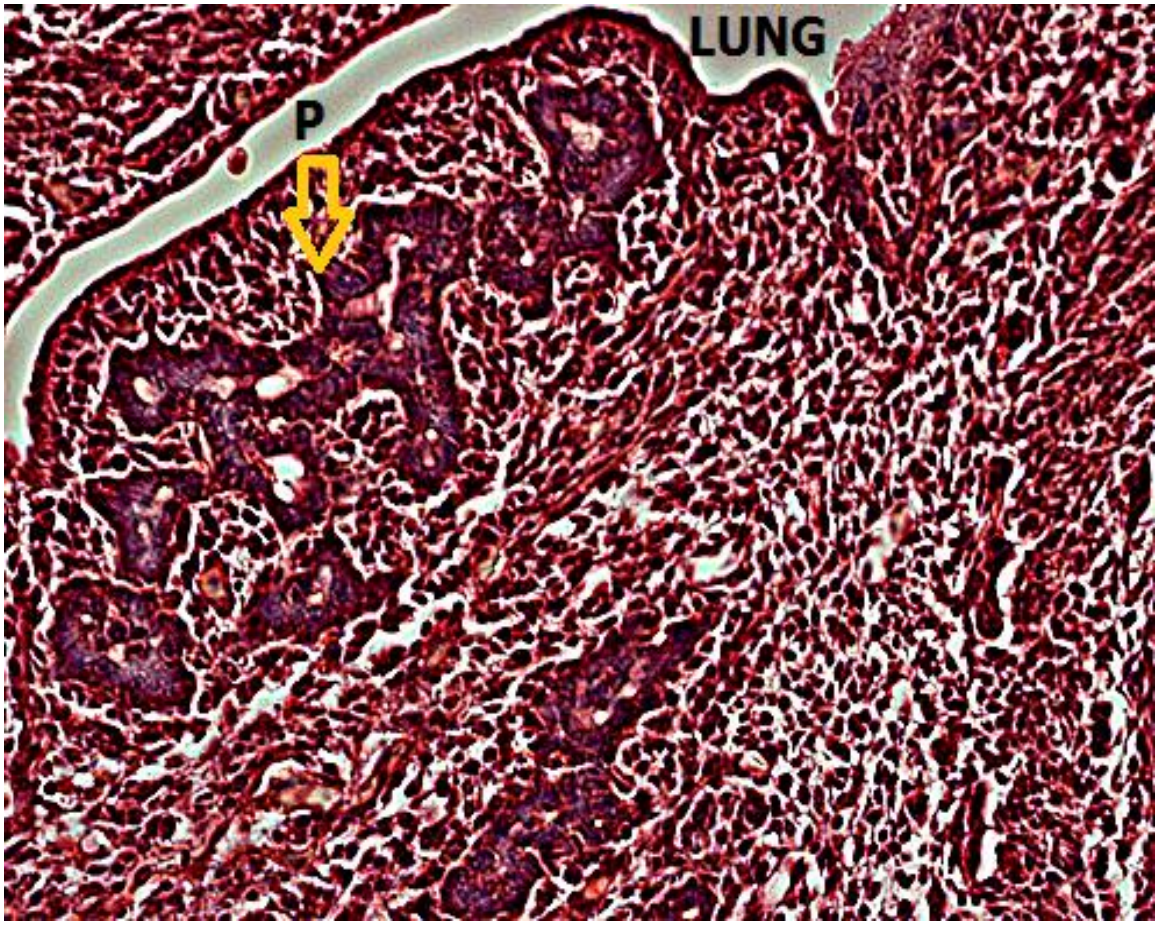

Figure 3.37. Stage 33 Lung. Developing parabronchi appear as solid cord-like structures (arrow head P) X400

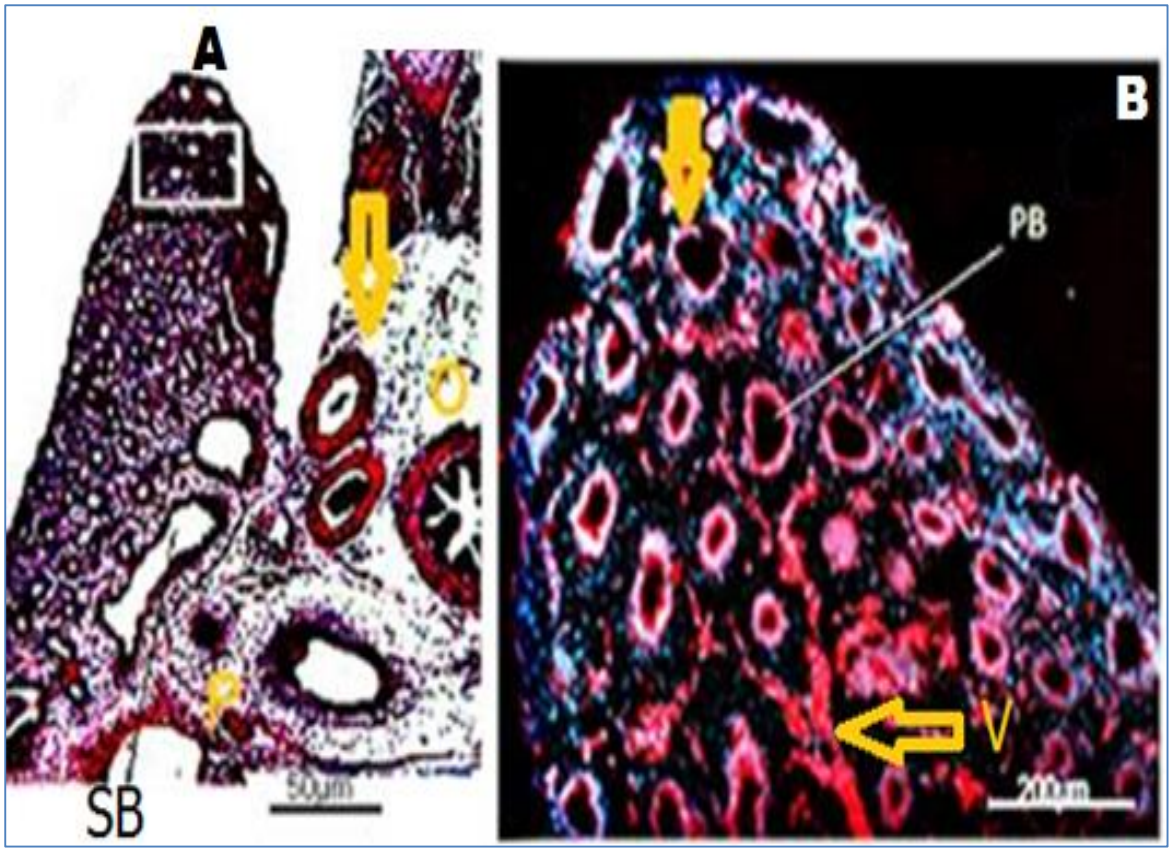

Figure 3.38. Stage 37 lung. The highlighted area in A) is seen in Image B). It shows a typical parabronchial lung with intrapulmonary vasculogenesis (V). PB-parabronchi, SB-secondary bronchi. Parabronchial epithelium expressed VEGF.

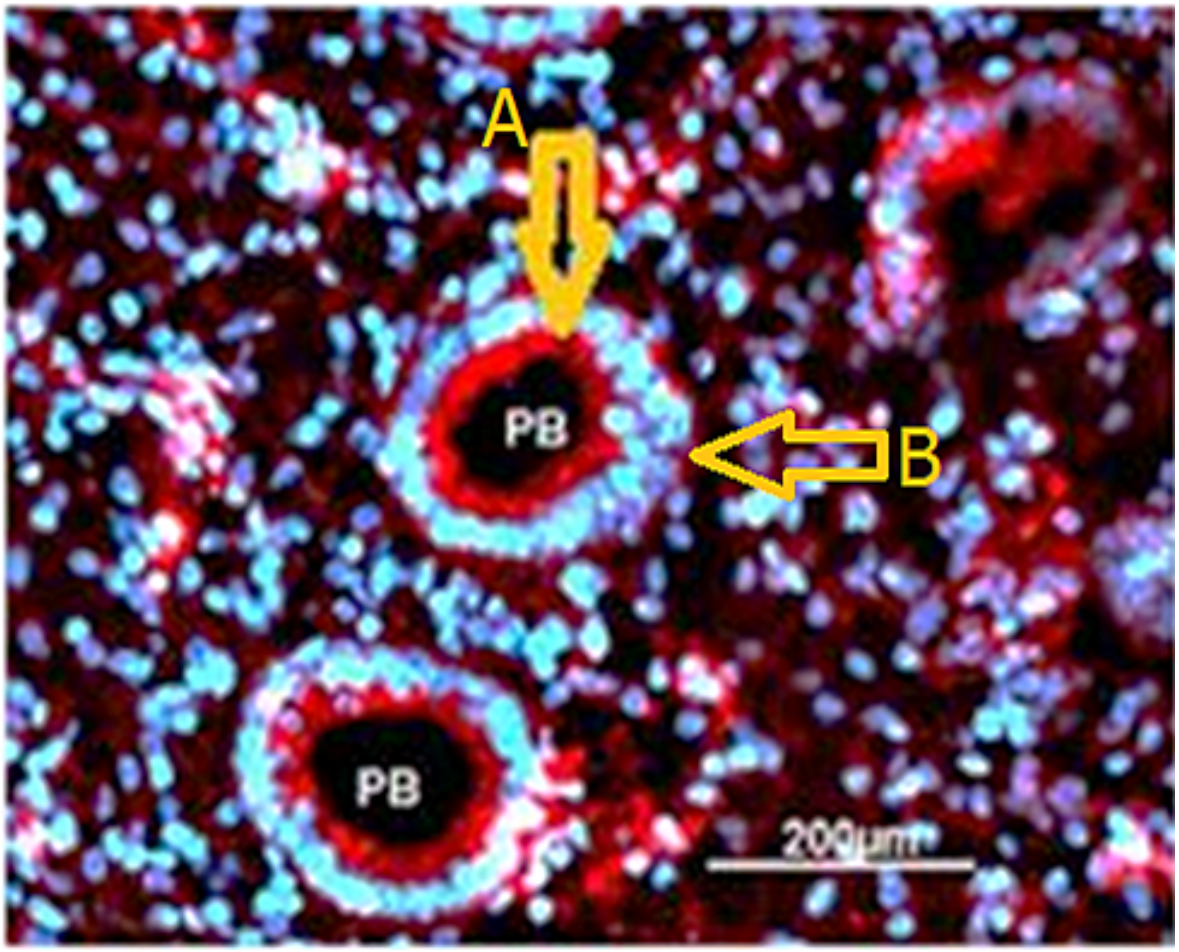

Figure 3.39. Parabronchi (PB) at stage 37 lung showing marked expression of VEGF at both the apical (Arrow heads A) and basal (Arrow head B) boarders.

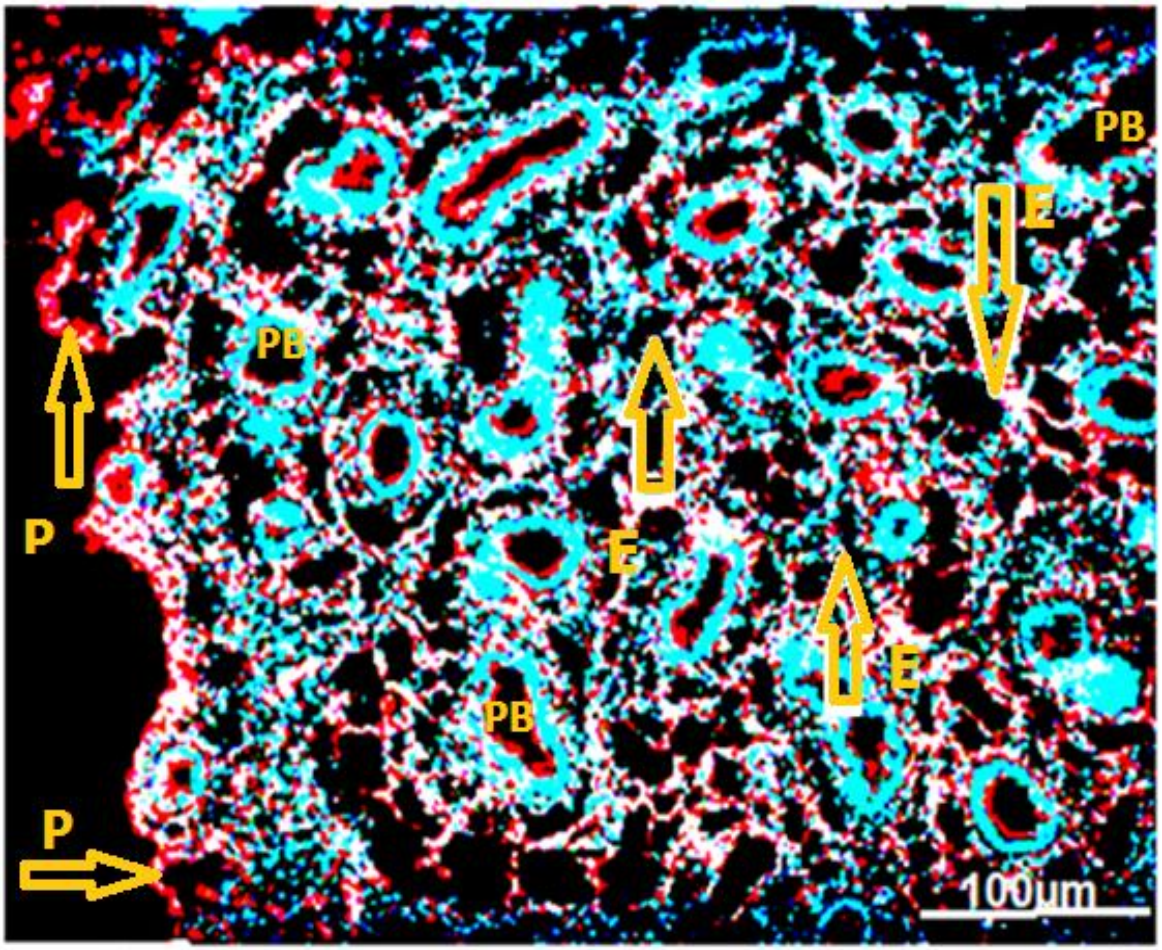

Figure 3.40. Stage 38 lung. Note numerous parabronchi (PB). Arrow heads (E) show gas-exchange tissues. These become thinner with lung maturity. The pleural covering (P) of the lung (P) also shows VEGF expression

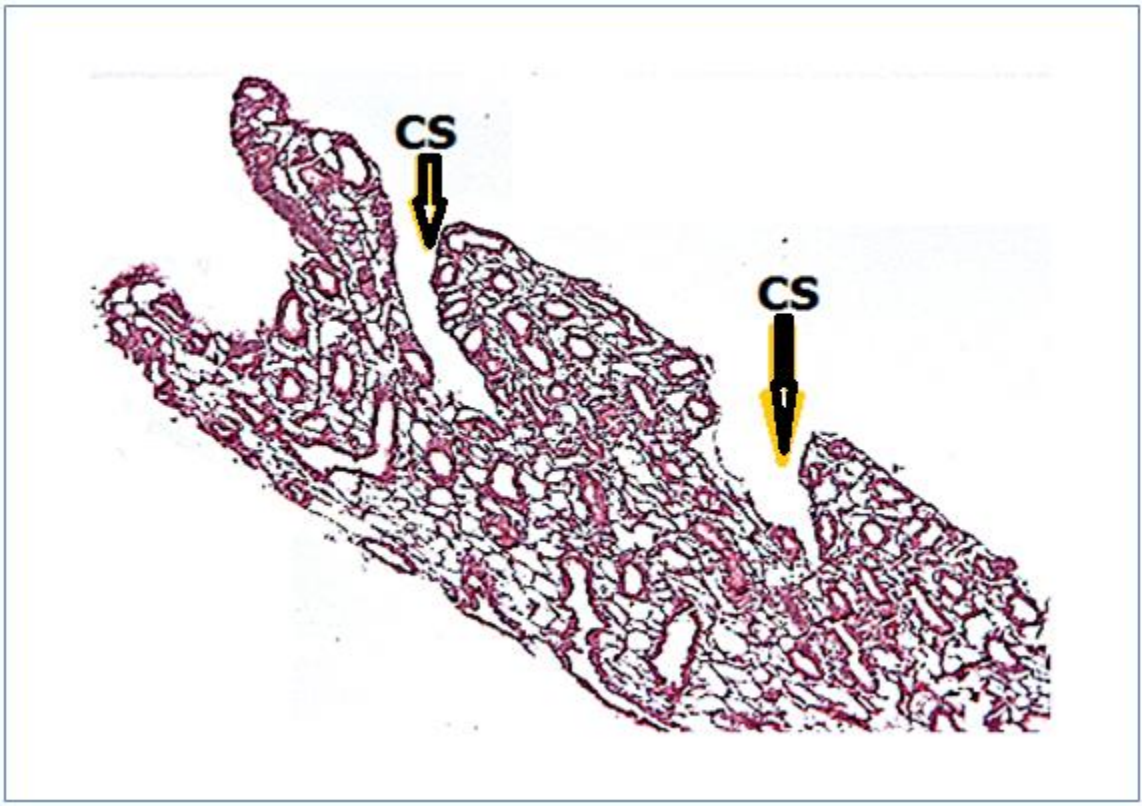

Figure 3.41. A stage 39 (day 13) showing a typical avian (parabronchial) lung. X10. Note the distinct costal sulci (CS) at the dorsal aspect of the lung

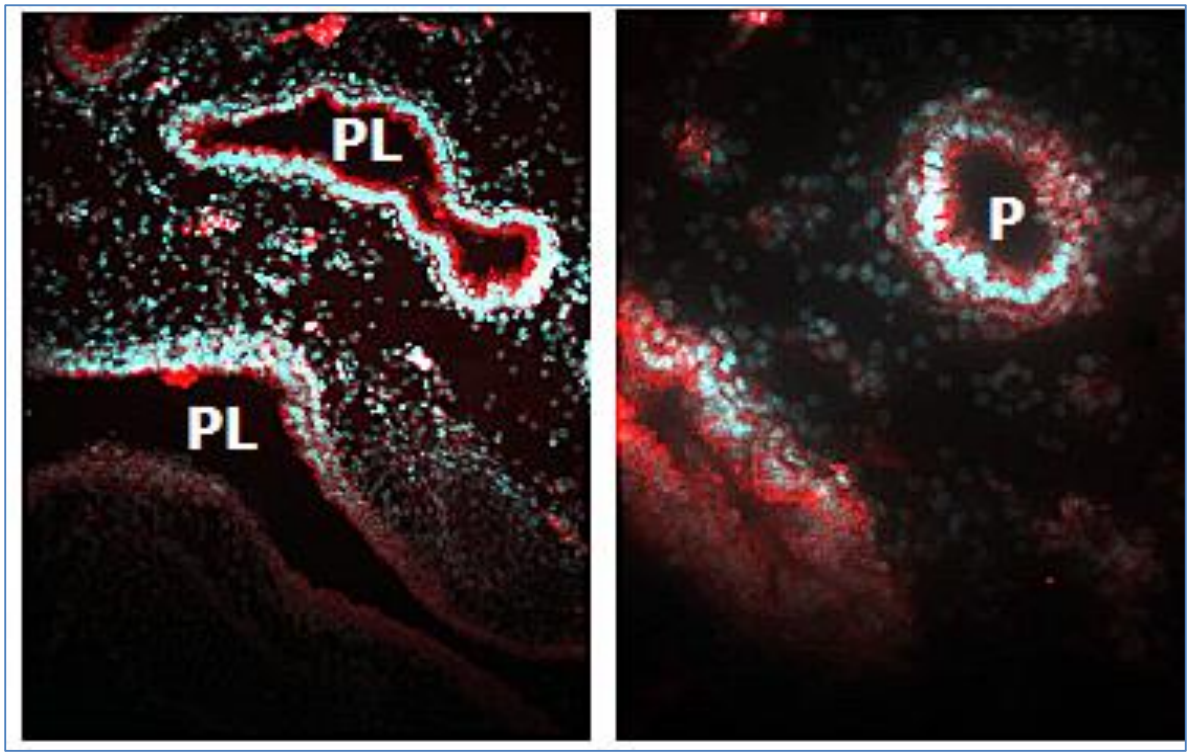

Figure 3.42. Stage 40 Lung. This section shows a typical parabronchi (P) and longitudinal sections through parabronchi (PL). Parabronchial epithelial expresses VEGF at both the apical and basal aspects. X200.

