The Pathogenesis and Prevention of Endometrial Polyps

Thesis submitted for the degree of Doctor of Medicine at the University of Leicester

By

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Abstract
The Pathogenesis and Prevention of Endometrial Polyps
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Endometrial polyps are bothersome lesions, commonly seen in clinical practice causing abnormal uterine bleeding and concern about the risk of malignant transformation. The mechanisms leading to the growth of polyps is unclear. The role of altered steroid receptor expression was studied using immunohistochemistry to examine the expression of ER-α ER-β PR-A and PR-B in polyps and endometrium in women treated with and without tamoxifen. A reduction in stromal ER-β expression in polyps from both groups compared to endometrium, and a reduction in stromal PR-B expression in tamoxifen polyps compared to tamoxifen endometrium suggests that an altered sensitivity to these estrogen and progesterone is key to polyp formation. In order to examine the genetic pathways that lead to polyp formation, a cross-platform microarray was carried out that similarly examined polyps and endometrium from women treated with and without tamoxifen. A subset of 3272 polyp genes was identified. Of these, 1659 genes were upregulated in polyps relative to the endometrium, and 1613 genes down regulated in polyps relative to the endometrium.

The mechanisms by which endometrial polyps are prevented in tamoxifen treated women using the LNG-IUS is thought to be via the mechanism of decidualisation. Firstly, decidualisation was confirmed in endometrial biopsy samples from tamoxifen treated women with the LNG-IUS in situ using immunohistochemistry to demonstrate an increase in IGFBP-1 expression in decidualised stromal cells. Finally, the effect of decidualisation on the expression levels 2 polyp genes, beta-catenin and Notch 2 in endometrial and polyp stromal cells, was examined by quantitative RTPCR using an in-vitro model to test the effects of medroxyprogesterone acetate, levonorgestrel and desogestrel treatment in combination with either estrogen or tamoxifen. This study demonstrated that progestin treatment did in-part reverse the polyp effect on these genes.
Declaration

None of the work presented in this thesis has been submitted for another degree at this or any other university

All the work in this thesis is my own unless acknowledged here, or by references:

1. Dr Panchal and Dr Taylor carried out the Affymetrix microarray examining postmenopausal and tamoxifen treated endometrium. They kindly provided the original data sets which was then formed an integral part of the cross-platform microarray study described in chapter 3.

2. Tim Gant from the University of Leicester, who has experience in high throughput genomics and bioinformatics, devised the methodology used in chapter 3 for normalizing the Affymetrix and illumina data sets prior to the cross-platform analysis.

3. Muna Abbas provided invaluable technical assistance in the latter stages of this research project. She completed the IGFBP-1 immunohistochemistry experiment described in chapter 4, the serum IGFBP-1 ELISA described in chapter 4, and the RT-PCR experiment described in chapter 5.
Acknowledgements

Firstly, my thanks go to the women who selflessly agreed to help in the studies that have contributed to this thesis. Their desire to help in finding answers to the questions posed here has motivated me to be a more diligent researcher.

I’m grateful to the staff in the Department of Genetics at the University of Leicester, most notably Henrik Townsend, whose support, advice and microarray expertise was invaluable. I would also like to acknowledge the help of Tim Gant who generously gave his time to help facilitate the cross platform microarray analysis, and Muna Abbas who kindly helped complete the ELISA and RT-PCR experiments.

My fellow clinical researchers Mohamed Mehasseb, Sam Engemise, and Vijay Kalathy were great colleagues to walk with through the journey of this thesis. I valued all of their support, humour, cups of coffee and most of all their ability to listen when it was needed.

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And finally, Mum and Dad, without whose love, support, encouragement and unquestioned faith in my ability I would never have made it.
Endometrial Polyps: A Literature Review

1. Polyps
2. Symptoms
3. The scale of the problem
4. Risk of Malignancy
5. Risk factors for endometrial polyps
   1. Tamoxifen
   2. Hormone replacement therapy
   3. Other factors

1.6 Pathogenesis
   1.1 Aromatase
   1.2 Origins in the Basalis Layer
   1.3 Monoclinality
   1.4 Human epidermal growth factor receptor 2 (c-erbB2)
   1.5 Kirsten ras proto-oncogene (K-ras) Mutation
   1.6 Steroid Receptors
   1.7 Proliferation and Apoptosis

1.7 Prevention of polyps

Page | 4
2  ER-alpha, ER-beta PR-A and PR-B Expression in Endometrial Polyps and Endometrium from Postmenopausal Women Treated with and without Tamoxifen 32

2.1 Background ........................................................................................................ 32

2.2 Aim ..................................................................................................................... 41

2.3 Materials and Method ....................................................................................... 42

2.3.1 Tissue collection ............................................................................................ 42

2.3.2 Immunohistochemistry .................................................................................. 43

2.3.3 Image Analysis ............................................................................................... 45

2.3.4 Statistical Methods ........................................................................................ 46

2.4 Results ................................................................................................................ 47

2.4.1 ER-alpha ......................................................................................................... 47

2.4.2 ER-beta .......................................................................................................... 51

2.4.3 PR-A ................................................................................................................. 55

2.4.4 PR-B ................................................................................................................. 59

2.5 Discussion ........................................................................................................... 63

3  A Cross-Platform Microarray of Endometrial Polyps and Endometrium from Postmenopausal Women Treated With and Without Tamoxifen .............. 68

3.1 Background ...................................................................................................... 68

3.2 Aim .................................................................................................................... 70

3.3 Experimental Design ......................................................................................... 70

3.4 Materials and Methods ...................................................................................... 73

3.4.1 Specimens ....................................................................................................... 73

3.4.2 RNA extraction ............................................................................................... 73

3.4.3 RNA Purification ............................................................................................. 74

3.4.4 RNA Quantification and Quality Assessment ................................................. 74
3.4.5 Polyp Microarray................................................................. 74
3.4.6 Polyp microarray gene expression analysis................................. 75
3.4.7 Endometrium Microarray ................................................................ 76
3.4.8 Endometrium microarray gene expression analysis......................... 76
3.4.9 Cross Platform Analysis.................................................................. 76
3.4.10 Pathway analysis........................................................................... 77
3.4.11 Preparation of cDNA for Quantitative RT-PCR .......................... 77
3.4.12 Quantitative RT-PCR ................................................................. 78

