

# **Association between endometriosis and heavy metals in a group of Sri Lankan women of reproductive age**

by

**Wedikara Arachchige Nalinda Yasanga Silva**



**Ph.D.**

**2012**

# **Association between endometriosis and heavy metals in a group of Sri Lankan women of reproductive age**

by

Wedikara Arachchige Nalinda Yasanga Silva

Thesis submitted to the University of Sri Jayewardenepura for the award of the Degree of Doctor of Philosophy in Physiology on “Association between endometriosis and heavy metals in a group of Sri Lankan women of reproductive age”, on 12<sup>th</sup> December 2012.

I certify that the candidate has incorporated all corrections, amendments and additions recommended by the examiners.

Sharmaine Fernando

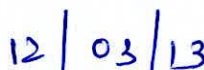
.....  
Professor Sharaine Fernando  
Professor in Physiology  
Department of Physiology  
University of Sri Jayewardenepura

Date 12.03.13 .....

The work described in this thesis was carried out by me under the supervision of Prof. Sharaine Fernando of University of Sri Jayewardenepura, Prof. Hemantha Senanayake and Prof. Kamani Tennekoon of University of Colombo, Dr. Roshini Peiris-John of University of Auckland, New Zealand and Prof. Rajitha Wickramasinghe of University of Kelaniya and a report on this has not been submitted in whole or in part to any University or any other institution for another Degree / Diploma.



.....  
W.A.N.Y Silva



.....  
Date

We certify that the above statement made by the candidate is true and that this thesis is suitable for submission to the University for the purpose of evaluation.

*S Fernando*

Professor Sharaine Fernando  
Professor in Physiology  
Department of Physiology  
University of Sri Jayewardenepura

Date *12.03.13*

*Hemantha Senanayake*

Professor Hemantha Senanayake  
Head  
Department of Obstetrics and Gynaecology  
Faculty of Medicine  
University of Colombo

Date *16.03.13*

*R. Peiris-John*

Dr. Roshini Peiris-John  
Research Fellow  
Section of Epidemiology and  
Biostatistics  
Faculty of Medical and  
Health Sciences  
University of Auckland  
New Zealand

Date *12.03.2013*

*Kamani Tennekoon*

Professor Kamani Tennekoon  
Professor of Molecular Life Sciences  
Institute of Biochemistry, Molecular Biology  
and Biotechnology  
University of Colombo

Date *12.03.2013*

*Rajitha Wickramasinghe*

Professor Rajitha Wickramasinghe  
Professor of Public Health  
Faculty of Medicine  
University of Kelaniya

Date *12/3/2013*



## TABLE OF CONTENTS

|   |             |
|---|-------------|
| <b>TABLE OF CONTENTS.....</b>                                     | <b>i</b>    |
| <b>LIST OF TABLES .....</b>                                       | <b>ix</b>   |
| <b>LIST OF FIGURES .....</b>                                      | <b>xi</b>   |
| <b>LIST OF ABBREVIATIONS.....</b>                                 | <b>xiv</b>  |
| <b>ACKNOWLEDGEMENTS.....</b>                                      | <b>xvii</b> |
| <b>ABSTRACT .....</b>   | <b>xx</b>   |
| <b>1. INTRODUCTION.....</b>                                       | <b>1</b>    |
| 1.1 Objectives.....   | 9           |
| 1.1.1 General objectives .....                                    | 9           |
| 1.1.2 Specific Objectives.....                                    | 9           |
| <b>2. LITERATURE REVIEW.....</b>                                  | <b>10</b>   |
| 2.1 Anatomy and physiology of the female reproductive system..... | 11          |
| 2.1.1 Anatomy of the female reproductive system.....              | 11          |
| 2.1.2 Physiology of the female reproductive system .....          | 12          |
| 2.1.2.1 Oestrogen structure, receptors and functions .....        | 12          |
| 2.1.2.1.1 Structure of oestrogen hormone .....                    | 12          |
| 2.1.2.1.2 Bio synthesis of oestrogens .....                       | 13          |
| 2.1.2.1.3 Oestrogen receptors .....                               | 14          |
| 2.1.2.1.4 Biological actions of oestrogens .....                  | 21          |
| 2.1.3 Summary .....   | 23          |
| 2.2 Endometriosis .....   | 24          |
| 2.2.1 Introduction .....  | 24          |
| 2.2.2 Incidence of endometriosis.....                             | 24          |
| 2.2.3 Impact of endometriosis.....                                | 25          |