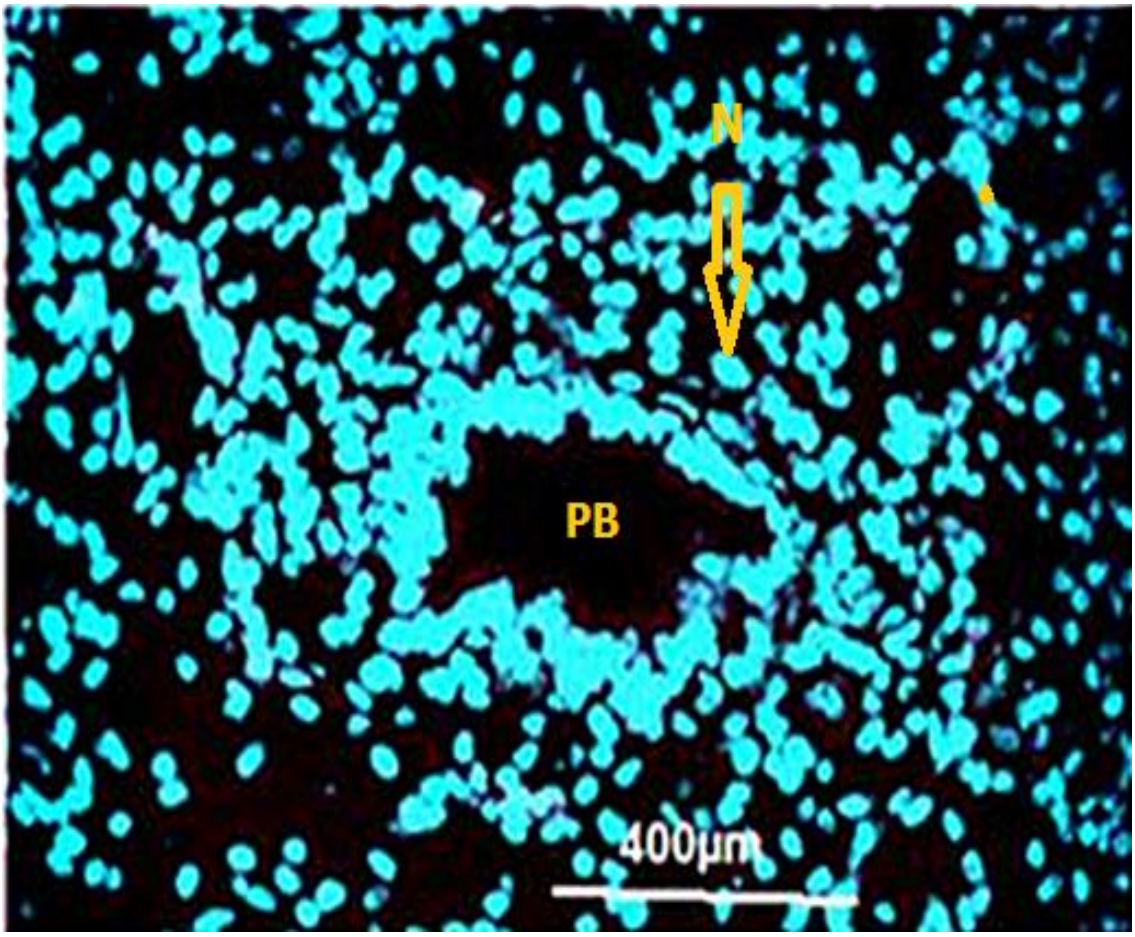

Figure 3.43. Negative section performed on a stage 40 embryonic lung. P-Parabronchus. Note the numerous nuclei stained with DAPI (e.g. arrow head, N).

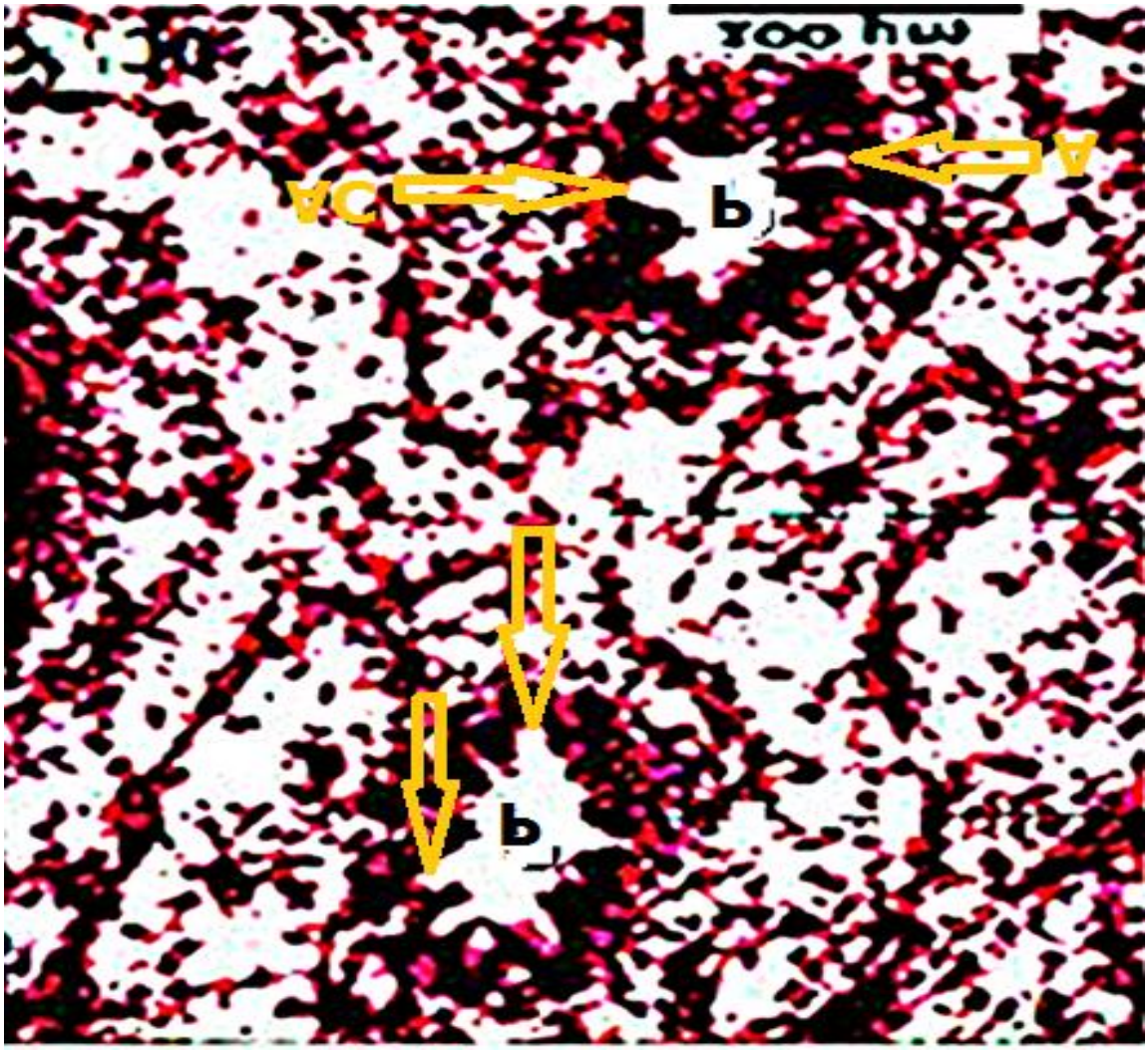

Figure 3.44. Stage 40 Lung with air capillaries (AC) (arrow heads). Atria (A) and infundibulum form as diverticula from the air capillaries (AC)

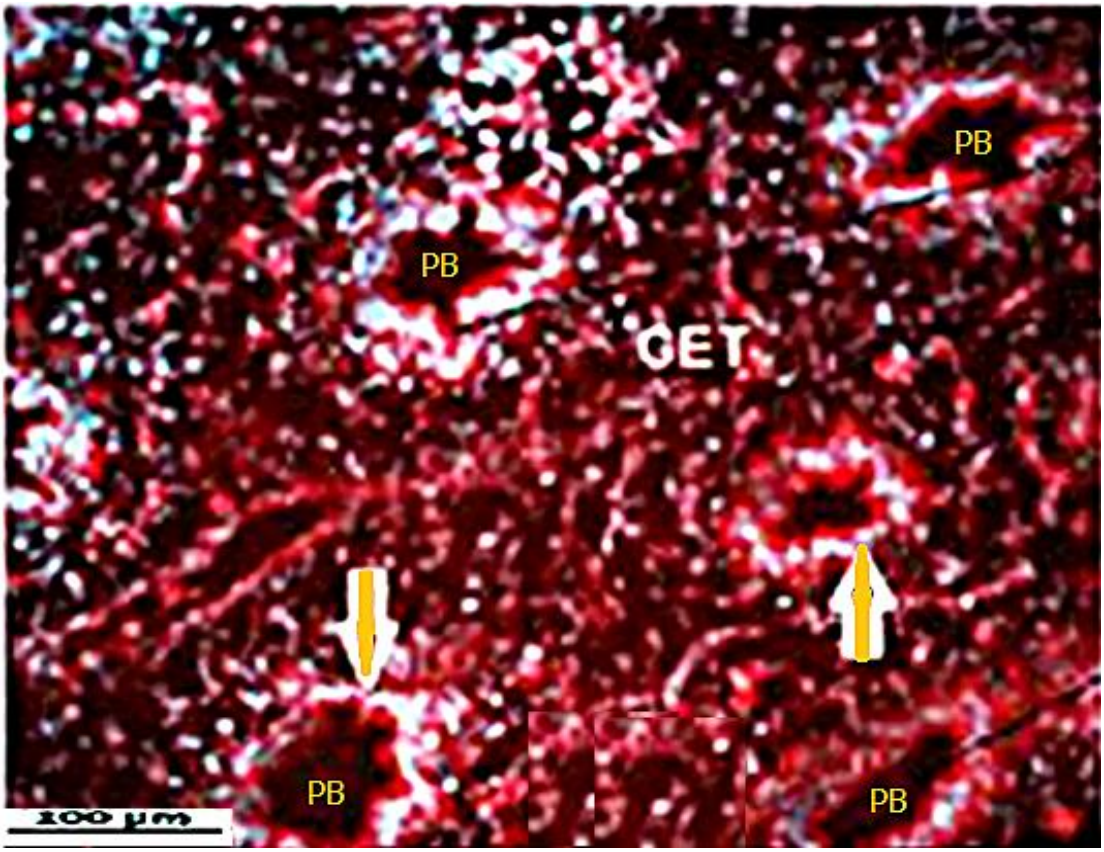

Figure 3.45. Stage 40 lung at X400 magnification showing parabronchi (arrow heads PB) with VEGF expression in both their apices and bases. GET-Gas exchange tissue

3.11. Discussion

At stage 19, the airway progenitor (foregut tube from which the laryngo-tracheal diverticulum develops is seen. The lung primordium forms at stage 20. At stage 22 (day 3.5) the lung bud is quite evident. It is at this stage that VEGF expression occurs, seen in a diffuse manner within the developing lung tissue and the surrounding mesenchyme. Blood islets with no defined boundaries are seen at day 5 (stage 27), with vasculogenesis occurring in different regions of the developing lung. At stage 37 (day 11), the blood islands are both numerous and quite distinct, forming a beautiful pattern of vessels. Moreover, secondary bronchi (SB) began to appear at stage 27 (day 5), becoming fully formed by stage 33 (day 8), the same stage at which PB began to form. Parabronchi first appearing as solid cords of cells, and later acquire a lumen. Maina (2003b) reported that parabronchi develop by budding from secondary bronchi (SB), growing into the adjacent mesenchyme, increasing in size and number. And that they are canalized by day 10 (stage 36). By day stage 39 (day 13), primary bronchi are lined by columnar epithelium. Maina (2003b), also reported that atria, infundibulae and air capillaries form on days 15, 16 and 18 (stages 41, 42 and 44) respectively).

At stage 39 (day 13) the typical parabronchial pattern that is characteristic of the avian lung is seen. But atria and infundibulae which are diverticuli from the luminal surface of PB are distinct at stage 14 lung. They are seen as irregularity in the periphery of the parabronchial lumen. The primitive pulmonary vascular plexus later link up to the main circulation that forms from the sixth pharyngeal arch, coming from the heart (Stenmark and Mecham, 1997). Although by day 10, the pulmonary vasculature is largely formed, complete pulmonary circulation occurs only after the development of the air capillaries on day 18 (Maina, 2004).

3.12. Conclusion

The early VEGF A expression in the avian lung shows that, as is the case with the mammalian lung, VEGF plays a normal role in early avian lung endothelial biology.

CHAPTER FOUR

4.0. COMPARATIVE EFFECT OF EFAVIRENZ ON ANGIOGENESIS IN CHICK CHORIOALLANTOIC MEMBRANE

4.1. Pulmonary Angiogenesis and Lung Development

Angiogenesis involves the formation of vascular sprouts from pre-existing vessels with resultant formation of a highly branched vascular plexus. This is a fundamental process in both development (Hyder and Stancel, 1999) and wound healing. Under these conditions, the process of angiogenesis is highly regulated (Liekens *et al.,* 2001); it is a complex multi-step process involving the release of proteases from "activated" endothelial cells, breakdown of the basement membrane and migration of the cells to the interstitial space, where they undergo proliferation. The cells acquire a lumen and pericytes are then recruited for vascular remodelling, after which a new basement membrane is formed. The newly formed vessels then fuse leading to initiation of blood flow (Mignatti and Rifkin, 1996, Liekens *et al.,* 2001).

The term “Vascular morphogenesis” describes a time-bound set of morphological changes in endothelial cells in order to generate vascular networks during organogenesis (Holderfield and Hughes, 2008). Mesenchyme gives rise to blood vessels initially by vasculogenesis and later by angiogenesis, through the formation of a capillary bed from endothelial cells that have differentiated from mesenchymal precursors (Makanya *et al.,* 2007). Vascular morphogenesis is necessary for tissue expansion. It occurs during organismal growth, in response to increasing higher metabolic demands, particularly as growth outstrips the available supply of oxygen. In response to hypoxia, those tissues release vascular endothelial growth factor (VEGF). In an attempt to restore an adequate blood supply and return local oxygen tension to an acceptable level, VEGF triggers the activation of local endothelial cells (ECs) and initiates the angiogenic response (Olsson *et al.,* 2006). Capillary formation is a complex process and in addition to VEGF,

is also regulated by other growth factors e.g. BMP, TGFß and notch signaling. Capillary formation involves endothelial cell activation, migration, alignment and proliferation, followed by the formation of the capillary tube. Further growth occurs by branching, anastomosis with other vessels and formation of a new basement membrane. VEGF is implicated in the development of the pulmonary vascular system (Ferrara, 2000, D'Angio and Maniscalco, 2002). It stimulates proliferation, differentiation and migration of endothelial cells leading to formation of the primitive vascular plexus in the mesenchyme of the airway (Acarregui *et al.,* 1999, Miquerol *et al.,* 2000). VEGF is a critical regulator of foetal pulmonary mesenchymal proliferation and development, enhancing foetal lung maturation (Chen *et al.,* 2007). Furthermore, VEGF is necessary for achieving the normal lung architecture (Majka *et al.,* 2006). A survey of VEGF localization in adult mice showed that it stabilizes mature vessels (Maharaj *et al.,* 2006). It has been demonstrated that deletion of a single allele of VEGF is lethal (Carmeliet *et al.,* 1996). And in mice, inactivation of VEGF or its receptor led to death *in utero* (Ferrara, 2000). Death occurs as a result of non-development of blood islands in the yolk sac and disorganization of blood vessels at all developmental stages of the embryo (Shalaby *et al.,* 1997). Cross-talk between epithelium and mesenchyme is essential in the lung (Bellusci *et al.,* 1996) for development of the gas exchange tissues. And mesenchyme - epithelium interaction is a complex process that if disrupted will cause anomaly. Development of epithelial structures such as the airway or limb that develop by epithelial budding, require a cohesive relationship between epithelium and blood vessels (e.g. limb bud, the lung). In the development of such organs, disruption of angiogenesis presents with obvious consequences (Seifert and Sobin, 1992). This was the case with thalidomide administration in the 1960s.

4.2. Role of VEGF in Carcinogenesis

VEGF is the key trigger in pathological angiogenesis (Goodsell, 2003). Inhibitors of VEGF signaling pathway are currently being studied for the treatment of lung cancers. A review of the

multiple functions of VEGF-A and its role in clinical oncology showed that it is widely expressed by tumour cells and it acts on many body cells and vascular endothelial. Serum levels of VEGF-A is therefore a good prognostic tool for clinical assessment of cancer patients and, if targeted, will promote patient recovery (Jarvis *et al.,* 2010). Moreover, it has been documented that VEGF is a ligand for neuropilins (Jarvis *et al.,* 2010) and particularly that, in addition to VEGFR-1 and VEGFR-2 receptors, VEGF-A is mediated by neuropilin (Dvorak, 2002). Neuropilins are transmembrane glycoproteins that are usually present in two forms (NRP-1 and NRP-2). Although they act mainly as co-receptors for another class of proteins called semaphorins, they act as receptors in neurons, where they enhance axon function.

4.3. Angiogenesis Inhibitors

Anti VEGF antibodies block its receptor binding, a process that has been exploited to block tumorigenesis (Goodsell, 2003). D'Amato et al. (1994) studied the effect of some angiogenesis inhibitors; thalidomide, some antimitotic agents, retinoic acid and tamoxifen on rabbit cornea. Thalidomide had an added advantage over the others in that; it is effective even with oral administration, and also relieved bFGF (FGF2)-induced corneal neovascularization in rabbit cornea. Electron microscopic examination of the corneal tissues showed that thalidomide-induced vascular changes were similar to those seen in the deformed limb vasculature of thalidomide-treated embryos. And according to Sampaio et al. (1991), thalidomide produces its anti-angiogenic effect by antagonizing the effect of tumour necrosis factor alpha (TNF- α); TNF-α increases endothelial cell expression of VEGF, VEGF receptors, FGF-2 and interleukin-8 (IL-8) (Yoshida *et al.,* 1997).

Thalidomide (Contergan) was developed by a German company called Chemie Grunenthal, as an anticonvulsant drug in 1954, acquiring a twenty-year patent (Eriksson *et al.,* 2001). It was first synthesized for its weak antihistamine properties but found to have profound sedative effect. The

anti-emetic effect of thalidomide was used to treat morning sickness in pregnant women in the 1960s, with gross teratogenic consequences such as failure of limb development (McBride, 1961, Smithells and Newman, 1992). Unfortunately, between 1957 and 1961, thalidomide had been used in Canada, Europe, Australia and Japan resulting in development defects in 10,000 babies, with death occurring in 40% of them, before the drug was withdrawn from the market worldwide. Between 1957 and 1961, thalidomide had been used in Canada, Europe, Australia and Japan as an anti-emetic. It had caused gross congenital malformations in 10,000 babies that led to death in 40% of them, before the drug was withdrawn from the market worldwide (Smithells and Newman, 1992). In the new born, thalidomide caused a variety of severe limb deformities such as amelia and phocomelia; both upper extremities showed absence of fingers, fusion at the elbow, and absence of the radius, and shortening of the ulna (Smithells and Newman, 1992). However, thalidomide is now being recognized as a useful drug in the treatment of pathologic angiogenesis, such as is found in solid tumours, diabetic retinopathy and macular degeneration. Orally administration had a high efficacy in inhibition of angiogenesis, and so, is a good long-term drug (D'Amato *et al.,* 1994). Continuous use of low doses of thalidomide in patients with a variety of advanced cancers showed improvement and stabilization of the disease for up to 6 months (Eisen *et al.,* 2000). In addition, the thalidomide derivative, pomalidomide has anti-inflammatory properties that have been recently used to treat multiple myeloma. The drug directly suppresses multiple myeloma in affected cells. Another drug, lenalidomide has since 2012 been licensed for the treatment of relapses in multiple myeloma and is currently used in the management of diseases such as malignant lymphomas, myelodysplastic syndrome, and chronic B lymphocytic leukaemia. Moreover, when used daily in combination with 50 mg of thalidomide, it inhibits progression of angiomatosis (Adam *et al.,* 2012). Anti-VEGF medicines have also been used in the treatment of wet age-related macular degeneration (AMD), a disease caused by abnormal growth of blood vessels under the retina (with resultant blindness if untreated). The growth of these vessels is usually triggered by VEGF. Examples of the drugs are Bevacizumab (Avastin), Pegaptanib (Macugen) and Ranibizumab (Lucentis).