3.5 Results................................................................................................ 79
3.5.1 Cross Platform Analysis............................................................... 79
3.5.2 Pathway Analysis .......................................................................... 87
3.5.3 Quantitative RT-PCR .................................................................... 90

3.6 Discussion.......................................................................................... 92

4 Stromal Decidualisation and IGFBP-1 expression in Endometrium and Serum and of Tamoxifen Treated Women Exposed to Intrauterine Levonorgestrel via the LNG-IUS 100

4.1 Background......................................................................................... 100
4.1.1 Decidualisation.............................................................................. 100
4.1.2 Markers of Decidualisation......................................................... 101
4.1.3 Levonorgestrel............................................................................. 102
4.1.4 Endometrial Effects of Levonorgestrel........................................ 103
4.1.5 The Effect of Levonorgestrel on Tamoxifen Treated Endometrium... 104

4.2 Aims.................................................................................................... 105

4.3 Materials and Methods...................................................................... 106
4.3.1 Tissue and Serum Samples.......................................................... 106
4.3.2 Immunohistochemistry................................................................. 107
4.3.3 ELISA............................................................................................ 108

4.4 Results................................................................................................ 110
4.4.1 Immunohistochemistry................................................................. 110
5 Effect of decidualisation on Beta-catenin and Notch-2 Expression in Tamoxifen and Estrogen-Primed Endometrial and Polyp Stromal Cells .................................. 119

5.1 Background ........................................................................................................ 119

5.2 Aim ..................................................................................................................... 123

5.3 Materials and Method .......................................................................................... 124

5.3.1 Specimens ....................................................................................................... 124

5.3.2 Preparation and Culture of Stromal Cells ......................................................... 124

5.3.3 ELISA ............................................................................................................. 126

5.3.4 RNA Extraction ............................................................................................. 127

5.3.5 RNA purification ............................................................................................ 128

5.3.6 RNA Quantification and Quality Assessment .................................................. 128

5.3.7 Preparation of cDNA for Quantitative RT-PCR .............................................. 128

5.3.8 Quantitative RT-PCR .................................................................................... 128

5.4 Results ............................................................................................................... 130

5.4.1 IGFBP-1 ELISA ............................................................................................ 130

5.4.2 RT-PCR .......................................................................................................... 133

5.5 Discussion .......................................................................................................... 143

6 Discussion of Findings and Future Work .............................................................. 148

6.1 Discussion of Findings ....................................................................................... 148

6.2 Future Work ...................................................................................................... 159

6.2.1 Endometrial origins of polyp pathogenesis .................................................... 159

6.2.2 Progestins and polyps ................................................................................... 159

6.2.3 LNG-IUS and polyps in postmenopausal women without tamoxifen treatment .......................................................... 159

7 Appendix 1: Ethics approval ................................................................................. 160
| Table 1.1 | 17 |
| Table 2.1 | 37 |
| Table 2.2 | 43 |
| Table 2.3 | 49 |
| Table 2.4 | 53 |
| Table 2.5 | 57 |
| Table 2.6 | 61 |
| Table 3.1 | 78 |
| Table 3.2 | 81 |
| Table 3.3 | 84 |
| Table 3.4 | 88 |
| Table 3.5 | 90 |
| Table 4.1 | 107 |
| Table 4.2 | 111 |
| Table 4.3 | 113 |
| Table 5.1 | 126 |
| Table 5.2 | 129 |
| Table 5.3 | 131 |
| Table 5.4 | 138 |
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 1.1</td>
<td>13</td>
</tr>
<tr>
<td>Fig. 2.1</td>
<td>47</td>
</tr>
<tr>
<td>Fig. 2.2</td>
<td>50</td>
</tr>
<tr>
<td>Fig. 2.3</td>
<td>51</td>
</tr>
<tr>
<td>Fig. 2.4</td>
<td>54</td>
</tr>
<tr>
<td>Fig. 2.5</td>
<td>55</td>
</tr>
<tr>
<td>Fig. 2.6</td>
<td>58</td>
</tr>
<tr>
<td>Fig. 2.7</td>
<td>59</td>
</tr>
<tr>
<td>Fig. 2.8</td>
<td>62</td>
</tr>
<tr>
<td>Fig. 3.1</td>
<td>72</td>
</tr>
<tr>
<td>Fig. 3.2</td>
<td>80</td>
</tr>
<tr>
<td>Fig. 3.3</td>
<td>91</td>
</tr>
<tr>
<td>Fig. 3.4</td>
<td>92</td>
</tr>
<tr>
<td>Fig. 3.5</td>
<td>97</td>
</tr>
<tr>
<td>Fig. 4.1</td>
<td>111</td>
</tr>
<tr>
<td>Fig. 4.2</td>
<td>112</td>
</tr>
<tr>
<td>Fig. 4.3</td>
<td>114</td>
</tr>
<tr>
<td>Fig. 5.1</td>
<td>120</td>
</tr>
<tr>
<td>Fig. 5.2</td>
<td>132</td>
</tr>
<tr>
<td>Fig. 5.3</td>
<td>134</td>
</tr>
<tr>
<td>Fig. 5.4</td>
<td>135</td>
</tr>
<tr>
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<td>139</td>
</tr>
<tr>
<td>Fig. 5.6</td>
<td>140</td>
</tr>
<tr>
<td>Fig. 5.7</td>
<td>141</td>
</tr>
<tr>
<td>Fig. 5.8</td>
<td>142</td>
</tr>
</tbody>
</table>
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<td>AMV-RT</td>
<td>Avian myeloblastosis virus reverse transcriptase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AUB</td>
<td>Abnormal uterine bleeding</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5'-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribose nucleic acid</td>
</tr>
<tr>
<td>c-erbB2</td>
<td>Human epidermal growth factor</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary ribonucleic acid</td>
</tr>
<tr>
<td>DAB</td>
<td>3',3'-Diaminobenzidine</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DES</td>
<td>Desogestrel</td>
</tr>
<tr>
<td>DMEM:F12</td>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-di-amine-tetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>ER-alpha</td>
<td>Estrogen receptor subtype alpha</td>
</tr>
<tr>
<td>ER-beta</td>
<td>Estrogen receptor subtype beta</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin Growth Factor</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>Insulin-like growth factor binding protein 1</td>
</tr>
<tr>
<td>IMB</td>
<td>Inter menstrual bleeding</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirit</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In-vitro</em> fertilisation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IVT</td>
<td><em>In-vitro</em> transcription</td>
</tr>
<tr>
<td>k-Ras</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LNG</td>
<td>Levonorgestrel</td>
</tr>
<tr>
<td>LNG-IUS</td>
<td>Levonorgestrel releasing intra uterine system</td>
</tr>
<tr>
<td>MPA</td>
<td>Medroxyprogesterone acetate</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCB</td>
<td>Post coital bleeding</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMB</td>
<td>Postmenopausal bleeding</td>
</tr>
<tr>
<td>PR-A</td>
<td>Progesterone receptor subtype A</td>
</tr>
<tr>
<td>PR-B</td>
<td>Progesterone receptor subtype B</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA Integrity number</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SHG</td>
<td>Sonohysterography</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TVS</td>
<td>Transvaginal ultrasound scan</td>
</tr>
<tr>
<td>TX</td>
<td>Tamoxifen</td>
</tr>
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</table>
1 Endometrial Polyps: A Literature Review