|   |    |
|---|----|
| 2.2.3.1 Pain symptoms associated with endometriosis .....           | 25 |
| 2.2.3.2 Subfertility .....  | 27 |
| 2.2.3.3 Risk of malignancy .....                                    | 27 |
| 2.2.4 Economic burden .....   | 27 |
| 2.2.5 Diagnosis of endometriosis .....                              | 28 |
| 2.2.6 Treatment options for endometriosis .....                     | 31 |
| 2.2.7 History .....   | 32 |
| 2.2.8 Theories on the aetiology of endometriosis.....               | 33 |
| 2.2.8.1 Sampson's theory of retrograde transplantation .....        | 33 |
| 2.2.8.2 Metaplastic theory .....                                    | 34 |
| 2.2.8.3 In situ development theory .....                            | 34 |
| 2.2.9 Current concepts on the aetiopathology of endometriosis ..... | 34 |
| 2.2.10 Endogenous aetiological factors.....                         | 37 |
| 2.2.10.1 Eutopic endometrium in women with endometriosis.....       | 37 |
| 2.2.10.1.1 Adhesion .....   | 37 |
| 2.2.10.1.2 Invasion .....   | 38 |
| 2.2.10.1.3 Proliferation .....                                      | 39 |
| 2.2.10.1.4 Apoptosis.....   | 39 |
| 2.2.10.1.5 Angiogenesis.....  | 40 |
| 2.2.10.1.6 Protein profile .....                                    | 41 |
| 2.2.10.1.7 Gene expression .....                                    | 41 |
| 2.2.10.2 Ectopic endometrium .....                                  | 42 |
| 2.2.10.3 Inflammation and immune response .....                     | 43 |
| 2.2.10.4 Hormonal factors .....                                     | 45 |
| 2.2.10.4.1 Biosynthesis of oestrogens in endometriosis .....        | 45 |
| 2.2.10.4.2 Oestrogen receptors in endometriosis .....               | 47 |

|  |    |
|--|----|
| 2.2.10.4.3 Consequences of oestrogen activity in endometriosis ..... | 47 |
| 2.2.10.5 Genetics .....  | 48 |
| 2.2.11 Exogenous aetiological factors .....                          | 50 |
| 2.2.11.1 Demographic characteristics .....                           | 50 |
| 2.2.11.1.1 Age .....   | 50 |
| 2.2.11.1.2 Ethnicity .....   | 51 |
| 2.2.11.1.3 Level of education .....                                  | 52 |
| 2.2.11.1.4 Occupation.....   | 52 |
| 2.2.11.1.5 Socioeconomic status.....                                 | 52 |
| 2.2.11.2 Biological characteristics.....                             | 53 |
| 2.2.11.2.1 Body habitus .....  | 53 |
| 2.2.11.2.2 Menstrual and reproductive characteristics .....          | 54 |
| 2.2.11.3 Dietary characteristics .....                               | 55 |
| 2.2.11.4 Environmental factors .....                                 | 56 |
| 2.2.12 Summary .....   | 59 |
| 2.3 Heavy metals.....  | 60 |
| 2.3.1 Definition .....   | 60 |
| 2.3.2. Sources of heavy metals.....                                  | 60 |
| 2.3.3 Sri Lankan situation.....                                      | 62 |
| 2.3.4 Routes of entry into the human body.....                       | 63 |
| 2.3.5 Effects on the human .....                                     | 66 |
| 2.3.5.1 Toxic effects.....   | 66 |
| 2.3.5.2 Metalloestrogens.....  | 69 |
| 2.3.5.2.1 In vitro evidence.....                                     | 69 |
| 2.3.5.2.2 In vivo evidence .....                                     | 75 |
| 2.3.5.2.3 Metalloestrogens and endometriosis .....                   | 77 |



|  |            |
|--|------------|
| 2.3.6 Methods of evaluating the heavy metal status in humans .....       | 80         |
| 2.3.6.1 Assessment of exposure .....                                     | 80         |
| 2.3.6.1.1 Proximity to sources .....                                     | 80         |
| 2.3.6.1.2 Levels of metals in dietary items .....                        | 80         |
| 2.3.6.1.3 Food frequency questionnaire.....                              | 80         |
| 2.3.6.2 Measurement in biological fluids .....                           | 81         |
| 2.3.6.2.1 Sample collection and preparation .....                        | 81         |
| 2.3.6.2.2 Methods for quantitative determination .....                   | 82         |
| 2.3.6.3 Evaluation of symptoms due to chronic heavy metal exposure ..... | 83         |
| 2.3.7 Summary .....  | 86         |
| 2.4. In vitro models for investigating endometriosis .....               | 87         |
| 2.4.1 Chicken chorioallantotic membrane .....                            | 87         |
| 2.4.2 Human eutopic endometrial explants .....                           | 88         |
| 2.4.3 Invasion chambers.....   | 90         |
| 2.4.4 Monolayer cultures .....   | 91         |
| 2.4.4.1 Early attempts to culture human endometrial cells .....          | 91         |
| 2.4.4.2 Sources of endometrial cells .....                               | 92         |
| 2.4.4.3 Monolayer cell cultures in endometriosis .....                   | 93         |
| 2.4.4.3.1 Diversity of cell types .....                                  | 94         |
| 2.4.4.3.2 Applications of monolayer cell cultures in endometriosis ..... | 95         |
| 2.4.5 Summary .....  | 100        |
| <b>3. METHODS .....</b>  | <b>104</b> |
| 3.1 Phase I.....   | 104        |
| 3.1.1 Study design .....   | 104        |
| 3.1.2 Study setting.....   | 104        |
| 3.1.3 Study population.....  | 104        |