4.4. Antiretroviral therapy: HAART

Based on the guidelines for the management of HIV-1-Infected Adults and Adolescents by the United States Department of Health and Human Services (2015), six classes of Antiretroviral (ARV) drugs have been used in combination therapy of HIV infection. The drugs are classified based on the phase of the retrovirus life-cycle that they inhibit. A typical choice of drug combinations will therefore include two Nucleoside reverse transcriptase inhibitors (NRTI) as a "backbone" along with a Non-Nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI) or Integrase inhibitors (also known as integrase nuclear strand transfer inhibitors or INSTIs) as a "base". The treatment to all patients with the HIV virus with combination of three or more drugs is recommended by both the United States Department of Health and Human Services (Guidelines for HIV management by US Department of Health and Human Services 2015), and the World Health Organization (World Health Organization, 2016).

HIV infection has been particularly associated to Kaposi's sarcoma (KS), cervical cancer and non-Hodgkin's lymphomas. KS presents normally with angio-proliferative lesions (Monini *et al.,* 2004). Many researchers have documented the regression of Kaposi's sarcoma after HAART administration (Burdick *et al.,* 1997, Aboulafia, 1998, Benfield *et al.,* 1998, Lebbé *et al.,* 1998, Cattelan *et al.,* 1999).The term 'highly active antiretroviral therapy' (HAART) refers to the use of multiple drugs in the management of HIV infection with the aim of reducing the viral load, maintaining the patient's immune function, and decreasing the incidence of drug resistance (Moore and Chaisson, 1999). Krown (2004) observed that the treatment regime, 'Highly active antiretroviral therapy' (HAART) in addition to improving the prognosis of HIV infection, also caused tumour regression and so, increased the rate of patient survival among HIV patients with pulmonary Kaposi Sarcoma (Holkova *et al.,* 2001). HAART also lengthens the time from onset of treatment to treatment failure (Bower *et al.,* 1999). HAART has therefore changed for good the natural history of AIDS- cancers.

Furthermore, it is now known that HIV-1 protease inhibitors (PI) are anti-tumour molecules that have been associated with regression of Kaposi sarcoma, since they possess anti-angiogenic properties. Because appropriate HIV management generally requires administration of HAART, distinguishing the antitumour effects of HAART from those induced by other agents in the management of KS treatment presents with difficulties (Krown, 2004). But the antitumour effects (e.g. anti-angiogenic effect) of component drugs in HAART could be easily compared to effects of known anti-cancer agents such as thalidomide.

4.5. **Statement of the Problem**: Certain cancers have been associated with HIV infection; Kaposi's sarcoma, non-Hodgkin's lymphomas and cervical cancer. It is therefore a significant finding that the incidence of these tumours in HIV-infected patients decreases significantly with the use of highly active antiretroviral therapy (HAART) (Monini *et al.,* 2004). Because Kaposi's sarcoma presents normally with angio-proliferative lesions the use of anti-angiogenic drugs may suppress its spread and promote patient survival. Anti-angiogenic drugs cut off vascular supply (and nourishment) to cancer cells. This is more so in that neovascularization is a necessity for the growth of neoplastic tissues (Pepper, 1997, Carmeliet and Jain, 2011). Furthermore, it has been noted that HIV-infected individuals with lung cancer received less attention for cancer treatment when compared to cancer patients that were HIV-negative (Suneja *et al.,* 2013).

4.6. **Justification**

It should be noted here that a Pubmed search for research comparing Efavirenz to thalidomide as an anti-angiogenic treatment yielded no results. This work therefore seems represent the first such report of the anti-angiogenic properties of Efavirenz

(McNeil *et al.,* 2017). Moreover, there is no publication on Efavirenz in relation to anti-metastasis of cancers. The uncertainty on the role of ARV in HIV and cancer patients is the underlying basis for this work:

4.7. **Aim**: To investigate the anti-angiogenic property of a first line ARD (Efavirenz)

4.8. Efavirenz

Efavirenz is a NNRTI that is marketed as Sustiva and Stocrin. It is used in combination with other antiretroviral drugs in adults and children with HIV infection (Coffey, 2011) and is recommended in treating both previously treated and untreated people (Staszewski *et al.,* 1999). Generic versions of Efavirenz are used only as part of the U.S. President's Emergency Plan for AIDS Relief's (PEPFAR) in countries with limited resources (Coffey, 2011).

4.9. Mechanism of action

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) of human immunodeficiency virus type 1 (HIV-1). Its activity is mediated mostly by non-competitive inhibition of HIV-1 reverse transcriptase (RT). HIV-2 RT and human cellular DNA polymerases α, β, γ, and δ are not inhibited by EFV. As a NNRTI, Efavirenz is a Cytochrome P450 3A Inducer, Cytochrome P450 2B6 Inducer, Cytochrome P450 2C9 Inhibitor, Cytochrome P450 2C19 Inhibitor, and Cytochrome P450 3A4 Inhibitor. Cytochromes are a number of compounds that consist of haem bonded to a protein. They function as electron transfer agents in many metabolic pathways involved with cellular respiration (Kim *et al.,* 2015). Efavirenz is therefore recommended for antiviral combination treatment HIV-1 infected adults, adolescents and even children 3 months of age and older with a weight of at least 3.5 kg. (NIH Fact sheets, 2018).

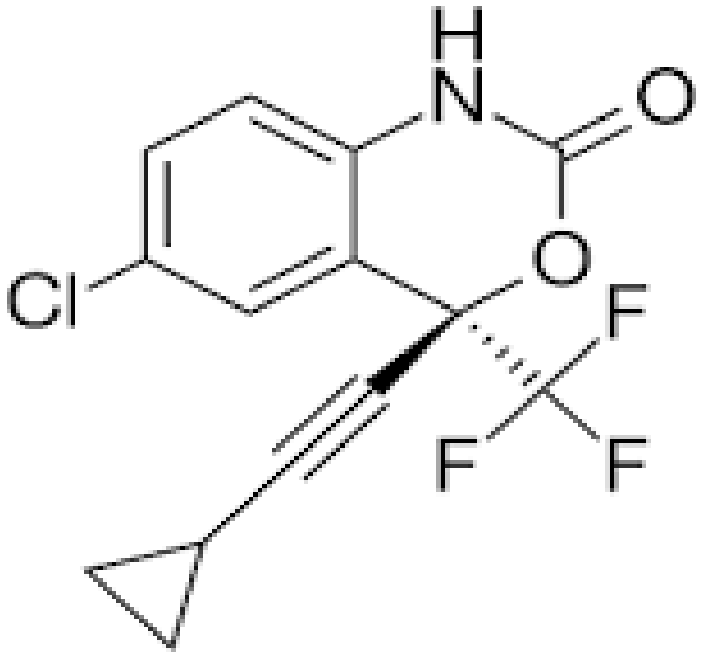

Figure 4.1. The structure of Efavirenz

Efavirenz is particularly effective when included in the initial treatment of HIV in that it suppress the viral load and so, helps increase the CD4 count (van Leth *et al.,* 2004) particularly when given in combination with nucleoside backbones (DeJesus *et al.,* 2004, Gulick *et al.,* 2004). Because Efavirenz is associated with significantly severe side effects such as mental disturbances and rash, and suppresses blood methadone levels *(a drug* used as a pain reliever and as part of the management for *drug* addiction detoxification (Coffey, 2011), it is necessary to use it with care e.g. give significant amounts of methadone along with Efavirenz therapy according to the need of the patient (Clarke, 2001). Nevertheless, Efavirenz is the most widely used non-nucleoside reverse transcriptase inhibitor (NNRTI) in the treatment of HIV infection (Gomez-Sucerquia *et al.,* 2012). Antitumour effects of antiretroviral therapy has been mentioned; moreover, protease inhibitor (PI) indinavir (also known as saquinavir), when administered to mice blocks Kaposi sarcoma-like lesions and suppresses bFGF and VEGF-induced angiogenesis in the chorioallantoic membrane assay. The potency of PI has been equated with that of paclitaxel (PTX), an anticancer drug (marketed as Taxol) that is used in chemotherapy of many human

malignancies; breast, pancreatic, lung, ovarian, cervical cancers and Kaposi sarcoma ((Horwitz 1994; Sgadari *et al.,* 2002). Moreover, PI prevented tissue invasion by endothelial cells and Kaposi Sarcoma cells invasion, and inhibit the growth and invasion of an angiogenic tumour-cell line *in vivo.* They have been advocated in the treatment of non-HIV Kaposi Sarcoma and in other HIV-associated tumours (Sgadari *et al.,* 2002.

4.10. **Ethics Clearance**

The work was carried out at the Wits Medical School (Ethics clearance certificate no. 2008/7/1). This work was funded by the University of the Witwatersrand Health Sciences Faculty Research Committee (FRC).

4.11. **Objectives**

1. To make direct visual observations of embryonic CAM
2. To evaluate the anti-angiogenic effect of Efavirenz on chick CAM
3. To compare the anti-angiogenic effect of Efavirenz to thalidomide in chick CAM
4. To ascertain if exogenous administration of VEGF enhances CAM angiogenesis

4.12. **Materials and Methods**

This experiment was done after the technique of Henshel *et al.,* 1997. The air cell on average occupies 2.5% of the volume of fresh eggs (Brand *et al,* 2013). The chorioallantoic membranes (CAM) of thirty (30) fertile eggs were used as *in vivo* vascular systems to study angiogenesis. Using this system the effect of the anti- retroviral drug Efavirenz on CAM angiogenesis was studied and compared to that of the known angiogenic drug (VEGF) and anti-angiogenic drug (thalidomide). All reagents were purchased sterile and all equipment used was autoclaved.

4.13. Experimental Groups and Treatment

70% ethanol was used as diluent for the stock solutions. Further dilutions were done using physiological saline. All drugs were given as a single injection into the air cell using needles and syringes. The eggs were incubated at C in a humidified incubator, randomly divided into five (5) treatment groups, and treated on day 3 of incubation (assumed to be at Hamilton and Hamburger developmental stage 21) as follows:

Control 1 Embryos (Solvent control group) (n=6): These embryos were treated with an equal volume of the diluent (0.2ml of 70% ethanol in 0.8 ml physiological saline). And like all treatment groups (except Control 2), on day 5 of treatment, the eggs each had an observation window cut in the egg shells.

Control 2 Embryos (n=6): These had normal development with no interference; no windows were cut in the egg shell.

VEGF-treated group (n=6): Positive control group. From a stock solution of human recombinant VEGF protein (R&D Systems), diluted in 70% ethanol, 200 µg in 1ml physiological saline /embryo was used (Rak *et al.,* 1995, Weisz *et al.,* 2001, Ferrara *et al.,* 2003).

Thalidomide-treated group (n=6): Negative control group. Thalidomide was prepared as a stock of 100 mg/ml. 20 mg in 1ml physiological saline was given to each embryo (D'Amato *et al.,* 1994, Neben *et al.,* 2002). Thalidomide dosage and schedule are still controversial. Teratogenic effect has been experimentally induced in pregnant rabbits at an oral dose of 100-300 mg/kg per day (Sterz *et al.,* 1987, Helms and Wetzel, 1996). A study showed the effect of thalidomide in disease treatment is dose dependent and that the outcome, in form of overall survival, improves when dosage is adjusted to reflect body size parameters such as body surface area, height, and weight (Neben *et al.,* 2002). D'Amato et al. (1994) in their study of thalidomide teratogenicity found that thalidomide is useful for the treatment of diseases that are dependent on angiogenesis.

Efavirenz-treated group (n=6): This is the test group. A 3 mg/ml of ethanol stock solution was made after the work of Gomez-Sucerquia et al. (2012) they ascertained that the solutions are stable, as tested by High Performance Liquid Chromatography (HPLC). 200 µg Efavirenz in 1ml physiological saline was given to each embryo. Efavirenz was obtained from Sequoia Research Products (Pangbourne, UK). Calculation of dosages: Doses were calculated from the recommended dosage approved by the U.S. Food and Drug Administration (FDA), for the treatment of HIV infection in adults and in children 3 months of age, and older who weigh at least 7 pounds 12 ounces (3.5 kilograms). WHO also recommends the dosage of 600mg/day for efavirenz in the simplified dosing (child-friendly solid formulations) (WHO 2013).

Route of Drug Administration: No paper or report on the use of efavirenz in chick air cell has been published in literature. The method of treatment was designed in consultation with Prof John Solly of the Faculty of Veterinary Science of the University of Pretoria, Onderstepoort campus in Pretoria, South Africa. The blunt side of the eggs were sterilized by swabbing with cotton wool soaked in 70% ethanol, and drugs were each instilled by needle puncture into the air cell, (between the outer and inner layers of the membranes). The puncture wound was then sealed using candle wax in order to prevent bacterial invasion. The eggs were then returned to the incubator. At this time, no other papers or reports on use of efavirenz in chick air cell has been published in literature. It is also recommended that the dose in mg can be dispensed in any combination of capsule strengths and capsules may be administered by sprinkling of the contents into an age-appropriate food. For adolescent (weighing ≥40 kg) and adults, the dose of Efavirenz is 600 mg once daily (NIH Fact sheets, 2018).

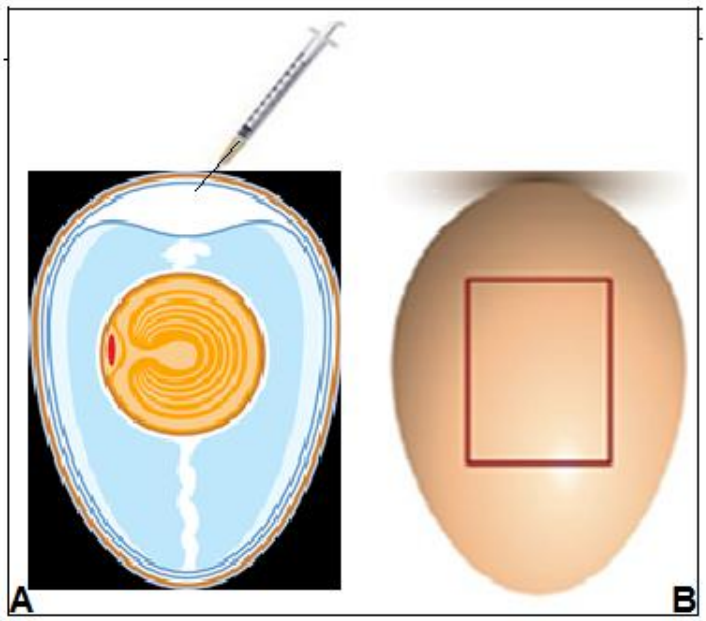

Figure 4:2. Figure A) is an illustration of how the eggs were treated.

The drugs treatment technique was designed in consultation with Prof John Solly of the Faculty of Veterinary Science of the University of Pretoria, Onderstepoort campus; the drugs were injected into the air cells. Figure B) shows the positioning of the observation window that was cut into the egg shells.

Procedure for cutting observation windows into the egg shells

With gloves worn, the egg shells were first wiped down with ethylated spirit on cotton wool. The egg shell was removed (as illustrated in figure 4.1B) and the inner shell membrane exposed. The inner shell membrane was then carefully removed.

On day 8 of incubation (day 5 of treatment), which is assumed to be Hamilton Hamburger developmental stage 31, windows of about 1 x 2cm were cut into the egg shell. Drops of antibiotics (Penstrept (50 µl/50ml chick ringer) were instilled into the windows for sterility and to help the CAM to sink below the egg shell. Through the windows CAM blood vessels were observed and images were captured using a Nikon microscope camera. The windows were again instilled with 2-3 drops of pen/strep solution and the eggs returned to the incubator until day 15 of treatment the CAM images were again captured and the embryos harvested. At the end of the

experiment, the thoracic cavities of the embryos were opened and the developing lungs photographed. The results were then compared to those of the control embryos.

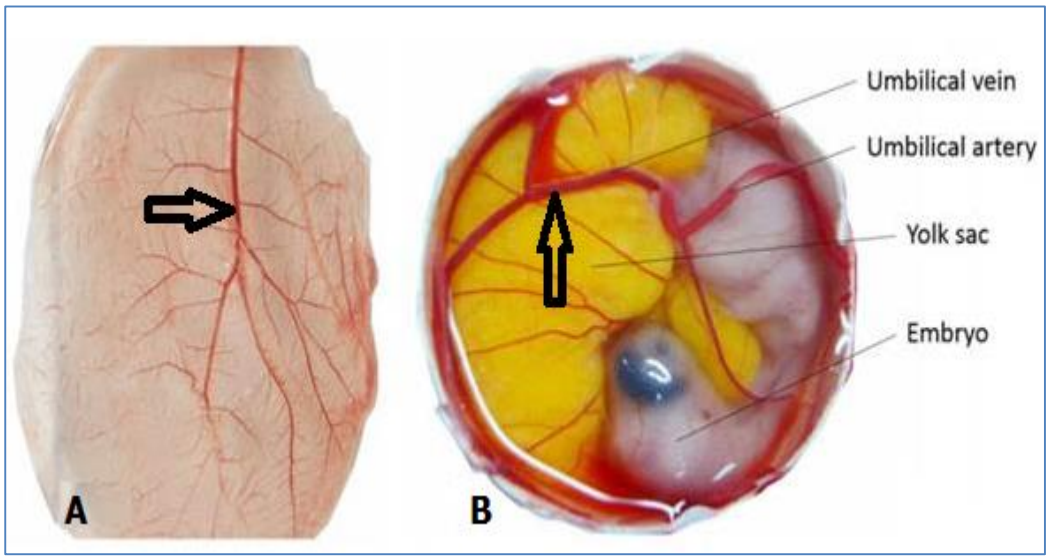

Figure 4:3. This is an image of the CAM Capillary plexus of a 10-day old chick embryo. Figure A) shows the umbilical artery (arrow); Figure B) shows free-floating vessels within the allantoic vesicle (arrow) (Yuan *et al.,* 2014).

4.9.1. **Experimental Groups and Treatment**

4.9.2. **Experimental Groups and Treatment**

4.14. Results

CAM blood vessels were scored as either “present” or “absent”. Images of embryonic CAMS were digitally captured using a Nikon Microscope camera. Results are as shown in table 4.1 below; they were analysed using one-way analysis of variance (ANOVA). Fisher exact test was used to determine association between treatment drugs and growth of CAM blood vessels, where $p < 0.05$ was considered as significant.