1.1 Polyps

Endometrial polyps are described as sessile or pedunculated projections of the endometrium (1). A polyp forms a circumscribed mass composed of varying amounts endometrial glands and thick walled blood vessels in a fibrotic stroma covered by epithelium. Characteristically, polyps have a central fibro-vascular core and according to the structure of glands within an endometrial polyp, these lesions can be classified histologically as ‘glandular’, ‘glandulo-cystic’, ‘adenomatous’, and ‘fibrous-cystic’ (2). Polyps can also be classified according to size and location within the endometrial cavity (3). Due to advancements in ultrasound and hysteroscopic technology enabling direct visualisation of the endometrial cavity, endometrial polyps are an increasingly common finding. In 1953, Scott published an article entitled “The Elusive Endometrial Polyp”, and concluded that “the endometrial polyp remains an enigma as far as frequency, bleeding potential and possibility of malignant transformation are concerned” (4).

Figure 1.1

Photographs of endometrial polyps taken at hysteroscopy at Leicester Royal Infirmary demonstrating the variation in size, shape and macroscopic appearance of endometrial polyps.
1.2 Symptoms

Endometrial polyps are often identified in women presenting with heavy periods, irregular periods, inter-menstrual bleeding or postmenopausal bleeding, which suggests that polyps may contribute to these symptoms. In support of this is the finding of tentative evidence that polypectomy may improve symptoms of abnormal bleeding (5). However, the incidence of polyps in some studies involving asymptomatic women is similar to that reported in symptomatic premenopausal women, although a recent population study from Denmark showed a lower incidence of abnormal bleeding in women with polyps compared to women without polyps (6).

It may seem intuitive that larger or more numerous polyps are more likely to cause abnormal bleeding but no significant association has been demonstrated between polyp size, number or site within the cavity and the presence of symptoms. Additionally, histological features identified in benign polyps have been shown to bear little relation to clinical signs and symptoms and have no implications regarding prognosis (7, 8). However, in the presence of abnormal uterine bleeding, and regardless of HRT use, co-existing diabetes or hypertension, endometrial polyps appear more likely to be malignant (9, 10).

Polyps have been implicated in the aetiology of infertility and miscarriage, being identified in 15% of 235 infertile women compared to an incidence of 3% in a control group of 31 fertile women requesting reversal of sterilisation (11). Sub fertility in women with endometrial polyps may arise due to blockage of sperm passage or embryo implantation.

1.3 The scale of the problem

The prevalence of endometrial polyps is difficult to ascertain. Firstly, because much of the literature refers to retrospective series with poorly defined study population. Secondly, many published series are not primarily concerned with reporting the prevalence of polyps. Thirdly, a range of diagnostic techniques have been used. Older
series have relied on a histological diagnosis of endometrial polyps in biopsy material obtained at dilatation and curettage, a technique likely to miss a significant proportion of polyps. The introduction of hysteroscopy, allowing direct visualisation of the uterine cavity, led to a higher reported incidence of intrauterine abnormalities than previously published \((12, 13)\); sonohysterography allows more accurate diagnosis of polyps compared to ultrasound alone. The use of such expensive and invasive diagnostic tests however is difficult to justify in an asymptomatic population where no clinical indication for their use exists. Hysteroscopy with resection allowing for confirmatory histological analysis is widely accepted as the gold standard for the diagnosis of endometrial polyps \((14, 15)\).

Table 1.1 summarises the available published series and suggests that the prevalence of polyps may be higher in symptomatic compared to asymptomatic women. Two studies reported the incidence of polyps in a small group of asymptomatic women. The first reported a 10% incidence of polyps in 100 premenopausal asymptomatic volunteers who were investigated after exclusion of women with prolonged bleeding from a group selected through advertisement \((16)\). This contrasts with the much lower incidence (1.2%) reported in premenopausal women undergoing hysteroscopic sterilisation \((17)\).

A population study from Denmark where women aged between 20 and 74 years were invited to undergo transvaginal ultrasound scan and sonohysterography found that more women (82%) who had histologically verified polyps were asymptomatic than symptomatic \((6)\).