|   |     |
|---|-----|
| 3.1.4 Sample size .....   | 105 |
| 3.1.5 Data collection.....  | 106 |
| 3.1.6 Collection of blood and tissue samples .....                                    | 108 |
| 3.1.7 Chemical digestion of blood and tissue samples .....                            | 110 |
| 3.1.7.1 Principle .....   | 110 |
| 3.1.7.2 Procedure.....  | 110 |
| 3.1.7.3 Measures to minimize metal contamination during the chemical digestion .....  | 111 |
| 3.1.8 Determination of metal levels .....   | 112 |
| 3.1.8.1 Determination of metal levels using Total-reflection X-ray Fluorescence ..... | 112 |
| 3.1.8.2 Determination of cadmium levels .....   | 115 |
| 3.1.8.3 Quality control and validation.....   | 117 |
| 3.1.9 Data analysis .....   | 119 |
| 3.1.9.1 Statistical packages.....   | 119 |
| 3.1.9.2 Data analysis .....   | 119 |
| 3.1.9.3 Analysis of blood and tissue metal levels.....                                | 120 |
| 3.1.10 Ethical clearance.....   | 120 |
| 3.2 Phase II.....   | 121 |
| 3.2.1 Collection of samples .....   | 121 |
| 3.2.1.1 Study participants .....  | 121 |
| 3.2.1.2 Ethical considerations .....  | 122 |
| 3.2.1.3 Collection of eutopic endometrial tissue samples .....                        | 122 |
| 3.2.2 Isolation of endometrial stromal cells.....                                     | 123 |
| 3.2.2.1 Collagenase digestion.....  | 123 |
| 3.2.2.1.1 Preparation of collagenase solution.....                                    | 123 |
| 3.2.2.1.2 Incubation with Collagenase .....   | 123 |
| 3.2.2.2 Isolation of stromal cells with filtration and centrifugation.....            | 124 |

|  |     |
|--|-----|
| 3.2.2.3 Determination of cell number .....   | 124 |
| 3.2.2.4 Determination of cell viability .....                                      | 125 |
| 3.2.3 Establishment of primary endometrial stromal cell (ESC) cultures.....        | 125 |
| 3.2.4 Establishment of sub cultures .....  | 126 |
| 3.2.5 Treatment of ESC cultures with metal compounds .....                         | 127 |
| 3.2.5.1 Preparation of metal solutions .....                                       | 127 |
| 3.2.5.1.1 Metal compounds .....  | 127 |
| 3.2.5.1.2 Preparation of cadmium solutions .....                                   | 127 |
| 3.2.5.1.3 Preparation of nickel solution .....                                     | 128 |
| 3.2.5.1.4 Preparation of lead solution.....  | 128 |
| 3.2.5.2 Treatment of the cell monolayers with metal compounds .....                | 129 |
| 3.2.6 Determination of relative cell proliferation .....                           | 130 |
| 3.2.7 Sulforhodamine B (SRB) assay .....   | 131 |
| 3.2.7.1 Principle.....   | 131 |
| 3.2.7.2 Assay procedure .....  | 131 |
| 3.2.8 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay..... | 132 |
| 3.2.8.1 Principle.....   | 132 |
| 3.2.8.2 Assay procedure .....  | 132 |
| 3.2.9 Immunohistochemistry .....   | 133 |
| 3.2.9.1 Principle.....   | 133 |
| 3.2.9.2 Preparation of cell monolayer for immunohistochemistry .....               | 134 |
| 3.2.9.3 Immunohistochemistry for vimentin and cytokeratin .....                    | 134 |
| 3.2.9.4 Immunohistochemistry for ER and PR .....                                   | 135 |
| 3.2.9. 5 Microscopic assessment of immunohistochemical staining .....              | 136 |
| 3.2.10 Statistical analysis .....  | 136 |
| 3.2.11 Maintenance of sterility .....  | 136 |



|  |            |
|--|------------|
| <b>4. RESULTS.....</b>   | <b>138</b> |
| 4.1. Results of the phase I.....   | 138        |
| 4.1.1 Demographical characteristics.....   | 138        |
| 4.1.2 Biological characteristics.....  | 140        |
| 4.1.3 Multivariate analysis of demographic and biological characteristics.....     | 142        |
| 4.1.4 Characteristics of women with endometriosis.....                             | 142        |
| 4.1.5 Association of endometriosis with sources of heavy metal exposure.....       | 144        |
| 4.1.5.1 Proximity to sources of environmental pollution.....                       | 144        |
| 4.1.5.2 Association of endometriosis with dietary items.....                       | 147        |
| 4.1.5.3 Association of endometriosis with drinking water and cooking utensils..... | 147        |
| 4.1.6 Symptoms of chronic heavy metal exposure.....                                | 150        |
| 4.1.7 Assessment of quality control and validation.....                            | 152        |
| 4.1.8 Heavy metal concentrations in blood.....                                     | 154        |
| 4.1.9 Heavy metal levels in ectopic endometrial tissue.....                        | 156        |
| 4.2 Results of Phase II.....   | 160        |
| 4.2.1 Characteristics of the participants.....                                     | 160        |
| 4.2.2 Characteristics of isolated endometrial stromal cells.....                   | 161        |
| 4.2.3 Establishment of endometrial stromal cell cultures.....                      | 161        |
| 4.2.3.1 Characteristics of primary endometrial cell cultures.....                  | 161        |
| 4.2.3.2 Characteristics of individual endometrial stromal cells.....               | 162        |
| 4.2.4 Subcultures/ passages of endometrial stromal cells.....                      | 162        |
| 4.2.5 Relative cell proliferation.....   | 166        |
| 4.2.6 SRB assay.....   | 168        |
| 4.2.7 MTT assay.....   | 169        |
| 4.2.8 Immunohistochemical analysis of endometrial stromal cells.....               | 171        |
| 4.2.8.1 Immunohistochemistry for oestrogen receptor.....                           | 171        |