Table 4:1: Summary of the effects of treatment on embryonic viability, CAM angiogenesis and erythropoiesis

Treatment Group (n=6)	**Viability (No of embryo(s) alive)**	**CAM Angiogenesis**	**CAM Erythropoiesis**
Control 1	6 (100%)	Yes	Yes
Control 2	6 (100%)	Yes	Yes
VEGF	5 (83.3%)	Yes	Yes
Thalidomide	1 (17%)	Yes	Yes
Efavirenz	0 (0.0%)	No	No

Table 4:2: Analysis of Results using ANOVA

Group	**Mean Angiogenic effect**	**Standard Deviation**	**F**	**P-value**
VEGF	0.83	0.41		
Thalidomide	0.17	0.41	9.306	*0.0005
Efavirenz	0	0		
Control 1	0.67	0.52		
Control 2	1	0		

*** indicates the p value is significant, $p < 0.005$**

From Table 4.2 we can say that, possessing 17% angiogenicity implies that thalidomide failed as an anti-angiogenic drug in the one embryo. Moreover, one of the embryos in the thalidomide group reached the end of the experiment but died within seconds of exposure. Its CAM had one short thriving blood vessel on the CAM surface (figure 4.5B below). In Efavirenz-treated CAMS, no blood vessels were seen and neither was there any sign of erythropoiesis (4.6A-D). Efavirenz had 0% angiogenicity, showing it to have absolute anti-angiogenic effect in chick CAM. Efavirenz therefore presented as a more potent anti-angiogenic drug than thalidomide. However, there was no statistical significant difference between the anti-angiogenicity of Efavirenz (100 %) and Thalidomide (83 %) (F = 1.000, p = 0.341). This is particularly because the anti-erythropoietic effect of the treatment drugs (which enhanced the effect of efavirenz) was not considered in the analysis. This will be further explored in future work.

Table 4:3: Fisher's exact test

Treatment Drugs	**CAM blood vessel present**	**CAM blood vessel absent**	**Fishers exact test (p-value)**
	f %	f %	
VEGF	5 (31.3)	1 (7.1)	
Thalidomide	1 (6.3)	5 (35.7)	
Efavirenz	0 (0.0)	6 (42.9)	*0.001
Control 1	4 (25.0)	2 (14.3)	
Control 2	6 (35.5)	0 (0.0)	

*** p- 0.001 is significant Fisher exact test was used to determine association between treatment drugs and growth of CAM blood vessels (p < 0.05). A p value of < 0.05 was considered significant for all analysis.**

4.15. CAM Images Captured On Day 5 And 15 Of Treatment

Photographs of embryonic CAMs were taken using a Nikon Microscope camera. Control vessels were well formed (see figure 4.3A-D below).

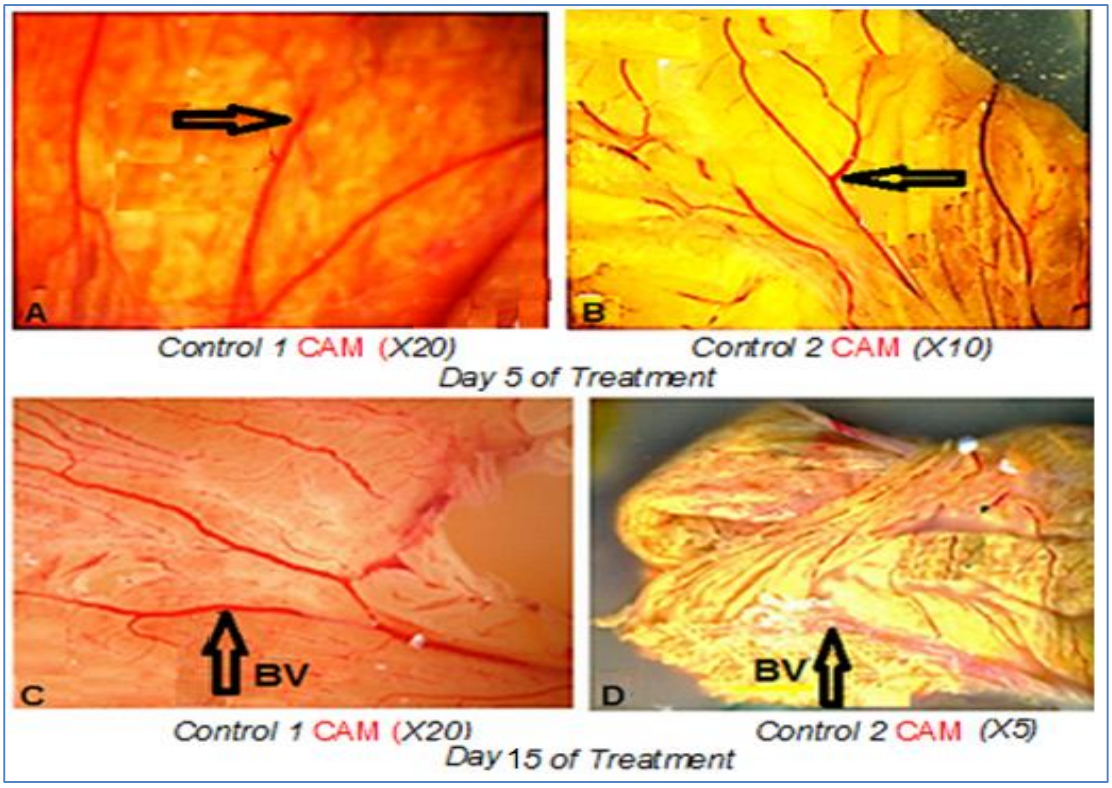

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Figure 4.4. Angiogenesis in control chick CAM. Blood vessels were well formed (see arrow heads and labels 'BV'). A= Control 1 CAM on day 5 of treatment; B= Control 2 CAM on day 5 of treatment; C= Control 1 CAM on day 15 of treatment D= Control 2 CAM on day 15 of treatment.

The embryos in the VEGF group responded to treatment in different ways: two had normal well-formed vessels (figure 4.4C), one embryo had vascular permeability leading to exudation and haemorrhage (figure 4.4A), one had increased CAM vascularity, in which the embryo had hyper dynamic circulation (for which the heart beat was captured on video (figure 4.4D). The 6th embryo died before the end of the experiment for an unknown reason, since it had well-formed CAM blood vessels (figure 4.4.F).

Day 5 of Treatment: Controls 1, in figure A) was the solvent control and Control 2, in figure B) had normal development (these subjects were not manipulated in any way). In both control groups, the CAM blood vessels (indicated by arrow heads), were well-formed.

Day 15 of Treatment: By the end of the experiment, CAMs of both control groups still had well-formed blood vessels (see arrow heads, BV). However, Control 1 CAM (figure C) had a rather reddish background, unlike the normal pale yellow colour of Control 2 (figure D). This indicated CAM congestion and is probably due to the presence of ethanol and/or instillation of Penstrept, being the solvent Control CAM. But since ethanol and Penstrept were also used in all other test groups, it will be right to rule this as a peculiar reaction by this subject.

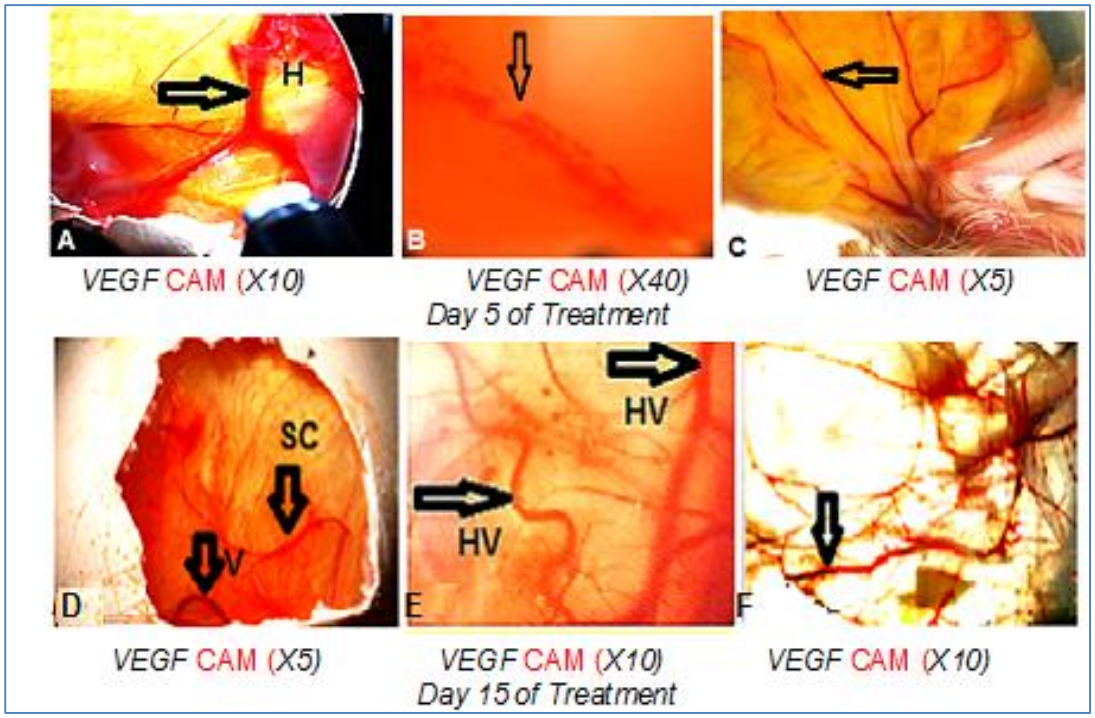

Figure 4.5. VEGF-A-treated CAM. Figure A) CAM had exudating ('leaky') blood vessels; Figure B) is an image at a higher magnification that showed VEGF-induced vascular wall defect; Figure C) appears to have normal angiogenesis; Figure D and E) shows a hyper vascular CAM. Note the increase in the number of blood vessels in figure E). Figure F) is the CAM for an embryo that died before the end of the experiment. The reason is not known and may be peculiar to the embryo.

Day 5 of Treatment with VEGF

These subjects had either normal CAM blood vessels (as in figure C), CAM with vascular haemorrhage (represented by CAM in Figure A), or hyper vascularization of CAM vessels, represented by Figure B. Vascular permeability is characteristic of VEGF, which is also known as permeability factor (Dvorak, 2002). Figure B) shows VEGF-induced vascular wall defect, as viewed at a higher magnification. Drake et al. (1995) reported that exposure to high levels of exogenous VEGF interfered with embryonic vascular assembly during the phase of neovascularization.

Day 15 of Treatment with VEGF

By day 15 of treatment VEGF-treated CAMs showed hyper vascularization (Figure D, arrow head labelled V). In addition, smaller calibre vessels (labelled SC) and at a higher magnification labelled ‘HV’ in figure E) were also found in the CAMs of subjects in this test group. The embryo with the CAM in figure F had died for an unknown reason. Nevertheless its CAM had well-formed blood vessels (as indicated by the arrow arrow).

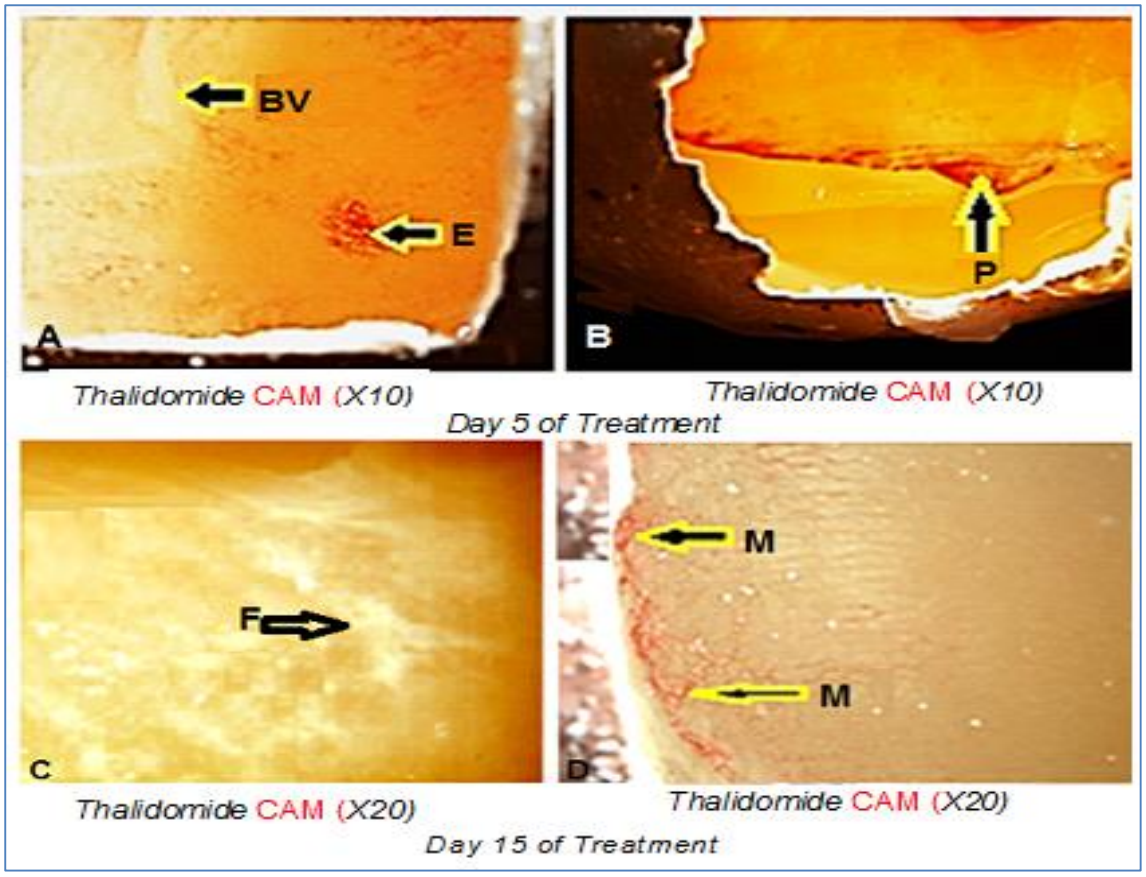

Figure 4.6. Angiogenesis inhibition as seen in chick CAMs treated with thalidomide (n = 6). The development of micro-vessels was reduced in all 6 subjects, by the presence of thalidomide. CAM in figure A) had an undeveloped blood vessel (BV) but had a patch of erythropoietic activity (E); figure B) shows the CAM with which the embryo thrived to end of the experiment; the CAM in Figure C) had excessive fibrotic tissue reaction (F) while the CAM in Figure D) had scanty erythropoiesis in the margin of the CAM (arrow, indicated M).

Day 5 of Treatment with Thalidomide

None of these CAMs had an established vascular network; the CAM (represented in figure A), had a solid white cord, probably the remnant of an uncompleted blood vessel (see arrow head, BV) and erythropoietic points, labelled 'E'. Patches of erythropoietic activities were seen in all thalidomide-treated CAMs. The most developed vessel in this treatment group was in the CAM in Figure B) (indicated by arrow head, P). Although a known angiogenic inhibitor used in

clinical practice, thalidomide failed in this one subject in that, the embryo was still alive at the end of the experiment.

Day 15 of Treatment with Thalidomide

At this time, Thalidomide- treated CAM had excessive tissue reaction (indicated by arrow head labelled F in Figure C). The subject in figure D) had scanty blood islands at the periphery of the embryonic CAM (arrow head M).

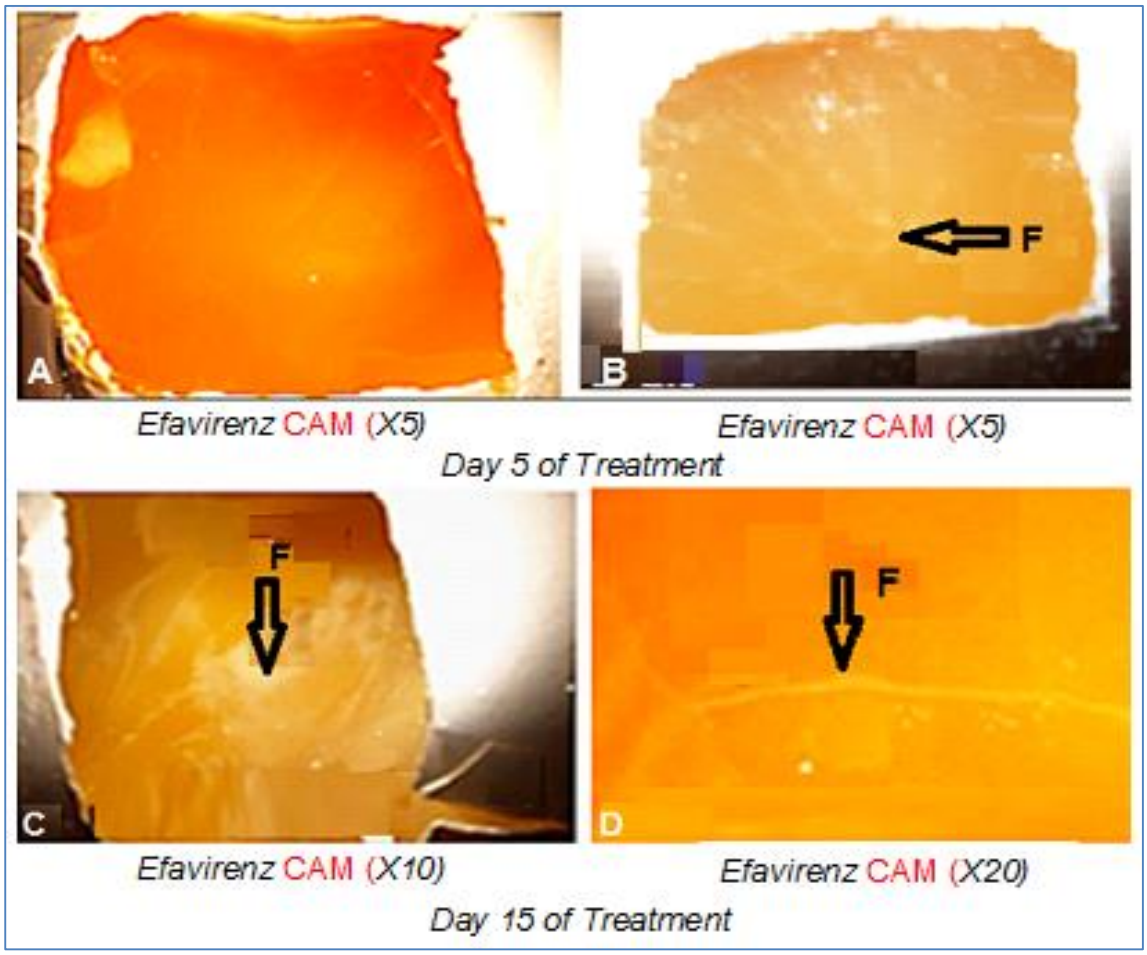

Figure 4.7. Absolute inhibition of angiogenesis as seen in chick CAM treated with Efavirenz. Figure A) shows a rather 'quiet' CAM. Figures B), C) and D) had excessive CAM tissue fibrosis (arrow, indicated as 'F'). The CAM in figure D) had a string on its surface that might be a suppressed CAM vessel. There was no angiogenic or erythropoietic activity seen in any CAM in this treatment group.

Day 5 of Treatment with Efavirenz

Treatment with Efavirenz suppressed formation of CAM vessels. These subjects had a characteristic “quiet” CAM as seen in the CAMs. Moreover, no red blood cells were seen in the CAMs; this implies that Efavirenz suppressed both angiogenesis and erythropoiesis. This caused failure to develop and thus an early embryonic death of all embryos in this test group.

Day 15 of Treatment with Efavirenz

Efavirenz treatment caused a distinct CAM pallor due to the absence of CAM vessels. There was exaggerated tissue reaction and poor CAM tissue patterning (fibrotic tissue indicated by ‘F’ in figure C). There was still no sign of erythropoiesis in any of the Efavirenz-treated CAMs.