Polyps are observed to be more frequent in older premenopausal women compared to younger women \((16)\). Of the 619 women evaluated in the Danish study, polyps were detected in 5.8% of pre- and 11.8% of post-menopausal women \((6)\). In asymptomatic premenopausal women the prevalence of polyps was 7.6%, while it was 13% in asymptomatic postmenopausal women. In this study, polyps were rare (0.9%) in women below the age of 30.
The prevalence of polyps in premenopausal women presenting with abnormal bleeding varied from 13% to 32.5% (16, 18). In the study by Nagele et al., the prevalence in pre and postmenopausal women presenting with menorrhagia, intermenstrual bleeding or postmenopausal bleeding was 11.3% (19). The lower estimate of 7.3% provided by Anastasiadis et al. may be due to their reliance on curettage for diagnosis (20). In women with postmenopausal bleeding, the prevalence of polyps was reported as 21% (19, 21). However, Anastasiadis et al. reported a prevalence of 10.3%, approximately half that seen in the other two studies (20). Again, this is likely to be due to the fact that polyps were diagnosed on the basis of curettage specimens. These data suggest that the prevalence of polyps in pre- and postmenopausal women is different and critically dependent upon the method of diagnosis.
### Table 1.1

A summary of the literature regarding the prevalence of endometrial polyps

**Abbreviations:** Pre = premenopausal; Post = postmenopausal; N = number of patients; HRT = hormone replacement therapy; AUB = abnormal uterine bleeding; IMB = intermenstrual bleeding; PCB = post coital bleeding; PMB = postmenopausal bleeding; TVS = transvaginal ultrasound; SHG = sonohysterography; +/- = with and without.

<table>
<thead>
<tr>
<th>Menopausal status</th>
<th>N</th>
<th>Clinical indications</th>
<th>Sampling technique</th>
<th>% with polyps</th>
<th>Ref</th>
</tr>
</thead>
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<tr>
<td>Pre and post</td>
<td>1415</td>
<td>AUB excluding women on HRT, the pill, or tamoxifen</td>
<td>Curettage</td>
<td>Pre: 7.3% Post: 10.3%</td>
<td>(20)</td>
</tr>
<tr>
<td>Pre</td>
<td>162</td>
<td>AUB</td>
<td>Hysteroscopy</td>
<td>10.5%</td>
<td>(22)</td>
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<tr>
<td>Post</td>
<td>510</td>
<td>Breast cancer</td>
<td>TVS +/- Hysteroscopy</td>
<td>16.7%</td>
<td>(23)</td>
</tr>
<tr>
<td>Pre</td>
<td>80</td>
<td>AUB</td>
<td>SHG</td>
<td>32.5%</td>
<td>(16)</td>
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<tr>
<td>100</td>
<td>Asymptomatic volunteers</td>
<td></td>
<td></td>
<td>10%</td>
<td></td>
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<tr>
<td>Pre</td>
<td>323</td>
<td>Asymptomatic requesting sterilization</td>
<td>Hysteroscopy</td>
<td>1.2%</td>
<td>(17)</td>
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<tr>
<td>Pre and post</td>
<td>686</td>
<td>Population study age 20-74</td>
<td>TVS and SHG</td>
<td>Pre: 5.8% Post 11.8%</td>
<td>(6)</td>
</tr>
<tr>
<td>Pre</td>
<td>433</td>
<td>AUB</td>
<td>TVS +/- SHG</td>
<td>13%</td>
<td>(18)</td>
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<tr>
<td>Pre and post</td>
<td>1468</td>
<td>Not specified</td>
<td>Hysteroscopy</td>
<td>7.1%</td>
<td>(24)</td>
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<tr>
<td>Pre and post</td>
<td>1120</td>
<td>AUB</td>
<td>Hysteroscopy</td>
<td>10%</td>
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<tr>
<td>501</td>
<td>AUB</td>
<td>Hysteroscopy</td>
<td>11.2%</td>
<td></td>
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<td>IMB or PMB</td>
<td>Hysteroscopy</td>
<td>16.8%</td>
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<td>Check after hysteroscopic surgery</td>
<td>Hysteroscopy</td>
<td>2.3%</td>
<td>(19)</td>
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<td>76</td>
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<td>3.9%</td>
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<td>29</td>
<td>Recurrent abortion</td>
<td>Hysteroscopy</td>
<td>Not reported</td>
<td></td>
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<td>72</td>
<td>Other</td>
<td>Hysteroscopy</td>
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<td>179</td>
<td>PMB</td>
<td>Hysteroscopy</td>
<td>21.2%</td>
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<tr>
<td>Pre and post</td>
<td>393</td>
<td>AUB</td>
<td>Hysteroscopy</td>
<td>14.5%</td>
<td>(25)</td>
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<tr>
<td>Pre and post</td>
<td>1305</td>
<td>‘Symptomatic’ women, selection criteria not reported</td>
<td>Curettage</td>
<td>23.8%</td>
<td>(21)</td>
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</table>
1.4 Risk of Malignancy

Endometrial polyps are believed to be a risk factor for endometrial cancer. They have been observed to be present in 12-34% of uteri containing endometrial cancer (26, 27). Peterson and Novak proposed that for an endometrial polyp to be considered as the origin of a primary endometrial cancer, the base (stalk) of the polyp and the surrounding endometrium must be free of malignancy (26). They observed that primary malignant transformation in an endometrial polyp is a rare occurrence and more recent literature seems to concur. These criteria, however, may underestimate the risk of malignancy in a polyp once the cancer has spread.

It is widely acknowledged that the majority of polyps are benign and whilst the natural history of these lesions is poorly understood, it has also been observed that small polyps do spontaneously regress (28). Despite these observations, the impetus to remove endometrial polyps once diagnosed is largely due to their risk of malignancy.

Studies that report the risk of malignancy in endometrial polyps based on curettage sampling are likely to have underestimate the true figure because of missed lesions. Additionally, it can be difficult to be certain of the origin of malignancy diagnosed by curettage. Only hysterectomy or hysteroscopic excision allow for removal of the entire polyp with its stalk leaving the adjacent endometrium intact, so that the exact origin of malignancy can be ascertained.