|   |            |
|---|------------|
| 4.2.8.2 Immunohistochemistry for progesterone receptor .....                    | 171        |
| <b>5. DISCUSSION .....</b>  | <b>180</b> |
| 5.1 Phase I.....  | 180        |
| 5.1.1 Demographic characteristics.....  | 180        |
| 5.1.2 Biological characteristics .....  | 184        |
| 5.1.3 Dietary characteristics .....   | 189        |
| 5.1.4 Association between heavy metal exposure and endometriosis .....          | 192        |
| 5.1.5 Whole blood heavy metal levels .....                                      | 193        |
| 5.1.6 Heavy metal levels in ectopic endometrial tissue .....                    | 196        |
| 5.2 Phase II.....   | 200        |
| 5.2.1 Establishment of primary endometrial stromal cell cultures .....          | 200        |
| 5.2.2 Effect of heavy metals on primary endometrial stromal cell cultures ..... | 207        |
| 5.2.2.1 Relative cell proliferation of ESC.....                                 | 207        |
| 5.2.2.2 SRB and MTT assays.....   | 208        |
| 5.2.2.3 Expression of ER and PR .....   | 212        |
| <b>6. CONCLUSIONS AND RECOMMENDATIONS .....</b>                                 | <b>216</b> |
| 6.1 Conclusions .....   | 216        |
| 6.2 Recommendations .....   | 217        |
| <b>7. REFERENCES.....</b>   | <b>218</b> |
| <b>8. APPENDICES .....</b>  | <b>265</b> |
| Appendix 1 .....  | 266        |
| Appendix 2.....   | 269        |
| Appendix 3.....   | 282        |
| Appendix 4.....   | 284        |
| Appendix 5.....   | 287        |



## LIST OF TABLES

|   | Page number |
|---|-------------|
| Table 2.1: Revised classification of endometriosis of the American Society for Reproductive Medicine .....  | 30          |
| Table 2.2: Characteristics of the main methods used in heavy metal analysis in biological samples .....   | 84          |
| Table 2.3: Features of chronic exposure to heavy metals .....   | 85          |
| Table 2.4: <i>In vitro</i> effects of currently used or novel compounds in endometriosis .....  | 101         |
| Table 4.1: Demographic characteristics of women with or without endometriosis .....   | 139         |
| Table 4.2: Menstrual and gynaecological characteristics of women with or without endometriosis .....  | 141         |
| Table 4.3: Summary of conditional logistic regression analysis .....  | 142         |
| Table 4.4: Stages of endometriosis among women with endometriosis   | 143         |
| Table 4.5: Modes of treatment received by women with endometriosis  | 143         |
| Table 4.6: Prevalence of symptoms associated with endometriosis in women with or without endometriosis .....  | 145         |
| Table 4.7: Association between close proximity (< 100m) to sources of environmental pollution and endometriosis .....                                     | 146         |
| Table 4.8: Association between food items and endometriosis .....   | 148         |
| Table 4.9: Sources of drinking water, utensils used for storage of drinking water and cooking in households of women with and without endometriosis ..... | 149         |
| Table 4.10: Prevalence of symptoms due to chronic heavy metal exposure in women with or without endometriosis .....                                       | 151         |
| Table 4.11: Comparison between certified and obtained values for the reference standards .....  | 153         |
| Table 4.12: Concentrations of heavy metals in whole blood ( $\mu\text{g/L}$ ) of women with or without endometriosis .....                                | 154         |

|  |     |
|--|-----|
| Table 4.13: Risk of endometriosis according to the tertile values of cadmium, lead, iron and zinc .....  | 155 |
| Table 4.14: Levels of heavy metals in ectopic endometrial tissue ( $\mu\text{g/Kg}$ ) and in whole blood ( $\mu\text{g/L}$ ) of women with endometriosis ..... | 157 |
| Table 4.15: Metal levels ( $\mu\text{g/Kg}$ ) of ectopic endometrial tissue in different sites in women with endometriosis .....                               | 158 |
| Table 4.16: Correlation between metal levels ( $\mu\text{g/Kg}$ ) in ectopic endometrial tissue .....  | 159 |
| Table 4.17: Stages of endometriosis in women with endometriosis .....  | 160 |
| Table 4.18: Cell number and viability of isolated endometrial stromal cells in women with and without endometriosis .....                                      | 161 |
| Table 5.1: Comparison of the demographic and biological characteristics of women with endometriosis .....  | 188 |