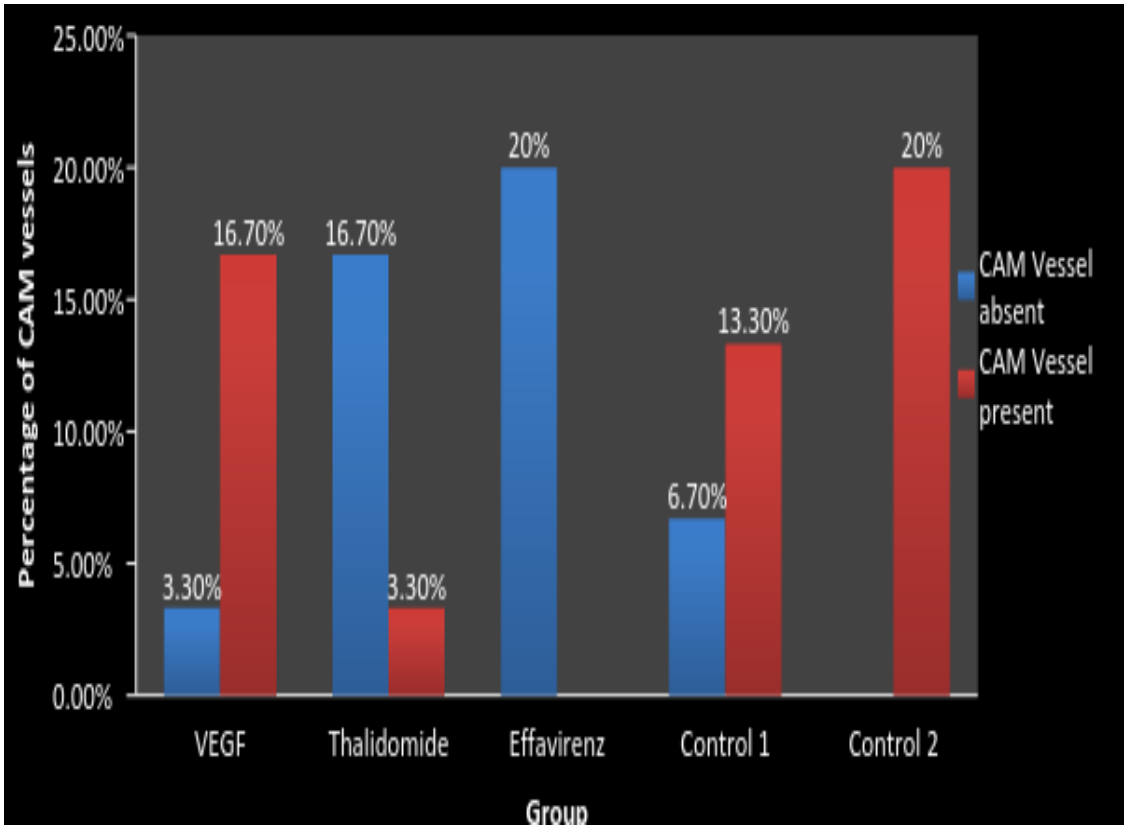

Figure 4.8. Bar graph representing the comparative efficacy of treatment drugs. The graph reflects the total percentage efficacy of all the drugs used. Since each of the 5 treatment groups had 6 subjects that were randomly allocated, each group represented 20% of all the subjects used (n=30).

When experimental groups are compared, control 2 presents with 100% angiogenic activity (20%), while VEGF treatment has the next highest score of 16.7% angiogenic efficacy. It failed as an angiogenic drug in one embryo that failed to develop adequately (awarding it an anti angiogenicity of 3.3%. Although the presence of VEGF is a major contributing factor in favour of CAM angiogenesis, exogenous VEGF did not seem to promote CAM angiogenesis beyond normal (as in control group 2). This suggests that administration of exogenous VEGF did not potentiate the effect of endogenous VEGF. However, this aspect of the work requires further experimentation. Thalidomide suppressed angiogenesis with a score of 16.7%. In one embryo however, angiogenesis was not suppressed resulting in a score 3.3% relative to the entire experiment (n=30). As is clearly evident from the bar graph Efavirenz was effectively totally anti-angiogenic, preventing the formation of blood vessels and vascularization of the CAM.

Comparison of the anti-Angiogenic effect of Efavirenz to Thalidomide.

Although Efavirenz suppressed blood vessel development (angiogenesis) and red blood cell formation (erythropoiesis), our work did not measure the anti-erythropoietic effect of treatment drugs. Considering angiogenesis alone, the results showed that there was no significant difference between the anti-angiogenic effect of Efavirenz and Thalidomide.

Table 4:4: Comparison of the anti-Angiogenic effect of Efavirenz to Thalidomide.

	N	**Mean**	**Standard deviation**	**F**	**p-value**
Thalidomide	6	0.17	0.41	1.000	0.341
Efavirenz	6	0.00	0.00		
Total	12	0.08	0.29		

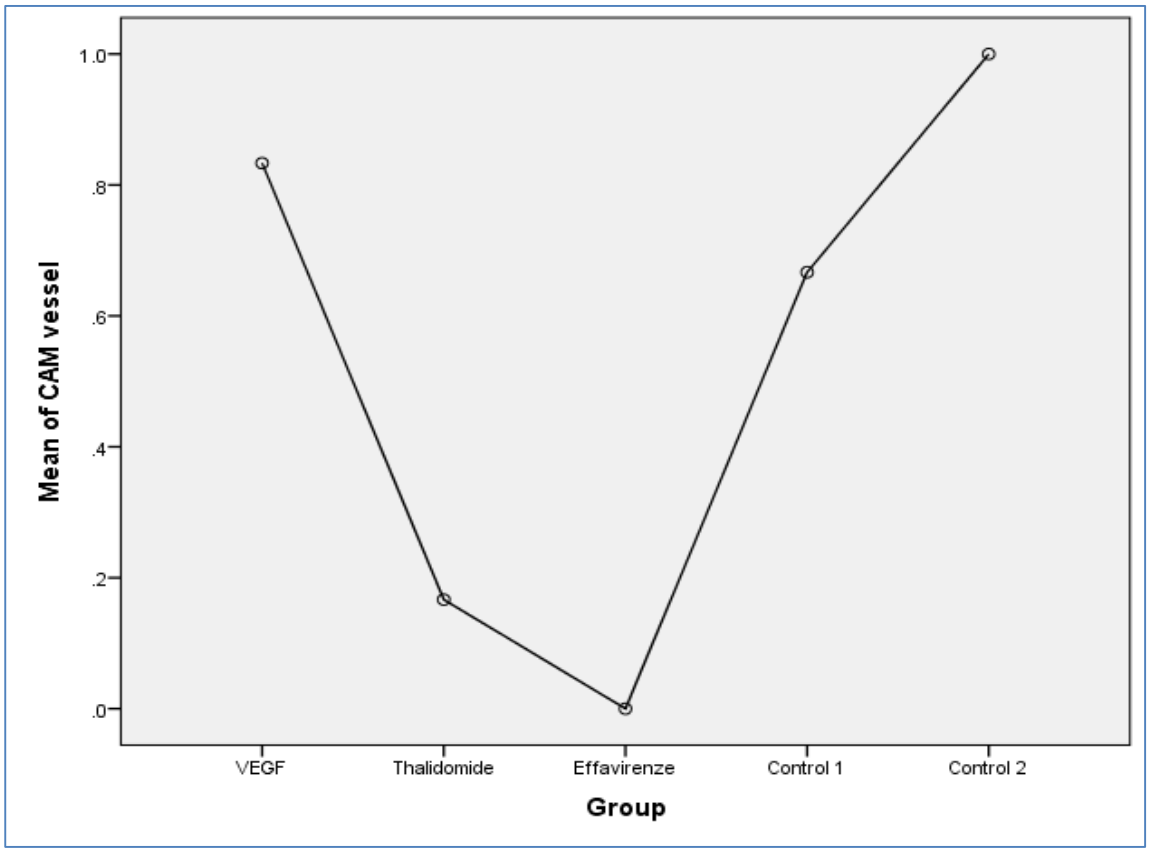

Figure 4.9. Mean Trend of CAM vessel formation across Treatment Groups

In order to show a visual representation of the relationship between the two variables (treatment drugs and presence or absence of CAM blood vessels), a trend line was fitted to the data and the R-squared value (coefficient of determination) was established.

The trend for the presence of CAM vessels in the various treatment groups was lowest for Efavirenz. Note that the value of “1” (for Control 2) represents a perfect association or fit between the lack of treatment or interference, and occurrence of development of CAM blood vessels). “0” (for Efavirenz) represents no statistical correlation between Efavirenz and CAM angiogenesis.

Dissection of Embryos on Day 15 of Treatment (Day 18 of Incubation)

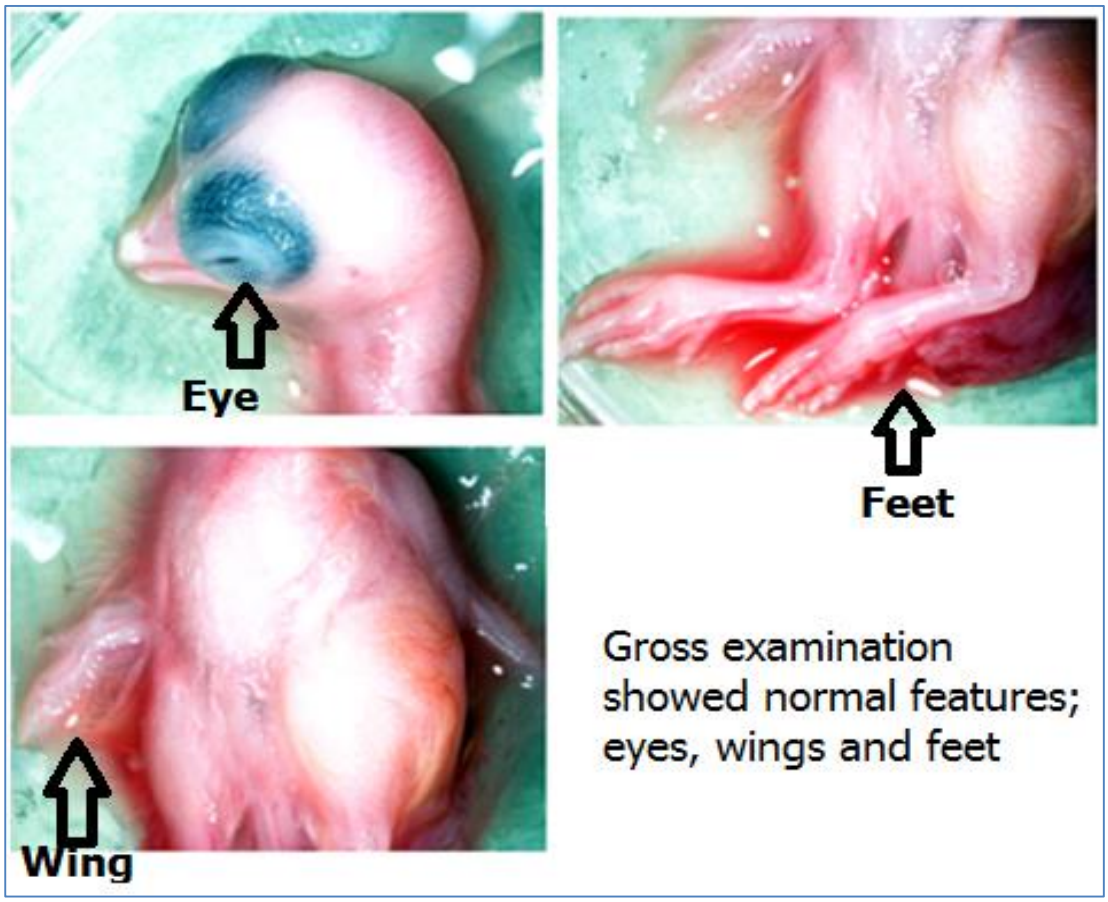

Figure 4.10. Control embryo. Magnification: X40. Gross examination of the embryos showed normal appearance of the head (A), trunk (B), and limbs (C).

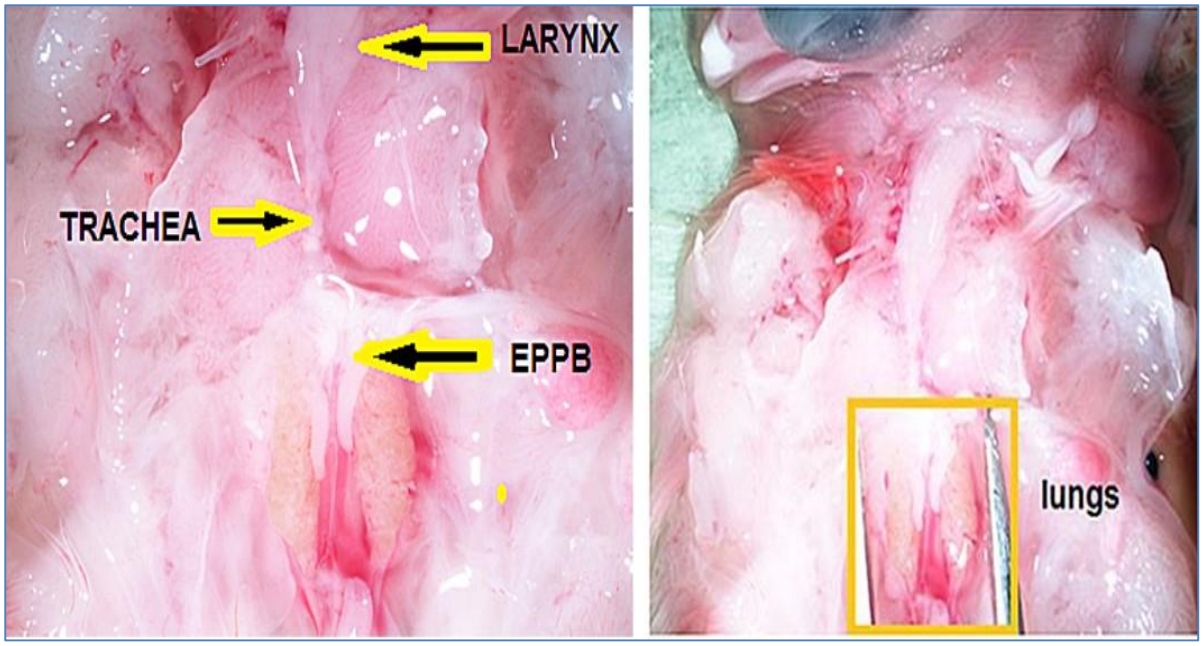

Figure 4.11. Dissected trunk of a Control embryo (Magnification: X40). Figure A) showed normal-looking thoracic structures such as the larynx, trachea and extra pulmonary bronchi (EPPB). Figure B) shows normal the gross appearance of the lungs as indicated in the region of interest. Note the normal pink colour of control embryonic tissues. "A" and "B" are not labelled on the Figures.

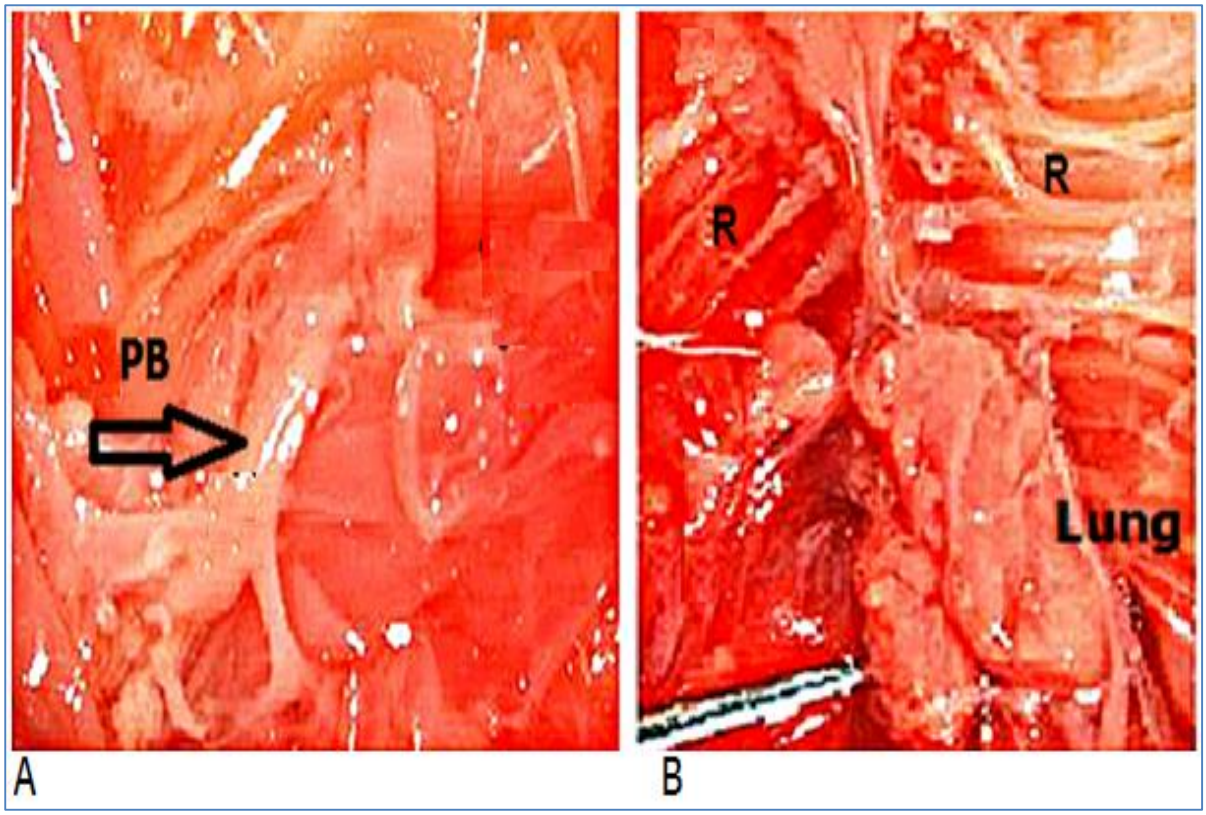

Figure 4.12. VEGF-treated embryo. Magnification: X40. There was excessive congestion (seen as redness) and fragility of embryonic tissues. Figure A) showed a dissected embryonic trunk in which the primary bronchi (PB) was well formed. Figure B) showed a relatively morphologically normal lung, but it appeared to be slightly hypertrophic when compared to that of control embryo. The ribs (R) were friable and rather deformed.

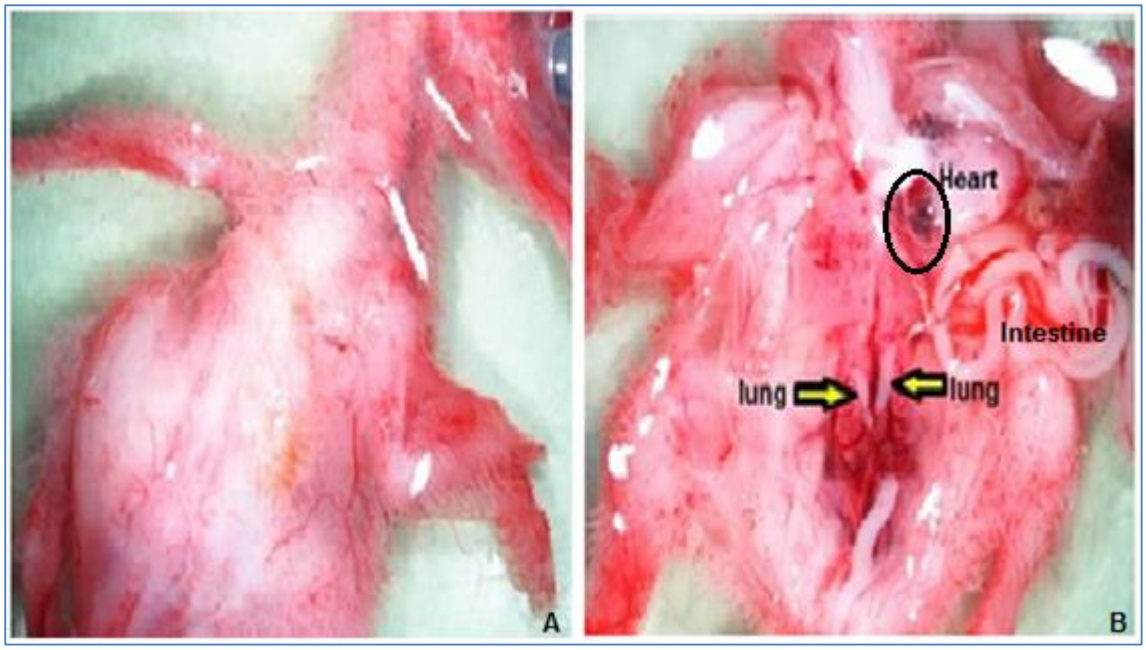

Figure 4.13. Dissection of the viable thalidomide-treated embryo. This embryo died as soon as it was exposed. Figure A) shows the dorsal aspect of the embryo, and B), the ventral aspect. Development of internal organs (heart and lungs) were suppressed. Note the haematoma in the cardiac wall (as depicted by the circle). Gross developmental anomalies seen include stunted growth, tissue fragility and redness (as a result of numerous tiny blood vessels on the skin).