A recent systematic review that sought the examine the oncogenic potential of endometrial polyps examined 17 studies published between 1999 and 2009 that altogether examined over ten thousand women undergoing polypectomy (10). They found the overall rate of malignancy (which includes the risk of atypical hyperplasia) amongst the women studied was 3.6%, with a rate of 1.7% amongst premenopausal women and 5.42% amongst postmenopausal women (relative risk 3.86; 95% CI 2.92-5.11). The risk of malignancy was significantly higher amongst postmenopausal women.
with abnormal uterine bleeding compared to asymptomatic postmenopausal women (4.47% relative risk 3.36 95% CI 1.45-7.80).

The risk of a concurrent endometrial carcinoma where atypical endometrial hyperplasia is present is high and this is also thought to be true where atypical hyperplasia is diagnosed in an endometrial polyp (29, 30). Mittal et al. examined 31 endometrial polyps that contained either adenocarcinoma, simple or complex atypical hyperplasia as well as the uterus subsequently removed at hysterectomy (31). In 66% of cases of complex atypical hyperplasia in polyps, there was either complex atypical hyperplasia or adenocarcinoma in non-polyp endometrium and in 90% of cases where polyps contained adenocarcinoma there was complex atypical hyperplasia or adenocarcinoma in the non-polyp endometrium.

The risk of malignancy associated with endometrial polyps therefore is likely to be low, but is associated with increasing age and menopausal status. Hyperplasia within a polyp has been demonstrated to be a strong marker of a wider endometrial pathology and when identified in postmenopausal women should lead to prompt consideration of hysterectomy.

1.5 Risk factors for endometrial polyps

A number of factors are thought to be associated with an increased risk of developing endometrial polyps. Because of the fact that polyps are rarely seen before menarche, and that they are more common in the peri-menopause it has been suggested that sex steroids may be involved in their aetiology. Additionally, exogenous estrogens, specifically tamoxifen and those used in hormone replacement therapy (HRT) may be initiators or promoters of polyp formation.

1.5.1 Tamoxifen

Tamoxifen is a triphenylethylene derivative and a selective estrogen receptor modulator. It was first described in 1966 and has been used in the treatment of breast
cancer for over 30 years (32). It is as a highly effective adjuvant treatment for women with hormone receptor positive breast cancer where five years of tamoxifen treatment reduces annual recurrence rate by nearly half and breast cancer mortality by a third compared to control groups (33). Whilst 5 years of treatment appears to be the optimal duration, the beneficial effects of treatment persist for 10 years after its cessation (33). Tamoxifen has also been shown to be beneficial for women with metastatic breast cancer, as well as being an effective prophylactic treatment for women at high risk of developing the disease (33, 34).

Estrogen receptor signalling is a promoter of tumour proliferation in up to 75% of breast cancers and tamoxifen is effective because it acts as a competitive inhibitor of the estrogen receptor (35). It binds to and induces conformational changes that prevent interaction of the receptor with co-activator proteins (36). In doing so it interrupts key growth factor pathways required for cell proliferation (37).

Although tamoxifen inhibits breast cancer growth via an anti-estrogenic mechanism, and the vasomotor symptoms associated with its use also supports an anti-estrogenic mechanism of action, it exerts estrogenic effects on the endometrium, vaginal epithelium, bone and on serum lipids in postmenopausal women. These effects account for its protective effects against both cardiovascular disease and osteoporosis in breast cancer patients (38, 39). Adverse effects include an increase in the occurrence of deep vein thrombosis or pulmonary embolism (40).

Despite tamoxifen’s beneficial effects on hormone receptor positive breast cancer, it is known to increase the risk of endometrial cancer (41). The increased risk in endometrial cancer is most marked in postmenopausal women and this is considered to be a function of tamoxifen’s estrogenic effect (42). A greater risk of aggressive uterine endometrial cancer subtypes has been observed in tamoxifen users, especially malignant mixed Mullerian tumours, papillary serous and clear cell tumours. The risk appears to increase with the duration of exposure to tamoxifen (43).
The use of tamoxifen is also associated with an increased frequency of ‘proliferative’ endometrial lesions including hyperplasia, polyps and cancer and the presence of these is often interpreted as evidence of the estrogenic effects of tamoxifen. Ismail reported polyps in 70% of tamoxifen treated uteri removed at hysterectomy (44). Endometrial polyps are the most common endometrial pathology associated with postmenopausal tamoxifen exposure, with a reported prevalence that varies between 8 and 36% and is higher in symptomatic (36%) than in asymptomatic (13%) women (45). Endometrial polyps associated with tamoxifen have a greater malignant potential than those found in women not on tamoxifen. Silva et al. observed that 77% of postmenopausal women who developed endometrial carcinoma after treatment with tamoxifen had endometrial polyps, whereas 34% of women in a comparable group who did not receive tamoxifen had endometrial polyps (46). The reported rate of malignant transformation in polyps of postmenopausal breast cancer and tamoxifen-treated patients is 3-11% (45).

Compared with ‘typical’ polyps, tamoxifen associated polyps are often larger, multiple and exhibit greater stromal fibrosis and appear more translucent (45). Tamoxifen treatment is associated with a number of features not seen in HRT-associated polyps or in ‘non-hormonally’ associated polyps, such as the presence of polarised glands that appear orientated along the long axis of the polyp. Tamoxifen treatment also produces ‘staghorn’ glands caused by focal crowding of the stroma and glandular clefting, a defined cambium layer due to stromal crowding around the glands, and frequent and diverse metaplasia (47).

1.5.2 Hormone replacement therapy

HRT with or without progestin has been associated with the development of new polyps and the growth of pre-existing polyps. The data from a number of studies are supportive of the role of the estrogen as a promoter of polyp formation, but a clear causal link is lacking due to poorly conducted studies. For example, in the study by Maia et al. 46% of women with abnormal uterine bleeding on four different HRT preparations (n=41) were found to have polyps (48). The incidence is greater than that observed in symptomatic
women not receiving HRT, but as these women were not assessed prior to starting HRT, a causative link between HRT use and polyp formation cannot be drawn.