## LIST OF FIGURES

|   | Page number |
|---|-------------|
| Figure 1.1 Gaps in scientific knowledge addressed by the present study                                    | 8           |
| Figure 2.1 Organizational structure of the literature review.....   | 10          |
| Figure 2.2 Female reproductive system .....   | 11          |
| Figure 2.3 Structures of naturally occurring oestrogens.....  | 12          |
| Figure 2.4 Synthesis of naturally occurring oestrogens.....   | 13          |
| Figure 2.5 Structure of the oestrogen receptor .....  | 14          |
| Figure 2.6 Diversity of the ligands that activate the oestrogen receptor.....                             | 17          |
| Figure 2.7 Mechanism of oestrogen receptor activation.....  | 18          |
| Figure 2.8 Current concepts on the aetiopathology of endometriosis.....                                   | 36          |
| Figure 2.9 Complex interplay of endogenous aetiological factors in the pathogenesis of endometriosis..... | 49          |
| Figure 2.10 Sources of heavy metals and their routes of entry into humans.....                            | 65          |
| Figure 2.11 <i>In vitro</i> metalloestrogenic effects of cadmium .....                                    | 74          |
| Figure 3.1 Summary of the methodology for the data and sample collection in phase I.....                  | 109         |
| Figure 3.2 Sample preparation and metal analysis procedure.....   | 112         |
| Figure 3.3 Principle of TXRF.....   | 113         |
| Figure 3.4 A typical spectrum obtained using the TXRF.....  | 115         |
| Figure 3.5 Principle of GFASS.....  | 117         |
| Figure 3.6 Serial dilution of cadmium solutions (A to E).....   | 128         |
| Figure 3.7 Summary of the methodology in phase II.....  | 137         |
| Figure 4.1 Primary endometrial stromal cell cultures under the inverted phase contrast microscope.....    | 163         |



|   |     |
|---|-----|
| Figure 4.2 Endometrial stromal cells in long term cultures under the phase contrast microscope.....   | 164 |
| Figure 4.3 Endometrial stromal cells following immune histochemical staining using primary antibodies for (A) vimentin (B) cytokeratin and (C) universal negative control under the binocular microscope.....   | 164 |
| Figure 4.4 Endometrial stromal cells under the phase contrast microscope.....   | 165 |
| Figure 4.5 Endometrial stromal cell culture from a control at 90 days after the initial culture at the 17 <sup>th</sup> passage (subculture) following immunohistochemical staining using primary antibodies for (A) vimentin (B) cytokeratin and (C) universal negative control; under the binocular microscope..... | 165 |
| Figure 4.6 Endometrial stromal cell culture from a patient at 60 days after the initial culture at the 10 <sup>th</sup> passage (subculture) following immunohistochemical staining using primary antibodies for (A) vimentin (B) cytokeratin and (C) universal negative control; under the binocular microscope..... | 165 |
| Figure 4.7 Relative proliferation of Endometrial Stromal Cells from women without endometriosis and women with endometriosis after 24 hours and 48 hours of treatment with (A) 10 <sup>-6</sup> M Cd (b) 10 <sup>-9</sup> M Ni and (C) 10 <sup>-9</sup> M Pb.....   | 167 |
| Figure 4.8 Relative proliferation of endometrial Stromal Cells from women without endometriosis and women with endometriosis after 24 hours and 48 hours of treatment with a combination of 10 <sup>-6</sup> M Cd, 10 <sup>-9</sup> M Ni and 10 <sup>-9</sup> M Pb .....  | 168 |
| Figure 4.9 Results of SRB assay following 48 hours of incubation with different concentrations of Cd (10 <sup>-8</sup> M to 10 <sup>-3</sup> M) and a combination of metal compounds (10 <sup>-6</sup> M Cd, 10 <sup>-9</sup> M Ni and 10 <sup>-9</sup> M Pb) .....   | 169 |
| Figure 4.10 Results MTT assay following 48 hours of incubation with different concentrations of Cd (10 <sup>-8</sup> M to 10 <sup>-3</sup> M) and combination of metal compounds (10 <sup>-6</sup> M Cd, 10 <sup>-9</sup> M Ni and 10 <sup>-9</sup> M Pb) .....   | 170 |
| Figure 4.11 Immunohistochemical staining of endometrial stromal cells with primary antibody for oestrogen receptor, following treatment with 10 <sup>-6</sup> M Cd for 24 hours and 48 hours in (A) women with endometriosis (B) women without endometriosis .....  | 172 |