Efavirenz-treated Embryos

Unlike thalidomide, no embryo survived following treatment with Efavirenz. At the end of the treatment period, all embryonic tissues were fully resorbed.

4.15. **Discussion**

The embryos were treated on day 3 of incubation (estimated Hamilton and Hamburger stage 21) and observed on day 5 and 10 of treatment. There was no specific reason for the two days chosen for observation. On the 5th day of treatment, each CAM was observed and CAM images were digitally captured. The images were again captured on the 15th day of treatment (estimated Hamilton and Hamburger stage 45) after which the experiment was terminated.

4.15.1. Control Embryos

There were two control groups; Control 1 which was the solvent control, were treated with an equal volume of the diluent (70% ethanol). Each subject also had an observation window cut into the egg shells. Control 2 represented normal embryonic development wherein no treatment was given. This group did not have an observation window cut into their egg shells.

Viability: As expected, control subjects were fully viable (see figures 4.3A-D). However, Control 2 embryos had a higher viability than the solvent control group.

CAM Blood vessels: Both control group embryos had well-formed CAM blood vessels but there were differences in CAM colour; the solvent Control CAMs lost the normal pale yellow CAM colour; and their CAM environment appeared to be slightly congested (as seen by the reddish colour). However, CAM colour cannot be attributable to ethanol since there was no such finding in the thalidomide and Efavirenz groups) (figures 4.5, and 4.6).

4.15.2. Treatment with VEGF

Viability

The VEGF-treatment group appeared to be the most viable of the treated embryos. The addition of exogenous VEGF affected embryonic development, causing an exaggeration of CAM vascularization, increased CAM vascular permeability and haemorrhage, embryonic distress and the death of one embryo (16.7% of cases, n=6). Although VEGF-induced CAM angiogenesis surpassed that of the manipulated control (Control 1), it fell short of normal embryonic CAM development (as in Control 2).

CAM Blood vessels

Endogenous VEGF is required for normal vascular assembly (Goodsell, 2003). Human VEGF165 (VEGF A), which was the form used in this work) is usually secreted from many body cells as a soluble factor (Leung *et al.,* 1989). Although VEGF A is angiogenic in function, local exogenous administration enhanced arterial re-endothelialization *in vivo* (Van Belle *et al.,* 1997), increased microvascular permeability, and caused vascular wall defects (Ferrara, 2000). This agrees with the finding in this work; VEGF- treated CAM had vascular haemorrhage (see figure 4.4A). This characteristic of VEGF has earned it the name 'permeability factor' (Dvorak, 2002). Defects in CAM vascular walls were seen in VEGF-treated CAMs (figure 4.4B). And at the end of the experiment, 50% of the embryos in the group died within seconds of exposure. Poor viability might have resulted from vascular exudation, haemorrhage (figure 4.4A) and the resultant lack of nourishment. It could also be secondary to the hyper dynamic circulation, evident in the increased CAM vascularity (seen in figure 4.4 D). In figure 4.4E, unusually large vessels formed vascular loops on the CAM surface. Increase in vascular (blood) pressure can induce embryonic distress. This agrees with the work of Drake et al. (1995) who reported that exposure to high levels of exogenous VEGF interfered with embryonic neovascularization, in a way similar to 50% reduction in VEGF expression (similar to heterozygous deletion of the VEGF allele).

Nevertheless, the effect of VEGF on CAM angiogenesis surpassed that of the solvent control group, a finding that goes against embryonic manipulation.

4.15.3. Treatment with Thalidomide

Viability

Only one thalidomide-treated embryo reached the end of the experiment.

CAM Blood vessels

The CAM tissues had no established vascular network but erythropoietic islands were seen on CAM surfaces (figure 4.5A and B) and margins (figure 4.5D. Despite this finding one subject had sufficient nourishment to get to day 15 of treatment. This suggests that in this embryo, thalidomide failed as an anti-angiogenic agent (see CAM in figure 4.5B), providing a viability of 16.7% in relation to thalidomide treatment, but 0% anti-erythropoietic activity; thalidomide-treated CAMs all had angiogenic activities; blood islands, and patches of erythropoietic activity (as represented by figure 4.5A and 4.5B and 4.5D). This shows that thalidomide, a known anti-angiogenic drug that is currently used in clinical practice as an anti-cancer agent (Kumar *et al.,* 2002), failed as an angiogenic drug in one subject. Efavirenz therefore seems to be a more potent anti-angiogenic (and possibly anticancer) agent than thalidomide.

4.15.4. Treatment with Efavirenz

Viability

Efavirenz showed absolute anti-angiogenic effect. This group therefore had 100% mortality with complete foetal resolution.

CAM Blood vessels

All efavirenz-treated CAMs were pale (for lack of vascularity), fibrotic and had no sign of erythropoiesis. This explains why all the group subjects died early during development; lack of

circulation, and so, lack of oxygen and nourishment (Figure 4.6A, 4.6B, 4.6C and 4.6D). By the end of the experiment, there was therefore no remnant of embryonic tissue found in any of the eggs. By day 15 of treatment there was no sign of embryonic activity in any of the eggs.

Use of Efavirenz in Pregnancy

The findings from this work raise some concern on the use of efavirenz in pregnant women with HIV infection. The question is: Is efavirenz teratogenic? This will be discussed further in the next chapter (Discussion Chapter).

4.16. Conclusion

Angiogenesis inhibitors are potentially anti-cancer agents. This study showed Efavirenz to be a more potent anti-angiogenic agent than thalidomide, possessing an absolute anti-angiogenic effect in the developing chick CAM. Furthermore, it suppressed erythropoiesis. This implies a possible future clinical use for Efavirenz as an anti-cancer drug, and with no fear of adverse effects for pregnant women and their babies. This awards Efavirenz an additional score when compared to thalidomide.

CHAPTER FIVE

5.0. GENERAL DISCUSSION

5.1. Development of the avian lung

In this work, developmental stages of the embryos were based on days of incubation in association with Hamilton and Hamburger's normal tables of development (Hamburger and Hamilton, 1992). It was only at harvesting that the embryos could be developmentally staged. Nevertheless, some developmental stages were missed and could not be evaluated. It is noteworthy to add that environmental factors such as the egg supply, and experimental conditions, including handling, incubation and humidity were consistent for all of the eggs. In comparison to the mammalian lung, many researchers have observed the lack of data on development of lungs in birds, particularly morphogenesis (Maina, 2003a, Maina, 2003b, Maina *et al.,* 2003, Maina, 2005, Maina, 2006). Avian lung development has been described as "a complex and little-studied process" (Makanya *et al.,* 2007). Birds in flight have collided with airplanes at high altitudes (Sodhi, 2002, Manville II, 2005, Gard *et al.,* 2007). At such height mammals would die from hypoxia or eventually develop adaptive physiological measures such as polycythaemia or life-threatening conditions such as pulmonary hypertension and subsequent right heart failure (Sands *et al.,* 2011). The lung of the domestic fowl (*Gallus Gallus variant domesticus*) develops between day 3.5 to day 4 of incubation (Bellairs and Osmond, 1998, Maina *et al.,* 2003). It forms from two endodermal ridges that arise from the floor of the primitive pharynx, and fuse to form a single lung entity. This primitive lung bud bifurcates into the left and right primordial lungs on day 5 (at stages 27) (Hamburger and Hamilton, 1951). The airway progenitor is the foregut tube. The mammalian lung develops from the laryngo-tracheal tube, a diverticulum of the foregut; and in communication with it. Separation of the two lung buds occurs when the oesophagotracheal ridges fuse to form the oesophagotracheal septum. Two primary lung buds are identifiable by the fifth week of life (Ellis and Mahadevan, 2010). The foregut tube is illustrated in fig. 3.2 and 3.3 (at stage 19). Due to the common origin of the gastrointestinal and respiratory

tracts, the developing lung is closely associated with the oesophagus, with the trachea and bronchi, lying ventral to it and the bronchi are formed in the stage 25 embryo (see fig 3.6). However, the lung bud is only evident at stage 22 (day 3.5 post incubation) (see fig. 3.6), appearing as an epithelial sac just anterior to the developing kidneys (see figs. 3.4 and 3.5). At this time, the boundary of the developing lung is indistinct and not well defined from the surrounding mesenchyme.

5.2. Development of the airway

The walls contain hundreds of tiny, anastomosing 'air capillaries' surrounded by a profuse network of blood capillaries (Welty and Baptista, 1988). It is within these 'air capillaries' that the exchange of gases (oxygen and carbon dioxide) between the lungs and the blood occurs. The primary bronchi enter the lungs to become mesobronchi and then give off dorsobronchi which are connected by smaller calibre tubes called parabronchi. It is the dorsobronchi that continue into parabronchi so that after passing through the parabronchi, air moves into the ventrobronchi (Maina, 1989).

From the present study, the two (left and right) primary bronchi were seen in the stage 25 lung (day 4.5). The oesophagus was centrally placed, lying just dorsal to the developing lung (see fig. 3.6). The secondary bronchi (SB) began to appear at stage 27 on day 5 of development. In the stage 29 embryo, the lung is well formed, with secondary bronchi seen in the lung buds (fig. 3.8). SB were particularly distinct at stage 31 (day 7), becoming fully formed by stage 33, this being day 8, at which stage parabronchi (PB) were noticeable (see fig. 37). It has been reported that on day 13 (stage 39), primary bronchial epithelium is obviously columnar epithelium (see fig. 3.10). PB that extend from the dorsobronchi to the ventrobronchi (Gleed and Ludders, 2001), first appeared as solid cords of cells at stage 33 (see fig. 3.11B). However some PB at this

developmental stage had begun to acquire lumens, unlike the findings of Maina (2003a), who reported that they only began to canalize by stage 36, at day 10.

5.3. Development of parabronchi

Parabronchi are importantly the site for gas exchange (Welty and Baptista, 1988). Maina (2003a), reported that beginning on day 9, PB form by budding into adjacent mesenchyme from secondary bronchi (SB), with the PB increasing in size and number as the lungs mature. Similarly, this is reported in the present study, as can be seen in the structures of the developing lung from days 6 to 14. . As described previously, the avian 'unidirectional', 'flow-through' respiratory apparatus involving cross-current gas flow with pulmonary blood flow was demonstrated by Powell and Scheid (1989). Atria and infundibulae are diverticulae from the PB, leading into air capillaries that lie parallel to blood capillaries, thus forming the ultrathin avian blood-air barrier, typically < 0.2 µm thickness (Bernhard *et al.,* 2001). As reported in the literature the atria, infundibulae and air capillaries form at stages 41, 42 and 44, equivalent to days 15, 16 and 18, respectively (Maina, 2003a). However, from this study, atria were found at an earlier developmental stage, at stage 39 on day 13 of development, this being the stage at which the typical parabronchial lung pattern was seen. Atria and infundibulae appeared as an 'irregularity' in the periphery of PB lumen. These were most distinct at stage 14 lung (see fig. 3.14).

5.4. VEGF Expression in the developing avian lung

Embryonic vasculogenesis begins in the form blood islands, 26-29 hours after the eggs are laid (deMello *et al.,* 1997). Although haematogenic and vasculogenic cells that form from mesoderm, become ultrastructurally distinguishable in the lungs of the domestic fowl from as early as day 4 (stage 23) of development, pulmonary angiogenesis is seen only from day 7 (stage 31) (Maina, 2004). Subsequently vascular growth consists of increase in size through expansion (Hislop,

2005). This is called intussusceptive angiogenesis and it occurs around embryonic day 15 (Makanya *et al.,* 2007). Blood vessels develop from endothelial precursor cells (angioblasts) (Stenmark and Mecham, 1997). Thereafter, the formed vessels elongate by the augmentation and alignment of surrounding angioblasts (Maina, 2004) until the vascular network is established. For this to occur, there is also need for capillary migration and trans-differentiation to form arterioles and arteries, as well as anastomoses of the vessels being formed (Adams, 2002). The formed plexus eventually links up to the main circulation that develops from the sixth pharyngeal arch arising from the heart (Stenmark and Mecham, 1997). At this time, angiogenic factors such as VEGF are expressed in a tempero-spatial manner, for the appropriate regulation of angiogenesis leading to normal blood vessel formation (Orlandini and Oliviero, 2000). In the present study a scoring scheme was developed based on the intensity of the immunostaining for VEGF expression: where absence of VEGF immuno-reactivity was scored as 'zero' (0), '+' for low VEGF expression, '++' for moderate expression and tissues with marked immuno-reactivity were scored as '+++'. Pregnant mouse uterus, which was used as positive control tissues showed marked VEGF immunoreactivity (see fig. 3.15 and 3.16). This was as a result of endometrial angiogenesis associated with embryonic development. A critical level of VEGF is required for normal vascular development and mice lacking a single VEGF allele die at mid-gestation (Finkelstein and Poole, 2003).

5.4.1. Stage 19 - 22

There was no VEGF activity at stages 19 and 20 of development. If angiogenic cells were present, they were indistinguishable from the surrounding mesenchymal tissue. The earliest VEGF expression was seen in the stage 22 lung (day 3.5 post-incubation), (see fig. 3.17); the developing lung buds, heart, kidneys, notochord (that was seen in the midline just ventral to the developing spinal cord) and liver. The trachea expressed VEGF in both the apical and basal aspects of the epithelium (see fig. 3.20). Onset of VEGF expression was on day 3.5 of incubation (stage 22). This coincides with the appearance of the lung bud in the findings of Maina et al.

(2003), and Bellairs and Osmond (1998) who reported day 3.5 and day 4 of incubation respectively. Stage 25

5.4.2. Stage 25

VEGF expression at stage 25, was diffuse in the developing lung tissue and surrounding mesenchyme but was quite distinct in the renal tubules and in the developing spinal cord (fig. 3.22). Furthermore, some VEGF- containing cells that were seen as 'fluorescing' cells (see fig. 3.29C), were found in perineural tissues possibly migrating towards the developing spinal cord. This was more marked in the spinal cord at stage 29 of development (fig. 3.29C). These cells possibly represent on-going perineural angiogenesis.

5.4.3. Stage 27

Avian pulmonary vasculature arises by vasculogenesis (deMello *et al.,* 1997) later expanding by angiogenesis (Hislop, 2005). Embryonic mesoderm differentiates into angioblasts which initiate vasculogenesis in the lung (Ferguson *et al.,* 2005). Stage 27 embryonic lung bud was noted as ventral to the dorsal aorta (DA) which expressed VEGF even more markedly, with one secondary bronchus (SB) seen at this stage. In this study, the first clear sign of vasculogenesis was the appearance of blood islands in the lungs at stage 27 (day 5). The blood islands had no defined boundaries but were scattered in the developing lung (see fig. 3.24); the pulmonary vascular plexus is not well formed at this stage.

5.4.4. Stage 29

Apart from an increase in the size of the lung bud, blood island formation and VEGF localization were similar to stage 27 (see fig. 3.26). In addition, VEGF activity was appeared as a homogenous staining in the lung parenchyma and within the surrounding mesenchymal tissues. As seen in figure 3.27, of all the embryonic tissues, the developing kidney tubules expressed the most VEGF in the stage 29 embryo; and there was marked angiogenic activity around the spinal cord (see figs. 3.29 and 3.30).

5.4.5. Stage 31

By stage 31 (day 7) of development, the two primary bronchi had formed and both lungs had secondary bronchi at their lateral and medial borders of the lungs (figure 30). The primary bronchus contained distinct VEGF expression at both the apical and basal borders of the airway epithelium (fig. 3.31).

5.4.6. Stage 37

At stage 37, the avian lung was parabronchial with intrapulmonary vasculogenesis, with parabronchial epithelium expressing VEGF (see fig. 3.33). A higher magnification showed marked expression of VEGF at both the apical and basal borders (figure 3.34). At stage 37 the pulmonary vessels were seen as four prominent structures surrounding the developing lung at the level of tracheal bifurcation (e.g. figure). Intrapulmonary vasculogenesis became obvious at stage 37 (see fig. 33A). VEGF is stored in both the apices of airway epithelial cells and the sub-epithelial matrix (see fig. 3.34). From the trachea, the extrapulmonary principal bronchus continues as the intrapulmonary principal bronchus (also known as the mesobronchus). Parabronchi that range from 0.5 to 2.0mm in diameter (depending on the size of the bird) can be several millimetres long, connecting dorsobronchi to ventrobronchi to allow air flow from dorsobronchi to ventrobronchi (Maina, 1989). Furthermore, air sacs were very prominent at this stage. With the first inspiration, inhaled air enters the posterior air sacs and lungs while at the same time, “older air” moves from the lungs into the anterior air sacs. The air sacs diminish in volume during the second inspiration as air moves out of the body; from the posterior air sacs through the lungs and from the anterior air sacs into the trachea. At this stage, the intensity of VEGF expression is distinct in that the pulmonary vessels were quite distinct, forming a beautiful pattern around the parabronchi (see fig. 3.33).

5.4.7. Stage 38

Stage 38 lung had numerous parabronchi (PB) that expressed VEGF at the apical border. VEGF expression was also noted on the pleural covering of the lung (see fig. 3.35).

5.4.8. Stage 40

VEGF expression gradually increased with lung maturation and peaked at stage 40 (day 14) (figure 3.37), with a typical parabronchial lung is represented at stage 40 (figures 3.39 and 3.40). The primitive pulmonary vascular plexus later links up to the main circulation that forms from the sixth pharyngeal arch, arising from the heart (Stenmark and Mecham, 1997). In the avian lung gas exchange takes place across a blood-gas barrier that consists of three components, the epithelial cell, the basal lamina, and the endothelial cell (Maina and King, 1982) and is thin when compared to the mammalian blood - gas barrier (Makanya *et al.,* 2006). Although by day 10 the pulmonary vasculature is largely formed, complete pulmonary circulation occurs only after the development of the blood capillaries on day 18 (Maina, 2004).

5.4.9. VEGF expression in other organs

VEGF is reported to be highly expressed in epithelial organs such as the lung, kidney and the salivary gland (Gjorevski and Nelson, 2011). Further, the role of VEGF in reproduction and for normal mammalian development is so critical that the loss of a single VEGF allele results in vascular anomaly and early intrauterine death (Ferrara *et al.,* 2003). The extensive expression of VEGF in the developing lung and adjacent organs suggests that as in the mammalian lung, VEGF plays a significant role in early avian embryonic development and particularly in organogenesis. Organs such as the limb bud and the lung develop by epithelial budding and require a cohesive relationship between epithelium and blood vessels, along with a complex interaction between the mesenchyme and epithelium. Any disruption of this process will most likely cause congenital anomaly (Orlandini and Oliviero, 2000). Particularly at this time, the specific regulation of each angiogenic factor is very likely required to determine the correct succession and composition of the factors for early vessel pattern formation (Orlandini and Oliviero, 2000). While Makanya et al. (2007) demonstrated that sprouting and intussusceptive angiogenesis are major angiogenic mechanisms in vascular development in the avian lung at later stages (stage 41), this falls beyond the scope of the present study which incorporated stages 19-40.