Additionally, Van de Boesch et al. studied 238 women who had been taking oral HRT for an average of 23 months, with 64% having received sequential regimens comprising estrogen alone followed by estrogen and progestin in combination, 24% having received continuous-combined estrogen and progestin regimens and 12% having received continuous tibolone alone (49). All women underwent transvaginal ultrasound scanning and polyps were identified in 39 (16.3%) women, of whom 43.6% were asymptomatic. The incidence is not dissimilar to the prevalence of polyps in women of similar age who are not taking HRT, and again as no baseline examinations were done prior to commencing HRT, it is not possible to examine the question of causation.

More recently, Oguz et al. reported on a randomised double blind trial to evaluate the effect of three different ‘bleed-free’ HRT regimens (50). Postmenopausal women (n=374) who had no transvaginal ultrasound evidence of endometrial pathology were randomised to receive either 0.625mg conjugated equine estrogen with 2.5mg medroxyprogesterone (Premelle), 2mg estradiol with 1mg norethisterone (Kliogest) or 2.5mg tibolone (Livial). Participants underwent outpatient hysteroscopy after 18 months and every 6 months thereafter over a 3 year follow-up period; 17 new endometrial polyps (4.53%) were identified – mostly detected after 30 months of therapy. There were significantly more polyps noted in the Kliogest group compared with either the Premelle group or the Livial group, suggesting a relationship to the type and dose of steroids used. The overall low incidence of polyps is difficult to explain, but it is notable that they used a 1.2mm microhysteroscope; no data was provided on the percentage of satisfactory hysteroscopic examinations (50).

There is evidence to suggest that tibolone may induce endometrial polyp formation. More polyps have been observed amongst women who experience unscheduled bleeding whilst on tibolone compared to women with spontaneous postmenopausal bleeding (51, 52). In addition, significantly more new polyps developed amongst a
tibolone taking group compared with a group taking 1mg estradiol and 0.5mg norethisterone over a 3 year study period reported by Perez-Medina et al. (53).

The THEBES multi-centre study randomised 3240 women to receive either tibolone 1.25mg or 2.5 mg daily, or continuous-combined oral conjugated equine estrogens 0.625mg with medroxyprogesterone acetate 2.5mg (CEE/MPA). In the first year, endometrial polyps were found in 33 (2.6%) women in the combined tibolone group and 40 (3.1%) in the CEE/MPA group, the corresponding figures during the second year were 24 (2.5%) and 25 (2.5%). (54) This data appears to support the endometrial safety of tibolone, but as the study relied on histological assessment of blind endometrial pipelle suction curettes, the incidence of polyps is likely to be an underestimate.

1.5.3 Other factors

There is some evidence for an association between the occurrence of endometrial polyps and obesity, hypertension (50, 55).

1.6 Pathogenesis

A number of theories of endometrial polyp pathogenesis have been studied.

1.6.1 Aromatase

Aromatase (CYP19A1) is a P450 enzyme responsible for the conversion of C19 steroids into estrogen and is a major contributor to circulating estrogen concentrations in postmenopausal women (56). Deregulated aromatase expression has been suggested as a pathogenic feature in a number of estrogen-dependent disorders, including endometrial cancer (57, 58), adenomyosis (59), endometriosis (60-62) and fibroids (63, 64); its presence has also been demonstrated in endometrial stromal cells throughout the menstrual cycle (65, 66). Maia et al. compared expression of aromatase in polyps and the surrounding endometrium with that of ‘disease-free’ endometrium in 118 pre-menopausal women undergoing hysteroscopy as part of a diagnostic work-up for in-vitro fertilisation (IVF) treatment (67). The percentage of aromatase positive cases in
disease free endometrium was significantly lower than that observed in endometrial polyps. On the other hand, Pal et al. recently demonstrated heterogeneity in mRNA expression of CYP19A1 in endometrial biopsies taken during the follicular phase from three uterine sites (polyp, adjacent endometrium and remote endometrium) in premenopausal women (68). This suggests that the pathogenesis of some endometrial polyps may be independent of an aromatase-dependent estrogen excess, and that other mechanisms may be involved.

1.6.2 Origins in the Basalis Layer

Several authors raised the possibility that endometrial polyps in premenopausal women originate from the basalis layer. In 1954, Schroeder noted the presence of basal adenomas in the uterus that extend up thought the functioning layer to form polyps (69). Since these were composed of refractory basal endometrium, their architecture did not conform to the phase of the menstrual cycle. Shortly after, Peterson and Novak examined 1100 polyps, and found a histological pattern at variance with the menstrual cycle in the majority (60%) of cases (26) More recently, Reslova et al. developed this observation and proposed the existence of a ‘functional’ polyp that resembles its surrounding endometrium and a ‘hyperplastic’ polyp, derived from basal endometrium, which is dependent on estrogen for its development but refractory to the actions of progesterone (55).

The theory that endometrial polyps originate in the basal endometrium is also supported by the higher expression of the basal and reserve cell marker P63 membrane protein in polyps obtained from postmenopausal women compared to the surrounding endometrium (70). Since P63 is being used as a marker of uterine ‘stem cells’ (71), this suggests a ‘stem cell’ origin for the forming polyp and the possibility of clonal cell growth.

1.6.3 Monoclonality

Cytogenetic investigations of endometrial polyps have demonstrated the presence of non-random structural changes. Walter et al. studied the chromosomal features of a
single endometrial polyp, and found that 6 out of 20 cells showed an inv(12)(p11.2q13) chromosomal translocation. All of the other 14 cells had a normal female karyotype (72). The breakpoint at 12q13 lies in an area that has been shown to be involved in a variety of benign and malignant soft tissue tumours, suggesting that this chromosomal change could be associated with the aetiology of endometrial polyp formation.

Dal Cin et al. also found clonal chromosome rearrangements in 57% of polyps, and divided the cytogenetic abnormalities into three groups; those displaying rearrangements in the 6p21-p22 region, the 12q13-15 region, or the 7q22 region (73). Vanni et al. observed a t(6;14)(p21;q24) translocation in 3 endometrial polyps that all had diffuse stromal hyperplasia (74). The suggestion here is that these chromosomal rearrangements confer a growth or survival advantage for such clones.