|  |     |
|--|-----|
| Figure 4.12 Immunohistochemical staining of endometrial stromal cells with primary antibody for progesterone receptor, following treatment with $10^{-6}$ M Cd for 24 hours and 48 hours in (A) women with endometriosis (B) women without endometriosis.....  | 173 |
| Figure 4.13 Immunohistochemical staining of endometrial stromal cells with primary antibody for oestrogen receptor, following treatment with $10^{-9}$ M Ni for 24 hours and 48 hours in (A) women with endometriosis (B) women without endometriosis.....   | 174 |
| Figure 4.14 Immunohistochemical staining of endometrial stromal cells with primary antibody for progesterone receptor, following treatment with $10^{-9}$ M Ni for 24 hours and 48 hours in (A) women with endometriosis (B) women without endometriosis.....  | 175 |
| Figure 4.15 Immunohistochemical staining of endometrial stromal cells with primary antibody for oestrogen receptor, following treatment with $10^{-9}$ M Pb for 24 hours and 48 hours in (A) women with endometriosis (B) women without endometriosis.....   | 176 |
| Figure 4.16 Immunohistochemical staining of endometrial stromal cells with primary antibody for progesterone receptor, following treatment with $10^{-9}$ M Pb for 24 hours and 48 hours in (A) women with endometriosis (B) women without endometriosis .....   | 177 |
| Figure 4.17 Immunohistochemical staining of endometrial stromal cells with primary antibody for oestrogen receptor, following treatment with a combination of $10^{-6}$ M Cd, $10^{-9}$ M Ni and $10^{-9}$ M Pb for 24 hours and 48 hours in (A) women with endometriosis (B) women without endometriosis .....    | 178 |
| Figure 4.18 Immunohistochemical staining of endometrial stromal cells with primary antibody for progesterone receptor, following treatment with a combination of $10^{-6}$ M Cd, $10^{-9}$ M Ni and $10^{-9}$ M Pb for 24 hours and 48 hours in (A) women with endometriosis (B) women without endometriosis ..... | 179 |
| Figure 5.1 Interplay of demographic and biological characteristics in the present study  | 192 |



## LIST OF ABBREVIATIONS

|                              |   |   |
|------------------------------|---|---|
| <b>AKT</b>                   | - | Threonine/serine kinases                |
| <b>AP-1</b>                  | - | Activating Protein-1                    |
| <b>As</b>                    | - | Arsenic                                 |
| <b>Au</b>                    | - | Gold                                    |
| <b>Bcl-2</b>                 | - | B-Cell lymphoma/leukaemia-2             |
| <b>BLyS</b>                  | - | B lymphocyte stimulator                 |
| <b>Ca</b>                    | - | Calcium                                 |
| <b>CAM</b>                   | - | Chicken chorioallantoic membrane        |
| <b>Cd</b>                    | - | Cadmium                                 |
| <b>CdCl<sub>2</sub></b>      | - | Cadmium chloride                        |
| <b>CEA</b>                   | - | Central Environmental Authority         |
| <b>Co</b>                    | - | Cobalt                                  |
| <b>Cr</b>                    | - | Chromium                                |
| <b>Cu</b>                    | - | Copper                                  |
| <b>E<sub>1</sub></b>         | - | Oestrone                                |
| <b>E<sub>2</sub></b>         | - | 17 $\beta$ -oestradiol                  |
| <b>E<sub>3</sub></b>         | - | Oestriol                                |
| <b>ECM</b>                   | - | Extracellular matrix                    |
| <b>EGFR</b>                  | - | Epidermal growth factor receptor        |
| <b>ER</b>                    | - | Oestrogen receptor                      |
| <b>ER<math>\alpha</math></b> | - | Oestrogen receptor alpha                |
| <b>ER<math>\beta</math></b>  | - | Oestrogen receptor beta                 |
| <b>ERE</b>                   | - | Oestrogen response elements             |
| <b>ERK</b>                   | - | Extra cellular signal-regulated kinases |

|                                |                                       |
|--------------------------------|---------------------------------------|
| <b>Fe</b>                      | - Iron                                |
| <b>FSH</b>                     | - Follicular stimulating hormone      |
| <b>GnRH</b>                    | - Gonadotropin releasing hormone      |
| <b>Hg</b>                      | - Mercury                             |
| <b>HPO</b>                     | - Hypothalamo-pituitary-ovarian axis  |
| <b>IHC</b>                     | - Immunohistochemistry                |
| <b>IL</b>                      | - Interleukin                         |
| <b>K</b>                       | - Potassium                           |
| <b>mER</b>                     | - Membrane oestrogen receptor         |
| <b>MAPK</b>                    | - Mitogen-activated protein kinase    |
| <b>MMP</b>                     | - Matrix metalloproteinase            |
| <b>NK</b>                      | - Natural killer                      |
| <b>Ni</b>                      | - Nickel                              |
| <b>NF-<math>\kappa</math>B</b> | - Nuclear Factor- $\kappa$ B          |
| <b>NSAID</b>                   | - Nonsteroidal antiinflammatory drug  |
| <b>OD</b>                      | - Optical density                     |
| <b>L1 CAM</b>                  | - L1 cell adhesion molecule           |
| <b>LH</b>                      | - Luteinizing hormone                 |
| <b>P</b>                       | - Phosphorus                          |
| <b>Pb</b>                      | - Lead                                |
| <b>PF</b>                      | - Peritoneal fluid                    |
| <b>PMC</b>                     | - Peritoneal mesothelial cell         |
| <b>PR</b>                      | - Progesterone receptor               |
| <b>PTWI</b>                    | - Provisional tolerable weekly intake |
| <b>S</b>                       | - Sulphur                             |