5.5. Effects of angiogenesis inhibition in chick CAM

In the second aspect of this work, fertile eggs were used as *in vivo* systems to study CAM angiogenesis and in particular, the effect of the antiretroviral drug, Efavirenz, on chick CAM angiogenesis. This treatment was compared to both that of the pro-angiogenic protein, VEGF and to the known anti-angiogenic drug, thalidomide. Blood cells and vessels are both mesodermal in origin; mesenchymal cells differentiate to give rise to the precursor cells (angioblasts and haematoblasts respectively. These cells are ultrastructurally distinguishable in the developing lungs of the domestic fowl at stage 23 (day 4) of development (Maina, 2004).

Blood vessels initially form by vasculogenesis and later grow in size and multiply by angiogenesis, through the formation of a capillary bed number as endothelial cells differentiate from mesenchymal precursors. Vasculogenesis involves formation of blood islands which coalesce to form sinusoidal spaces (deMello *et al.,* 1997). At stage 31 (day 7), at which time, endothelial cells differentiate into erythrocytes and drop into the lumen of the developing blood vessels (Maina, 2004). In chicks, the establishment of pulmonary vascular network relies on capillary migration and trans-differentiation (Adams, 2002). The airways act as a template for pulmonary angiogenesis (Hislop, 2005). At this time, drugs may interfere with this process; the development of epithelial structures can therefore be disrupted if angiogenesis is interrupted (Seifert and Sobin, 1992). This is the case with thalidomide. Although the exact mechanism of action for thalidomide is unknown (Stephens *et al.,* 2000) it is a known anti-angiogenic drug that is used to control ocular angiogenesis and to treat pathologic angiogenesis such as found in solid tumours, for example in Kaposi's sarcoma, and in diabetic retinopathy (D'Amato *et al.,* 1994).

5.5.1. Five days of Angiogenesis inhibition

From the results obtained, the % total angiogenicity per group were: Control 2=100%, Control 1=67%, VEGF=83%, Thalidomide= 17% and Efavirenz= 0 % (n = 30). As expected, control CAMs had well-formed blood vessels (Figure 4.3A-D). When experimental groups were compared to

control groups, the control group presents the highest angiogenic effect of 20%. VEGF- treated embryos had the second score of 17% angiogenic efficacy. In VEGF-treated embryos CAM blood vessels were well formed with adequate branching (Figure 4.4C and D). However, there was an increased vascular permeability with exudation due to a possible vascular wall defect (see fig.4.4B) and hypervascularization of CAM tissues. In contrast, thalidomide-treated CAMs lacked an established blood vessel network; and CAM blood vessels were scanty (figure 4.5A, B and D). The overall effect of thalidomide was as an absolute anti-angiogenic agent. But the test drug, Efavirenz was 100% anti-angiogenic, representing the most potent anti-angiogenic drug in the study. The development of angiogenesis within the CAM was totally suppressed with Efavirenz treatment, together with a lack erythropoiesis. This developmental block resulted in embryonic death. Moreover, treatment with Efavirenz appeared to cause distinctive CAM pallor (Figure 4.6 A- D).

5.5.2. 15 days of Angiogenesis inhibition

After 15 days of treatment, the control embryos had well-formed blood vessels (Figure 4.3 C and D). In comparison, treatment with either Efavirenz or Thalidomide caused embryonic death in 100% and 80% of the embryos, respectively. Also, there was full embryonic tissue resolution at the end of the experiment. This was reflected in the pale colour and physiological inactivity of the CAM environment (Figures 4.5 and 4.6).

5.6. Use of Efavirenz in Pregnancy

Suppressive antiretroviral therapy (ART) reduced genetic evidence of viral replication in tissues (Kearney *et al.,* 2014) and in addition to ART, delivery by elective caesarean section and bottle feeding (rather than breastfeeding) have reduced mother to child transmission (MTCT) below 1-2% in developed countries (Thorne and Newell, 2003). After several clinical trials with antiretroviral drugs, first-line treatment for HIV infection currently comprises the combination of a non-nucleoside reverse transcriptase inhibitor such as Efavirenz, or one or two protease inhibitors (Volberding and Deeks, 2010). Efavirenz is a potent ARD and so, is

particularly an important first line drug (ENCORE1 Study Group, 2015). As it is one of the most prescribed antiretroviral drugs (Kryst *et al.,* 2015). Its dosage and safety is of great concern (ENCORE1 Study Group, 2015). Questions have been raised concerning foetal exposure to Efavirenz in the first-trimester, particularly on the risk of developing congenital anomalies of the central nervous system (CNS) (Ford *et al.,* 2014). Furthermore, despite limited clinical evidence, mitochondrial disease has been reported in children prenatally exposed to antiretroviral therapy; eight cases of mitochondrial dysfunction were reported in France, secondary to prenatal zidovudine exposure, and although uninfected, two of the eight children later died (Thorne and Newell, 2003). Other concerns raised include genetic variations in Efavirenz metabolism, its drug interactions with anti-tuberculous drugs and the effect of pregnancy on Efavirenz pharmacokinetics (Dooley *et al.,* 2014, ENCORE1 Study Group, 2015). Data from Africa are generally limited (ENCORE1 Study Group, 2015). But WHO data shows that in 2016, on the average globally, 76% of all pregnant women living with HIV were placed on antiretroviral drugs (ARDs) to prevent MTCT. This therefore calls for more efficient monitoring of ARD toxicity. Unfortunately, because the focus on ART is to treat and prevent spread of HIV infection, some side effects may not be widely reported (World Health Organisation, 2014). The impact of ART should not overshadow morbidity or life-threatening conditions to the embryo or foetus or mother. In Soweto, South Africa, Dooley et al. (2014) evaluated a cohort of HIV infected pregnant women at their 37th week of gestation. Out of 97 women who participated, 44 had tuberculosis. They found that pregnancy increased the risk of low Efavirenz concentrations but it did not increase MTCT and that while isoniazid (that is used for both TB treatment and prophylaxis) reduced the rate of Efavirenz clearance, this was not the case with rifampin (Dooley *et al.,* 2014). Also, genetic factors have been associated with variability in plasma Efavirenz concentrations in HIV-infected black South African adults and children. Using a double-blind, placebo-controlled trial, ENCORE1 Study Group (2015) compared the safety of efavirenz 400 mg with the standard 600 mg dose, combined with tenofovir and emtricitabine (that was the first-line HIV therapy at 38 clinical sites in 13

countries). The patients' profiling was as follows: HIV-infected patients not younger than16 years of age, with no previous antiretroviral therapy, but a CD4 cell count of 50-500 cells per µl, and plasma HIV-1 viral load of at least 1000 copies per ml. The patients were then given a fixed-dose daily tenofovir 300 mg and emtricitabine 200 mg but either 400 mg daily or 600 mg daily efavirenz and monitored for 96 weeks. Results showed that efavirenz 400 mg is non-inferior to the standard dose of 600 mg in combination with tenofovir and emtricitabine as initial HIV therapy. Instead, there was the added advantage of fewer efavirenz-related side effects with the 400 mg dose. These findings supported the routine use of the lower dosage (ENCORE1 Study Group, 2015).

The first consolidated guidelines for the use of antiretroviral drugs (ARV) to treat and prevent HIV infection, was published only in 2013 (World Health Organisation, 2013). The report insisted on the need to document ARD toxicity particularly in the following conditions: introduction of new drugs, ARDs use in children, during breastfeeding and in pregnant women. WHO also stressed on the need to identify people groups with risk factors, and to develop preventive measures. It was also noted that, although HAART has reduced the mother-to-child transmission rates to around 1-2%, it is not always available or affordable in low and middle-income countries (Siegfried *et al.,* 2011). In randomised controlled trials Siegfried et al. (2011) therefore compared antiretroviral regimen used to decrease MTCT with a placebo or no treatment, or other antiretroviral regimen, and weighed the gain (decrease in transmission risk) to the loss (what effect these interventions have on maternal and infant mortality and morbidity). Their results showed that the timing for treatment and the specific ARD used were key factors. More interesting was the work of Ford et al. (2014); a systematic review and meta-analysis of twenty-three studies with birth outcomes among women exposed to efavirenz during the first trimester of pregnancy recorded 2026 live births and 44 congenital anomalies including one neural tube defect. There was no statistical difference in the overall risks of congenital anomalies between Efavirenz and non-Efavirenz regimens. It was based on this review, that the revised WHO guidelines on ART was created, recommending efavirenz

as a first-line drug in HIV management therapy for all ages, gender and gestational age (World Health Organisation, 2013). Furthermore, a retrospective, multicentre cohort study later carried out in Pointe Noire and Republic of Congo (from September 2005- June 2012) found no congenital anomalies in both Efavirenz and non-Efavirenz subjects (Bisio *et al.,* 2015). Nevertheless, WHO recommends that ARD toxicity be well monitored, and a comprehensive pregnancy record be kept at all treatment centres, with spontaneous reporting of all birth defects but pregnancy outcome and safety issues for children exposed to prenatal ARD or chronic use need to be long term because the possibility that such children may be at risk of developing cancers at older ages must be excluded (Thorne and Newell, 2003).

5.7. The role of VEGF in carcinogenesis

VEGF is the key trigger in pathological angiogenesis such as occurs in cancers (Goodsell, 2003, Carmeliet, 2005), driving endothelial cell proliferation, migration and survival, while at the same time, increasing vascular permeability (Moghaddam *et al.,* 2012). VEGF enables tumour cells undergo an “angiogenic switch” with resultant angiogenesis that provides more nutrients and oxygen, and that also rid them of their waste products (Folkman, 1971). By promoting tumour angiogenesis and increasing vascular permeability, VEGF has been associated with peritoneal carcinomatosis such as ovarian cancers. Both experimental and clinical studies have shown that VEGF levels are inversely correlated with patient survival (Moghaddam *et al.,* 2012). Unfortunately, the scope of this work does not provide any relationship between the mechanism of action of VEGF and Efavirenz. But the early expression of VEGF stage 22/day 3.5 of development emphasises its significant role in early pulmonary vasculogenesis and normal lung maturation. Subsequently VEGF-treated CAM had tissue fragility (Figure 4.9), indicating that VEGF interfered with normal embryonic development. The relationship between each of the drugs and development or lack of formation of CAM blood vessels was evaluated by mathematically fitting a trend line to the

data, from which the coefficient of determination (R^2) was calculated. The trend for the development of CAM vessels in the various treatment groups was lowest for Efavirenz. Statistically Fisher's exact test demonstrated a significant association between the treatment drugs and the development of CAM angiogenesis; while Efavirenz had an absolute anti-angiogenic effect in chick CAMs, thalidomide was not wholly effective in 17% of treated embryos. The results thus showed Efavirenz is a more potent anti-angiogenic agent than thalidomide. However, there was no statistical difference between the two treatment drugs since the important anti-erythropoietic effect of Efavirenz on chick CAM was not considered in this study. This will be considered for future studies.

5.8. Use of Efavirenz in Cancer treatment.

Many researchers have claimed spontaneous regression of HIV-associated cancers with HAART, particularly with a reduction in KS in people taking anti-retrovirals that include Efavirenz (Murdaca *et al.,* 2002, Servato *et al.,* 2013). Of six Non-Nucleoside Reverse Transcriptase Inhibitors studied (Nevirapine, Etravirine, Efavirenz, Lersivirine, Delavirdine and Rilpivirine), Efavirenz had the highest anti-mitotic effect against pancreatic cancer cells, presenting it as a possible new option in the treatment of that cancer (Hecht *et al.,* 2015).

Houédé et al. (2014) evaluated the efficacy and safety of Efavirenz in metastatic prostate cancer that is resistant to orchidectomy (castration). They reported that the normal therapeutic dose of 600 mg Efavirenz did not statistically cause an improvement in serological profile but exploratory analysis showed increasing the dosage may be of potential benefit. On the other hand, Sikora et al. (2010), explored two known Efavirenz-induced side effects (breast hypertrophy and gynaecomastia) and reported the risk of breast cancer, due to Efavirenz directly modulating oestrogen receptors which promotes the incidence of breast cancer. This requires further investigation since it is contrary to the findings of Hecht et al. (2015) and Houédé et al. (2014) mentioned above. Lastly, efavirenz has been associated with anaemia

(deficiency of red blood cells (RBC). A 48-hour exposure of human RBC to ≥ 2 µg/ml concentrations of Efavirenz caused cell shrinkage and enhanced removal/clearance of deformed circulating erythrocytes by the lymphatic system (Bissinger *et al.,* 2015). This work is in a way related to the findings of the present study wherein Efavirenz suppressed not only angiogenesis but notably, also erythropoiesis.

5.9. Conclusion

In this work, Efavirenz has been shown to have significant effects on early embryonic development, preventing angiogenesis from occurring within chorioallantoic membrane of the domestic fowl (*Gallus Gallus* variant *domesticus*). Its anti-angiogenic properties seem to surpass that of thalidomide and can be potentially useful in clinical practice. However, in order to draw a conclusion to this observation, we may require larger numbers of treated embryos for comparison. Further, since we are not aware of any chance occurrences with the eggs prior to their purchase and incubation, it can be said that early environmental factors are also not accounted for in this work. Moreover, on delivery, the eggs were randomly allocated to the different experimental groups; for example, they were not weighed. Nevertheless, there is no doubt that in the future Efavirenz will play a more important role in the management (prevention and treatment) of HIV-infected patients. *And that this work has contributed to knowledge on Efavirenz, and the importance of developmental angiogenesis.*

Future work

It has been documented that cancer tissue requires increased blood supply for survival, a process called neovascularization, and that this process is mediated by VEGF. Although this work shows that Efavirenz inhibits the angiogenic process, its exact mechanism of action is not known, being beyond the scope of this work. This aspect of the drug will be investigated in the future e.g. how efavirenz reduces the expression of VEGF in embryonic tissues.

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APPENDIX SECTION

APPENDIX 1: ANIMAL ETHICS CLEARANCE

APPENDIX 2: IMMUNOHISTOCHEMISTRY DATA COLLECTION SHEET

APPENDIX 3: AUTOMATED TISSUE PROCESSING PROTOCOL

APPENDIX 4: RECIPES FOR SOLUTIONS AND REAGENTS

APPENDIX 5: IMMUNOFLUORESCENCE PROTOCOL

APPENDIX 6: RELATIONSHIP BETWEEN STAGES OF DEVELOPMENT

APPENDIX 7: STATISTICAL ANALYSIS OF RESULTS

APPENDIX 8: PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK

APPENDIX 1: ANIMAL ETHICS CLEARANCE

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2008/7/1

APPLICANT: Dr R McNeil

SCHOOL: School of Anatomical Science
DEPARTMENT:
LOCATION: Medical School

PROJECT TITLE: **The role and effect of fibroblast growth factor 10 and 7 and vascular endothelial growth factor on the developing lung of the domestic fowl (*Gallus varaint domesticus*)**

Number and Species

30 New Hampshire chicken and 1 mouse

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20080129. This approval remains valid until 20100129

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Signed: _____ Date: 05/03/2008
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 05/03/2008
(Registered Veterinarian)

cc: Supervisor:
Director: CAS

Works 2000/lain0015/AESCCert.wps

APPENDIX 2: DATA COLLECTION SHEET FOR IMMUNOHISTOCHEMISTRY

DATA COLLECTION SHEET
(IMMUNOHISTOCHEMISTRY)

DATE:	TITLE OF EXPERIMENT		RESEACHER
RESULTS			
Slide no	**Positive (Immunolocalization present)**	**Negative (Immunolocalization Absent)**	**Comment**
Conclusion			

Signature of Researcher: _____Date: _____/_____/_____

Name of Researcher: _____

APPENDIX 3: AUTOMATED TISSUE PROCESSING PROTOCOL

(Using automated tissue processor (Shanden, Citadel 1000)

Overview of the steps in tissue processing for paraffin sections

1. Obtaining a fresh specimen

Fresh tissue specimens will come from various sources. It should be noted that they can very easily be damaged during removal from patient or experimental animal. It is important that they are handled carefully and appropriately fixed as soon as possible after dissection. Ideally fixation should take place at the site of removal, perhaps in the operating theatre, or, if this is not possible, immediately following transport to the laboratory.

2. Fixation

The specimen is placed in a liquid fixing agent (fixative) such as formaldehyde solution (formalin). This will slowly penetrate the tissue causing chemical and physical changes that will harden and preserve the tissue and protect it against subsequent processing steps 2. There are a limited number of reagents that can be used for fixation as they must possess particular properties that make them suitable for this purpose. For example, tissue components must retain some chemical reactivity so that specific staining techniques can be applied subsequently.3 Formalin, usually as a phosphate-buffered solution, is the most popular fixative for preserving tissues that will be processed to prepare paraffin sections. Ideally specimens should remain in fixative for long enough for the fixative to penetrate into every part of the tissue and then for an additional period to allow the chemical reactions of fixation to reach equilibrium (fixation time). Generally, this will mean that the specimen should fix for between 6 and 24 hours. Most laboratories will use a fixative step as the first station on their processor.

Following fixation, the specimens may require further dissection to select appropriate areas for examination. Specimens that are to be processed will be placed in suitable labelled cassettes (small perforated baskets) to segregate them from other specimens. The duration of the processing schedule used to process the specimens will depend on the type and dimensions of the largest and smallest specimens, the particular processor employed, the solvents chosen, the solvent temperatures and other factors. The following example is based on a six-hour schedule suitable for use on a Leica Peloris™ rapid tissue processor.

3. Dehydration

Because melted paraffin wax is hydrophobic (immiscible with water), most of the water in a specimen must be removed before it can be infiltrated with wax. This process is commonly carried out by immersing specimens in a series of ethanol (alcohol) solutions of increasing concentration until pure, water-free alcohol is reached. Ethanol is miscible with water in all proportions so that the water in the specimen is progressively replaced by the alcohol. A series of increasing concentrations is used to avoid excessive distortion of the tissue.

A typical dehydration sequence for specimens not more than 4mm thick would be:

70% ethanol 15 min

90% ethanol 15 min

100% ethanol 15 min

100% ethanol 15 min

100% ethanol 30 min

100% ethanol 45 min

At this point all but a tiny residue of tightly bound (molecular) water should have been removed from the specimen.

4. Clearing

Unfortunately, although the tissue is now essentially water-free, we still cannot infiltrate it with wax because wax and ethanol are largely immiscible. We therefore have to use an intermediate solvent that is fully miscible with both ethanol and paraffin wax. This solvent will displace the ethanol in the tissue, then this in turn will be displaced by molten paraffin wax. This stage in the process is called “clearing” and the reagent used is called a “clearing agent”. The term “clearing” was chosen because many (but not all) clearing agents impart an optical clarity or transparency to the tissue due to their relatively high refractive index. Another important role of the clearing agent is to remove a substantial amount of fat from the tissue which otherwise presents a barrier to wax infiltration.

A popular clearing agent is xylene and multiple changes are required to completely displace ethanol.

A typical clearing sequence for specimens not more than 4mm thick would be:

Xylene 20 min

Xylene 20 min

Xylene 45 min

5. Wax infiltration

The tissue can now be infiltrated with a suitable histological wax. Although many different reagents have been evaluated and used for this purpose over many years, the paraffin wax-based histological waxes are the most popular. A typical wax is liquid at 60°C and can be infiltrated into tissue at this temperature then allowed to cool to 20°C where it solidifies to a consistency that allows sections to be consistently cut. These waxes are mixtures of purified paraffin wax and various additives that may include resins such as styrene or polyethylene. It should be appreciated that these wax formulations have very particular physical properties which allow tissues infiltrated with the wax to be sectioned at a thickness down to at least 2

μm, to form ribbons as the sections are cut on the microtome, and to retain sufficient elasticity to flatten fully during flotation on a warm water bath.

Histological waxes such as Paraplast™ are popular and multiple changes are required to completely displace the clearing agent.

A typical infiltration sequence for specimens not more than 4mm thick would be:

Wax 30 min

Wax 30 min

Wax 45 min

6. Embedding or blocking out

Now that the specimen is thoroughly infiltrated with wax it must be formed into a “block” which can be clamped into a microtome for section cutting. This step is carried out using an “embedding centre” where a mould is filled with molten wax and the specimen placed into it. The specimen is very carefully orientated in the mould because its placement will determine the “plane of section”, an important consideration in both diagnostic and research histology. A cassette is placed on top of the mould, topped up with more wax and the whole thing is placed on a cold plate to solidify. When this is completed the block with its attached cassette can be removed from the mould and is ready for microtomy. It should be noted that, if tissue processing is properly carried out, the wax blocks containing the tissue specimens are very stable and represent an important source of archival material.