Cells with a growth advantage grow at the expense of their neighbours forming clonal clusters derived from a common progenitor (75). Jovanovic et al. used inherited X-linked polymorphism and the Lyon principle (76) to study hysterectomy specimens that comprised adenocarcinoma, atypical hyperplasia and polyps, to assess whether ‘precursor’ lesions could be recognised by their monoclonal growth patterns and non-random X chromosome inactivation (77). The study included three endometrial polyps in women with well-differentiated endometrial cancer. Although the three polyps examined were monoclonal, the pattern of X inactivation skewing was opposite to that noted in the associated cancer, which suggested that polyps might not be precancerous. However, this was conclusion was based on a relatively small number of samples.

**1.6.4 Human epidermal growth factor receptor 2 (c-erbB2)**

Over-expression of the oncogenic human epidermal growth factor receptor gene c-erbB2 has been documented in various genital tract malignancies, and *in-vitro* studies have shown that c-erbB2 protein and mRNA levels increase in response to estrogen (78, 79). The exact role of c-erbB2 in the pathogenesis of endometrial polyps remains controversial. Maia et al. investigated the expression of this receptor in endometrial
polyps of postmenopausal women and found that c-erbB2 was over-expressed in 80% of polyps compared to the surrounding endometrium, and was confined to glandular epithelium being largely absent in stroma and that there was a positive correlation between the proliferation marker Mib-1 (Ki-67) and c-erbB2 expression (80). However, Inceboz et al. also studied c-erbB-2 expression in endometrial polyps and endometrium from postmenopausal women and found no difference in its expression between the two groups (81).

### 1.6.5 Kirsten ras proto-oncogene (K-ras) Mutation

K-ras encodes a GTPase protein (P21) located on inner plasma cell membrane that is involved in cell receptor signal transduction pathways (82). Mutations of the K-ras proto-oncogene have been detected in 15-30% of type 1 endometrial carcinomas and are thought to be correlated with phenotypic progression from complex atypical hyperplasia to endometrial carcinoma (83). Tamoxifen exposure, particularly amongst postmenopausal women with breast cancer, is associated with a greater risk of malignant degeneration of endometrial polyps, and Haschisuga et al. identified mutations in codon 12 of the K-ras gene in 7 out of 11 (64%) of tamoxifen related endometrial polyps (84). This incidence was greater than the incidence of the same mutation in ‘sporadic’ hyperplasia, suggesting that this K-ras mutation may be involved in the survival of endometrial polyps.

### 1.6.6 Steroid Receptors

The role of estrogen and progesterone in the pathogenesis or prevention of polyps remains unclear. There is evidence that these hormones influence endometrial polyps as estrogen receptors and progesterone receptors are found within polyps (85). It may be assumed that estrogen receptor mediates the pro-proliferative effect of estrogen, whilst reduced progesterone expression may render the polyp less responsive to progesterone differentiation, decidualisation and shedding. The resultant persistence of polyps may in turn increase its susceptibility to mutations, and potential malignant transformation.
Methodological differences make comparison between published studies on estrogen and progesterone receptor expression in endometrial polyps from premenopausal women difficult, and no published research has addressed the distribution of receptor subtypes in this group. Additionally, the cyclical changes in the endometrium renders studies of that tissue challenging and only a small proportion of endometrial polyps are hormone-responsive and show cyclical histological changes.

1.6.7 Proliferation and Apoptosis

Proliferation and apoptosis are relevant to the development and growth of endometrial polyps. In estrogen-responsive tissue, these processes are mediated in part by steroid hormones via their receptors (86-88). Risberg et al. reported that endometrial polyps had lower Ki-67 scores compared to proliferative, hyperplasic or malignant endometrium, but not secretory endometrium (89). In that study, expression in polyps of the anti-apoptotic protein B-Cell Lymphoma 2 (Bcl-2) was intense both in the glands and in the stroma. This was evident in both polyps with many glands (hyperplasic) and in those with a minor glandular component (atrophic), although few exceptional markedly dilated glands were Bcl-2-negative. Unfortunately, the polyps studied were heterogeneous with regard to menopausal status and phase of the menstrual cycle making interpretation more difficult.

Maia et al. demonstrated that Ki-67 and Bcl-2 expression in both polyps and normal endometrium vary concordantly throughout the menstrual cycle (90). The study by Taylor et al. support this finding, but only in part (91). They found no difference in Ki-67 expression when comparing polyps from premenopausal women with endometrium obtained from the corresponding phase of the menstrual cycle. However, they found that polyps taken from the proliferative phase had significantly elevated Bcl-2 expression compared with proliferative endometrium.

Inceboz et al. reported that Ki-67 and Bcl-2 were higher in both glands and stroma of polyps compared to postmenopausal endometrium (81). McGurgan et al. reported that
Ki-67 was statistically significantly higher in the stroma, but not in the glands and they found no difference in Bcl-2 expression when comparing polyps obtained from pre- and postmenopausal women (92). Unfortunately, these studies did not report the proliferation index and relied on more subjective immunohistochemical scores, thereby making these data difficult to compare with the preceding studies.

The expression of Ki-67 and Bcl-2 in polyps removed from women treated with tamoxifen is also controversial. Altaner et al. reported higher Ki-67 but not Bcl-2 in tamoxifen polyps compared to polyps from postmenopausal women, whilst the reverse was reported by McGurgan et al. (92, 93).

1.7 Prevention of polyps

Attempts to prevent polyps are limited to the use of the levonorgestrel intra-uterine system (LNG-IUS) in women on tamoxifen. A randomised controlled trial has shown that in postmenopausal women already on tamoxifen, 12 months of treatment with the LNG-IUS results in an endometrial decidual response and an inhibitory effect on polyp formation (94). Long term follow-up (average 26 months) of the same trial cohort (n=122) has, however, demonstrated that although there were 8 new polyps that developed in the control group, and 3 in the intervention group, the difference did not reach statistical significance (95). A recently published systematic review from 2015 using data from Gardner et al. and others has confirmed that the LNG-IUS does inhibit polyp formation in women using tamoxifen (96).