|                               |   |
|-------------------------------|---|
| <b>SERM</b>                   | - Selective oestrogen receptor modulator        |
| <b>Sn</b>                     | - Tin   |
| <b>TFF 1</b>                  | - Trefoil factor 1                              |
| <b>TIMP</b>                   | - Tissue inhibitors of matrix metalloproteinase |
| <b>TNF<math>\alpha</math></b> | - Tumour necrosis factor $\alpha$               |
| <b>TSP-1</b>                  | - Thrombospondin-1                              |
| <b>TXRF</b>                   | - Total-reflection X-ray Fluorescence           |
| <b>WHO</b>                    | - World Health Organization                     |
| <b>uPA</b>                    | - Urokinase plasminogen activator               |
| <b>UK</b>                     | - United Kingdom                                |
| <b>US</b>                     | - United States                                 |
| <b>UV</b>                     | - Ultraviolet                                   |
| <b>VEGF</b>                   | - Vascular endothelial growth factor            |
| <b>Zn</b>                     | - Zinc  |

## ACKNOWLEDGEMENTS

- Work described in this thesis constitutes support by many individuals in at least five different institutions where financial assistance was provided by four separate funding sources.
- The original research question on the association of heavy metals and endometriosis was first posed by Prof. Hemantha Senanayake of University of Colombo. Thus I would like to acknowledge Prof. Hemantha Senanayake for supervising me during this research project that was based on his original idea.
- Dr. Roshini Peiris-John who was always well tuned to conduct quality research inspired me in many ways. She opted to supervise me even after her resignation from the University of Sri Jayewardenepura despite a busy schedule.
- Meeting Prof. Rajitha Wickramasinghe and being supervised by him were indeed life time experiences for me. I would like to thank him for his input not only as a statistician but also as an internationally recognized researcher.
- The opportunity to work under Prof. Kamani Tennekoon as a clinical research assistant back in 2005 was a turning point in my professional life. Prof. Tennekoon was a pillar of strength for me during this project. I will eternally cherish the memories of Prof. Tennekoon correcting my thesis in her usual methodical manner despite physical constraints.
- I am indebted to Prof. Sharaine Fernando for stepping in as the internal supervisor at a very difficult stage of this project. Her assistance helped me in numerous ways to concentrate on the completion of the bench work and writing of the thesis. I deeply appreciate the personal interest taken by Prof. Fernando that enabled me to complete my PhD.



- Senior colleagues at the Department of Physiology Prof. Sivithri Wimalasekara, Prof. Priyadarshika Hettiarachchi, Dr. D.K. Ruberu, Dr. Chandana Hewage and Dr Himasu Waidyaseka helped me in many ways towards the successful completion of this study. I would like to thank them, especillly for allowing me to take study leave for one and a half years.
- I would like to thank the present Dean of Faculty of Medical Sciences Prof. Mohan De Silva for the assistance.
- Prof. Jayantha Jayawardana former Dean of Faculty of Medical Sciences is acknowledged for the support given during the initial stages of this study.
- In addition to granting me study leave, the University of Sri Jayewardenepura was the first funding source whose research grant (ASP/6/Re/2008/06) that enabled the initiation of this study. Dr. Narada Warsasuriya, Former Vice Chancellor of University of Sri Jayewardenepura is acknowledged for approving the above grant.
- I would like to express my deep sense of gratitude to Dr. N.L.A. Karunaratne, Vice Chancellor of University of Sri Jayewardenepura who never hesitated whenever a request was made for his assistance.
- University Grants Commission (UGC) is acknowledged for providing the grant that was instrumental in upgrading my MPhil to a PhD. I would like to thank the staff at the International Cooperation division of the UGC for all the support.
- I wish to express sincere thanks to the National Coordinating Committee on Reproductive Health Research of Sri Lanka (NCC-HRP/ WHO) for providing the necessary financial assistance that enabled the establishment of endometrial stromal cell cultures.



- International Training and research on environmental and occupational health (ITREOH) programme (NIH grant-Two5497-07) of the University of Alabama, Birmingham, USA is acknowledged for the financial assistance and training during this project.
- Staff at the Professorial Gynaecology unit of the National Hospital and staff of Ward 16 of the De Soyza Maternity Hospital Colombo are acknowledged for the cooperation during data collection.
- I wish to thank the staff of the Operating Theatre A of the National Hospital Colombo for the excellent support during sample collection.
- Mr. Vajira Waduge and the staff at the Atomic Energy Authority are acknowledged for all the support during preparation and analysis of samples.
- Dr. M.S.M Iqbal and Mrs. D Aluthpatabendi at the Institute of Fundamental Studies, Kandy are acknowledged for the assistance in cadmium analysis.
- I wish to thank Staff and colleagues at the Institute of Biochemistry, Molecular Biology and Biotechnology of University of Colombo, especially Mr. Sameera Samarakoon, Scientific Assistant (Cell culture) who made my stay at the IBMBB indeed a pleasurable one.
- My parents are fondly remembered for providing me love, care and a good education without which none of this would have been possible.
- Last but not the least; I would like to thank all the women who volunteered to participate in this study without whose support this thesis would not have been a reality.