APPENDIX 4: RECIPES FOR SOLUTIONS AND REAGENTS

PREPARATION OF FIXATIVE (4% PARAFORMALDEHYDE IN 0.1M PB FOR 1 LITER)

1. Heat 750 ml of distilled water to 60°C.
2. Add 40 grams of paraformaldehyde to the heated distilled water and stir until dissolved.
3. Add 250 ml of 0.4M PB to the dissolved solution.
4. Filter the new solution through filter paper and store in the refrigerator. The solution lasts a maximum of one week in the refrigerator.

PREPARATION OF 10L 4% PARAFORMALDEHYDE IN 0.1M PB

1. Heat 2.5L of distilled water to 60°C
2. Add 400g of paraformaldehyde to the heated distilled water and stir until dissolved
3. Filter the new solution through filter paper and store in the refrigerator. The solution lasts a maximum of one week in the refrigerator.
4. Add 2.5L of 0.4M PB and 5L of distilled water to the filtered solution.

PREPARATION OF GEL COATED SLIDES

0.5% Gelatine- coated slides (0.5% gel, 0.1% calcium chrome sulphate)

- Heat 600 ml distilled water to 60^{o}C
- Add 3g gelatine
- Add 0.6g calcium chrome sulphate
- Allow for 10 min in order to dissolve

- Leave to cool to room temperature

PHOSPHATE BUFFERED SALINE (PH 7.4) 10X

The following ingredients were dissolved in 800ml distilled H_2O.

- 80g of NaCl
- 2.0g of KCl
- 14.4g of Na_2HPO_4
- 2.4g of KH_2PO_4

pH was adjusted to 7.4.

And volume was adjusted to 1L with additional distilled H_2O.

Solution was then sterilized by autoclaving.

10M CITRATE BUFFER (PH 6.0)

There were 2 Stock Solutions: Solution A and Solution B

Preparation:

Solution A - 4.2 grams Citric Acid + 200ml ddH2O

Solution B – 14.7 grams Sodium Citrate

Working Solution – 18ml A + 82ml B fill to 1L ddH2O and pH to6.0

ANTI-BODY DILUTIONS

All the dilutions were done using 1% BSA in PBS). For antibody optimization, serial dilutions were used on the tissues and the working volumes per section were obtained and maintained.

Primary: 1 in 100 µl

Secondary: 1 in 50 µl

These are the final working concentrations that need to be maintained .For example

METHOD FOR MIXTURE OF TRITON X

Ingredients:

- PB 0.1 M -100 ml
- Triton X -100 0.25 ml

Preparation:

Heat PB at 60°C, and add Triton under gentle shaking.

Keep shaking until a clear solution is obtained.

Leave to get to room temperature before use.

Store in the fridge.

Duration: approximately one month.

PREPARATION OF 0.4M PHOSPHATE BUFFER (PB)

Ingredients:

- Distilled water 500 ml
- Sodium di-Hydrogen Phosphate 10.6 g
- Di-Potassium Hydrogen Phosphate anhydrous 56 g
- Distilled water 1 L

Shake to dissolve

Ensure pH is between 7.2-7.4

PREPARATION OF 0.1M PHOSPHATE BUFFER

0.4M PB	250 ml
Distilled water	750 ml

APPENDIX 5: IMMUNOFLUORESCENCE PROTOCOL

(By Dr Clem Penny and Dr Kate Theron)

1. DEPARAFFINIZATION AND REHYDRATION

- Incubate slides at 60^{o}C overnight.
- Deparaffinise in Xylene -5min at 2 steps.
- Rehydrate was done by passing the sections through graded series of alcohols for 1 min each – 100%, 95%, and 70% and gently flowing tap water-5mins each.

2. ANTIGEN UNMASKING

Sections were placed in plastic container and covered with Citrate Buffer (pH 6.0) and heated in microwave at medium heat (720W) for 5 min:

- Top up buffer was added and tissue heated for further 5 min
- Sections were allowed to cool for 20 min
- Excess fluid was aspirated from slides which were then rinsed in 1x PBS for 5 min

3. CELL PERMEABILISATION

This was done using Triton-X100:

- 0.1% Triton-X100 in 0.1% BSA/PBS was added to sections for 10 min
- Sections were rinse in 3 changes 0.1% BSA/PBS, each for 5 mins

4. PRIMARY ANTIBODY

Rabbit polyclonal antibodies was used1: 100 dilutions.

100µl Primary antibodies was diluted with 0.1%BSA/PBS

Sections were incubated overnight at 4^{o}c

Next Morning slides were washed in 0.1%BSA/PBS 3 times, 5 mins each time:

Add 50 µl secondary antibodies (Anti-phosphotyrosine (4G10) – Alexa Fluor 568 (red) diluted in PBS for 1 hour at room temp

5. SECONDARY ANTIBODY

4G10 – Alexa Fluor 568 (red) conc: 1:300

Sections were washed in 3 changes of PBS on a magnetic stirrer, covered with foil.

6. MOUNTING

Sections on slides were mounted using aqueous mounting medium + anti-fade, covered with coverslip and Viewed.

APPENDIX 6: RELATIONSHIP BETWEEN STAGES OF DEVELOPMENT

Relationship between Stages of Development and Days of Incubation at 39 ^{0}c

(Hamburger & Hamilton staging for chick embryos 1951, 1992)

Stage 1. Pre-streak period

Stage 2. Initial streak 6-7 hrs of incubation

Stage 3. Intermediate streak 12-13 hrs.

Stage 4. Definitive streak 18-19 hrs.

Stage 5. Head process19-22 hrs.

Stage 6. Head fold: (23-25 hrs.).

Stage 7. One somite: (23-26 hrs.)

Note: Stages 7 to 14 are based primarily on the numbers of pairs of somites which are clearly visible. A stage is assigned to every third pair of somites which is added

Stage 8. 4 somites: (26-29 hrs.).

Stage 9. 7 somites: (29-33 hrs.).

Stage 10.10 somites: (33-38 hrs.).

Stage 11. 13 somites: (40-45 hrs.)

Stage 12. 16 somites: (45-49 hrs.).

Stage 13. 19 somites: (48-52 hrs.).

Stage 14. 22 somites: (50-53 hrs.)

Beyond stage 14 the number of somites becomes increasingly difficult to determine with accuracy.

Stage 15. (Hamburger, ’38; Saunders, ’48, stage 1; 50-55 hrs.).

Stage 16. (Hamburger-Saunders stage 2; 51-56 hrs.).

Stage 17. (Hamburger-Saunders stage 3; 52-64 hrs.).

Stage 18. (Hamburger-Saunders stage 4; 65-69 hrs.).

Stage 19. (Hamburger-Saunders stage 5; 68- 72 hrs.).

Stage 20. (Hamburger-Saunders stage 6; 70 - 72 hrs.).

Stage 21. (Saunders stage 7; 3.5 days).

Stage 22 (Saunders stages 8 and 9 combined; 34 days).

Stage 23. (Saunders stage 10; 3.5- 4 days).

Stage 24. (4 days).

Stage 25. (4.5 days).

Stage 26. (4.5-5 days).

Stage 27. (5 days).

Stage 28. (5.5 days).

Stage 29. (6 days).

Stage 30. (6.5 days).

Stage 31. (7 days).

Stage 32. (7.5 days).

Stage 33. (7.5-8 days).

Stage 34. (8 days).

Stage 35. (8-9 days).

Stage 36. (10 days).

Stage 37. (11 days).

Stage 38. (12 days).

Stage 39. (13 days).

Stage 40. (14 days).

Stage 41. (15 days).

Stage 42. (16 days).

Stage 43. (17 days).

Stage 44. (18 days).

Stage 45. (19-20 days).

Stage 46. Newly-hatched chick (20-21 days).

APPENDIX 7: STATISTICAL ANALYSIS OF RESULTS

PREAMBLE

The data were analysed using the Statistical Package for Social Sciences (version 21 SPSS Inc., Chicago, IL) software program. While the one-way Analysis of Variance (ANOVA) was used to compare the anti-angiogenic efficacy of the drugs, at 0.05 significance level ($p \leq 0.05$), Fisher exact test was used to determine the association between treatment drugs and growth of CAM blood vessels. A p value of < 0.05 was considered significant for all analysis.

PART ONE: EXPRESSION PATTERN OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN THE DEVELOPING AVIAN LUNG

In the first aspect of the work, paraffin sections of embryonic lung buds (5µm thickness) at stages 19-40, were immunolocalized for VEGF. Negative control sections were incubated with primary antibody but the secondary antibody was omitted (replaced with 100µl of 0.1% BSA/PBS). Positive controls were performed on histological sections of pregnant mouse uterus (5µm thickness). Results were scored as VEGF present or absent. No statistical analysis was done.

Table 1: Scoring scheme for VEGF in embryonic lung tissue

SCORE	INTERPRETATION
0	No VEGF detected
+	VEGF mildly detected
++	VEGF moderately detected
+++	VEGF markedly/heavily detected

PART TWO: EFFECT OF EFAVIRENZ ON ANGIOGENESIS IN CHICK CHORIOALLANTOIC MEMBRANE

Table 2: Fisher's exact test

Treatment Drugs	**CAM blood vessel present**	**CAM blood vessel absent**	**Fishers exact test (p-value)**
	f %	f %	
VEGF	5 (31.3)	1 (7.1)	
Thalidomide	1 (6.3)	5 (35.7)	
Efavirenz	0 (0.0)	6 (42.9)	0.001
Control 1	4 (25.0)	2 (14.3)	
Control 2	6 (35.5)	0 (0.0)	

Table 3: Mean Angiogenic score (ANOVA)

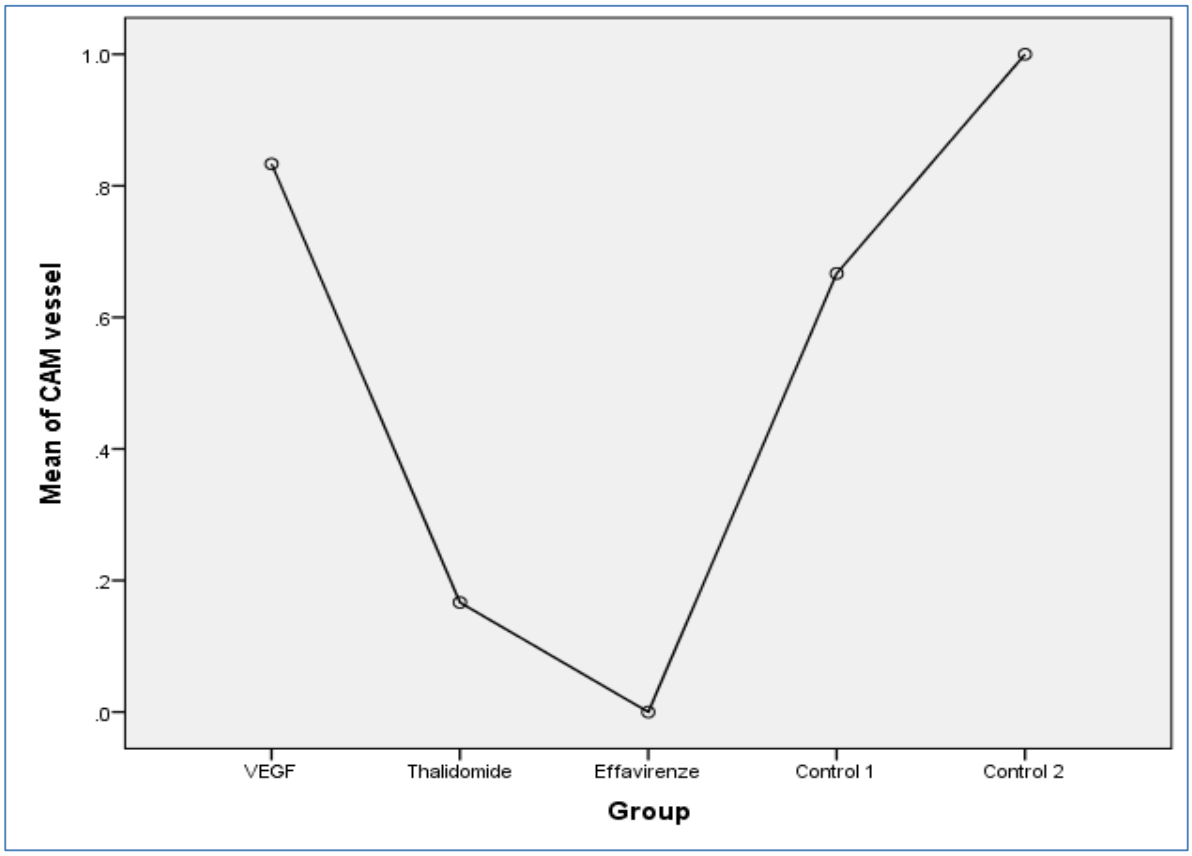
Efavirenz suppressed angiogenesis in all 6 subjects (showing absolute anti-angiogenicity and 0% angiogenicity). Thalidomide had mean angiogenicity of 0.17; Thalidomide failed as a negative control drug in that one embryo.

Group	**Mean Angiogenic Effect**	**Standard Deviation**	**F**	***P-value***
VEGF	.83	.41		
Thalidomide	.17	.41	9.306	.0005
Efavirenz	0	0		
Control 1	.67	.52		
Control 2	1	0		

Figure 1: Mean Trend of CAM vessel formation across Treatment Groups

Mean of CAM vessels

In order to show a visual representation of the relationship between the two variables (treatment drugs and presence or absence of CAM blood vessels), a trend line was fitted to the data and the R-squared value (coefficient of determination) was established. The trend for the presence of CAM vessels in the various treatment groups was lowest for Efavirenz:



Note that the value of “1” (as for Control 2) represents a perfect association or fit between the treatment drug and occurrence of CAM angiogenesis). “0” (as for Efavirenz) represents no statistical correlation between Efavirenz and CAM angiogenesis.

Table 4: Comparing the anti-Angiogenic effect of Efavirenz to Thalidomide.

There was no significant difference between the anti-angiogenic effect of Efavirenz and Thalidomide. Although Efavirenz suppressed both blood vessel development (angiogenesis) and red blood cell formation (erythropoiesis), our work did not measure the anti-erythropoietic effect of treatment drugs.

	N	**Mean**	**Standard Deviation**	**F**	**p-value**
Thalidomide	6	.17	.41	1.000	.341
Efavirenz	6	.00	.00		
Total	12	.08	.29		

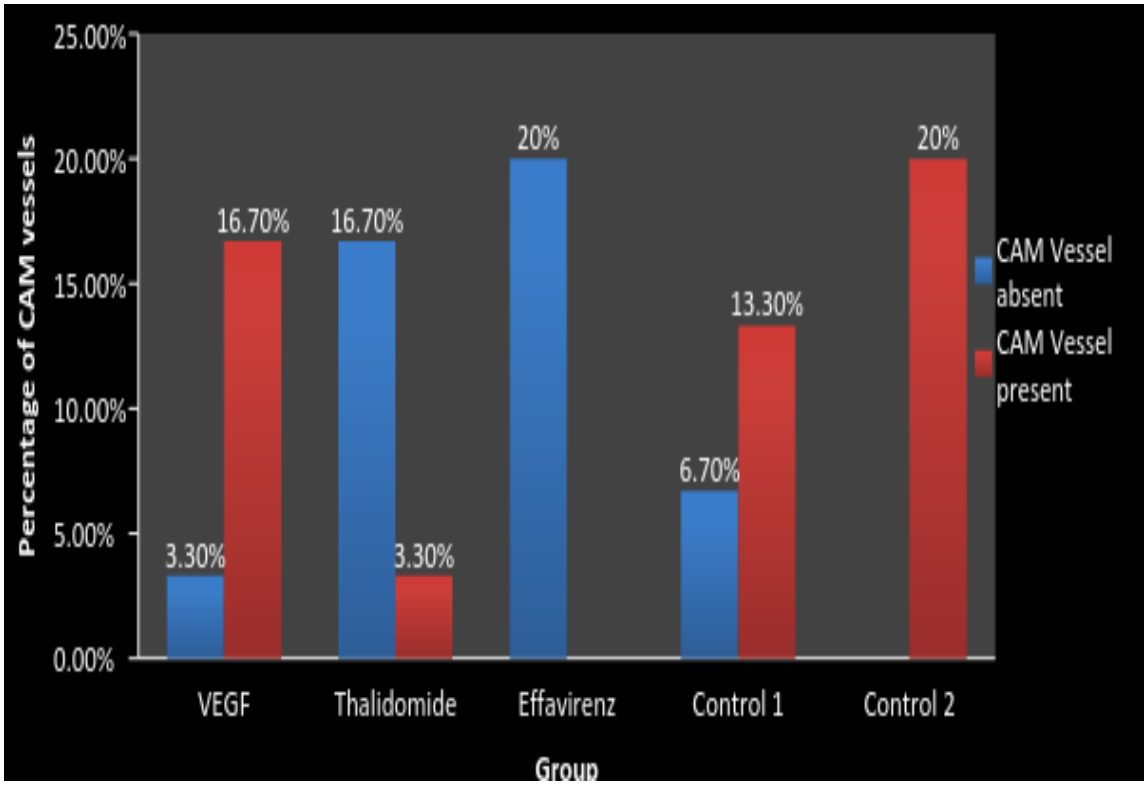


Figure 2: Comparative efficacy of treatment drugs

The figure reflects the total % efficacy of all the drugs used. Both their angiogenic and anti-angiogenic properties were evaluated per group (n=6). The % angiogenic and % anti-angiogenic properties of the treatment drugs were evaluated per group (n=6). Since each of the 5 treatment groups had 6 subjects that were randomly allocated, each group represented 20% of all the subjects used (n=30). It is seen that in 30 embryos, equally allocated to the 5 groups, when experimental groups are compared, control 2 presents with 100% angiogenic activity (20%). VEGF treatment has the next highest score of 16.7% angiogenic efficacy. It failed as an angiogenic drug in one embryo (awarding it an anti angiogenicity of 3.3%). Thalidomide failed to suppress angiogenesis in one subject out of 6 (3.3% angiogenicity in the whole experiment (n=30). That Effavirenz was 100% anti-angiogenic, means it had no angiogenic property. Use of ethanol as a diluent was common to all the treated groups. And although the presence of VEGF is a major contributing factor in favour of CAM angiogenesis, exogenous VEGF did not promote CAM angiogenesis beyond normal (as in control group 2). This may suggest that administration of exogenous VEGF did not potentiate the effect of endogenous VEGF. This aspect requires further experimentation in the future.

APPENDIX 8: PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK

PUBLISHED MANUSCRIPTS

1. 2017: ROSIE T MCNEIL, CLEMENT B PENNY, AND MARGOT J HOSIE (2017). Antiretroviral therapy and HIV-associated cancers: anti-angiogenic effect of Efavirenz on chick chorioallantoic membrane (CAM). Trop J Pharm Res 16 (11): 2741-2747
2. 2017: ROSIE THECLA MCNEIL, MARGOT JILL HOSIE AND CLEMENT BRIAN PENNY (2017). Developmental angiogenesis: expression of vascular endothelial growth factor (VEGF) in the developing avian lung. Int J Res Med and Health Sci 13(1): 1 -7

PUBLISHED ABSTRACTS

1. Rosie Thecla McNeil, Margot Jill Hosie and Clement Brian Penny (2016). Developmental angiogenesis: expression of vascular endothelial growth factor (VEGF) in the developing kidney. FASEB 2016
2. Rosie Thecla McNeil, Margot Jill Hosie and Clement Brian Penny (2017). Perineural angiogenesis: expression of vascular endothelial growth factor (VEGF) in the developing spinal cord. FASEB 2017