# **Association between endometriosis and heavy metals in a group of Sri Lankan women of reproductive age**

**Wedikara Arachchige Nalinda Yasanga Silva**

## **ABSTRACT**

Heavy metals such as cadmium, nickel and lead activate the oestrogen receptor (ER) to exert oestrogenic effects; thus implicated in the aetiology of endometriosis an oestrogen dependant disease. A purported role of heavy metals in the continuation of ectopic endometrial tissue could be hypothesized.

The objectives of this study were to compare the demographic, biological, exposure status of heavy metals, blood levels of heavy metals as well as the effects of heavy metals on endometrial stromal cell cultures between women with endometriosis and women with no evidence of the disease. In addition, presence of heavy metal in ectopic endometrial tissue was investigated.

In this case-control study women with endometriosis (n=150) were compared with age-matched controls (n=150) with no evidence of endometriosis, both confirmed by laparoscopy or laparotomy. A pre tested interviewer-administered questionnaire was used for data collection. In a sub population of women (n=50 in each group), venous blood samples and ectopic endometrial tissue samples were obtained and digested with supra pure 65% HNO<sub>3</sub>. Blood and tissue samples were analyzed for heavy metals using Total-reflection X-ray Fluorescence and graphite furnace atomic absorption spectroscopy. From both groups (n=10 in each group) eutopic endometrial samples were obtained to isolate endometrial stromal cells (ESC). Primary ESC cultures were established in RPMI medium and the cultures in the third passage were treated with Cd, Pb and Ni at concentrations of 10<sup>-6</sup> M, 10<sup>-9</sup> M and 10<sup>-9</sup> M respectively. At 24 h and 48 h

the relative cell proliferation was calculated using the Neubauer haemocytometer, while Progesterone receptor (PR) and ER expression were assessed by immunohistochemistry. SRB and MTT assays were performed following 48 hours of exposure of ESC cultures of both groups to Cd at concentrations of  $10^{-8}$  M to  $10^{-3}$  M.

Chi square tests and odds ratios (OR) (McNemar's) were used to determine associations between dichotomous variables. Conditional logistic regression analysis was done to adjust for potential confounding variables. Log transformed blood levels of metals were compared using paired t-test while blood and tissue metal levels were correlated using the spearman rank correlation. ANOVA was used to analyze relative cell proliferation, SRB and MTT results.

The risk of endometriosis was 2.084 times greater among those with an education above Advanced Level as compared to those having an education below Advanced Level ( $p=0.021$ ) after adjusting for confounding factors. Endometriosis was commoner among women living close to a main road (OR=1.694; 95 % CI=1.037-2.767) (unadjusted) as compared to the respective reference group. Women with endometriosis had significantly higher ( $P=0.011$ ) geometric mean (95% CI) blood nickel levels [2.65 (1.94-3.38)  $\mu\text{g/L}$ ] as compared to women without endometriosis [0.80 (0.70-0.90)  $\mu\text{g/L}$ ]. Blood levels [women with vs. without] of cadmium [0.84 (0.17-1.98) vs 0.85 (0.26-1.45)  $\mu\text{g/L}$ ] and lead [11.09 (0.67-13.36) vs 6.90 (1.72-8.09)  $\mu\text{g/L}$ ] were similar in the two groups ( $p=0.289$  and  $p=0.123$  respectively). The geometric mean (95% CI) levels of cadmium, nickel and lead detected in the ectopic endometrial tissue of women with endometriosis were 2.86 (2.13-3.60), 17.55 (13.15-21.94) and 25.78 (18.49-33.08)  $\mu\text{g/Kg}$  respectively with no significant correlation between blood and tissue levels of



cadmium, nickel or lead. Cd treatment increased the relative proliferation in ESC and at 48 h, cell proliferation was higher in cultures from women with endometriosis ( $p=0.02$ ) than in women with no evidence of disease. Treatment with Cd reduced expression of ER and increased expression of PR in the ESC from women with endometriosis when compared to normal women. Relative cell proliferation and alterations in ER and PR levels were most prominent in ESC of women with endometriosis at 48 h. In the MTT assay, while significant differences were noted at  $10^{-6}$  M of Cd there was no dose dependent response.

In this group of Sri Lankan women in the reproductive age, endometriosis is commoner among those who are more educated. Cadmium, nickel and lead were present in ectopic endometrial tissue and higher levels of nickel in blood were detected in women with endometriosis compared to women without endometriosis. Cadmium was capable of inducing oestrogenic effects in cultured endometrial stromal cells that were more prominent in women with endometriosis.