The mechanism whereby progestins interact with endometrial cells to inhibit tamoxifen-induced polyp formation is currently being investigated. Decidualisation may have a role. Whether or not the LNG-IUS can also prevent polyp formation in women who are not taking tamoxifen remains unanswered.
1.8 Conclusion

Endometrial polyps are commonly encountered in gynaecological practice although their exact prevalence is unknown. They may be identified incidentally or present with symptoms of abnormal uterine bleeding and serve as a risk factor for malignant transformation. Treatment by hysteroscopic resection has been shown to be effective at relieving symptoms, but the optimal management of the asymptomatic polyp is still uncertain. The pathogenesis and mechanisms leading to polyp formation and effective treatment are uncertain and require more investigation. Over the last 50 years our understanding of endometrial polyps has advanced, but Scott may still be correct and the endometrial polyp still in many ways, remains elusive (4).
1.9 Hypotheses and Experimental Approaches

The following hypotheses will be examined in this thesis:

Firstly, endometrial polyps from women with and without tamoxifen treatment will demonstrate similar patterns on estrogen and progesterone receptor isoform expression when compared with endometrium of the same tamoxifen status. Despite the phenotypic differences that exist between tamoxifen polyps and benign postmenopausal polyps, these lesions develop within the endometrium via similar mechanisms. This hypothesis will be tested using immunohistochemistry studies of estrogen and progesterone receptor isoform expression (ER-alpha, ER-beta, PR-A and PR-B) within tamoxifen polyps and tamoxifen endometrium, benign postmenopausal endometrial polyps, and postmenopausal endometrium.

Secondly, the formation of endometrial polyps in women with and without tamoxifen treatment is triggered in the endometrium by modulation of a common subset of ‘polyp genes’. These genes can be identified by comparing tamoxifen polyps with tamoxifen endometrium, and benign postmenopausal polyps with benign postmenopausal endometrium. This hypothesis will be tested using microarray analysis of tamoxifen polyps and tamoxifen endometrium, and postmenopausal endometrial polyps and postmenopausal endometrium. Findings will be validated using quantitative RT-PCR.

Thirdly, tamoxifen primed postmenopausal endometrium undergoes decidualisation with LNG-IUS treatment. This hypothesis will be tested using immunohistochemistry and ELISA studies of IGFBP-1 expression in endometrial biopsies and serum taken from women receiving tamoxifen as adjuvant therapy for breast cancer and those that also have the LNG-IUS in situ.

Fourthly, decidualisation of tamoxifen primed stromal cells in-vitro with levonorgestrel, medroxyprogesterone acetate and desogestrel results in the inhibition of key genes
responsible for polyp formation. This hypothesis will be tested using an *in-vitro* model for examining the paracrine effects of tamoxifen and levonorgestrel with that of tamoxifen and medroxyprogesterone acetate and/or tamoxifen and desogestrel on decidualisation and the expression of polyp genes within endometrial and polyp stromal cells.
## 2.1 Background

Endometrial polyps are a frequently observed endometrial pathology in postmenopausal women. They are associated with abnormal uterine bleeding, and may serve as a risk factor for endometrial cancer. Despite their prevalence, the pathogenesis of these lesions remains unclear. However, the observed influence of tamoxifen, menopausal status and HRT suggests that a hormonal stimulus is likely to influence polyp formation.

Estrogen and progesterone are critical to the maintenance and function of the endometrium. Their effects are mediated by specific nuclear receptor proteins, ER-alpha, ER-beta, PR-A and PR-B which are present in endometrial stromal and epithelial gland cells (97). The expression of these receptors in the glandular and stromal compartments is known to vary throughout the normal menstrual cycle and fluctuate in response to changes in the hormonal environment. In the functionalis layer, glandular ER-alpha, ER-beta and PR expression is high during the proliferative phase, and reduces in the secretory phase. Stromal ER-alpha and PR expression is high during the proliferative phase, and reduces in the secretory phase. Stromal ER-beta expression has been shown to remain unaltered (97-99). In contrast, ER-alpha ER-beta and PR expression have been shown to remain unchanged in the basalis layer (100). After the menopause, as estradiol levels fall, ER-alpha, ER-beta, PR-A and PR-B expression levels fall compared to that of the proliferative endometrium (101).

Tamoxifen, a selective estrogen receptor modulator, is known to be associated with a range of benign endometrial pathologies, including endometrial polyps, hyperplasia, endometrial cystic atrophy, adenomyosis and the growth of fibroids. In addition, it increases the risk of malignant transformation to endometrial carcinoma and uterine sarcoma (102). Endometrial polyps are the most commonly reported pathology associated with tamoxifen exposure (103). Polyps arising in tamoxifen treated women
tend to differ from those in women receiving no hormonal treatment. They are larger, more likely to be multiple, and have a translucent appearance with greater stromal fibrosis (45). Tamoxifen polyps have a greater risk of malignant transformation (10). The mechanisms behind the polyp inducing effects of tamoxifen are not well understood.

Expression of ER and PR in the endometrium has been shown to be altered in a range of pathological states including adenomyosis (104) and adenocarcinoma (105), and in response to tamoxifen treatment (106, 107). In addition, ER and PR expression in endometrial polyps has been reported in numerous studies, but findings from these studies vary, and differences in methodology make it hard to draw comparisons. The studies examining ER and PR expression using immunohistochemistry in polyps with and without tamoxifen are summarised in Table 2.1.

Five studies have investigated ER and PR distribution in polyp tissue in a range of hormonal environments. Firstly, McGurgan and Gul compared polyps from premenopausal women and postmenopausal women with no hormonal treatment (108, 109). McGurgan studied premenopausal polyps from the secretory phase of the menstrual cycle, whereas Gul studied premenopausal polyps from the proliferative phase. The differences in techniques for image analysis make these studies difficult to compare, however, both agree that ER and PR expression differs in polyps from these two groups. Secondly, Cohen and McGurgan studied the effect of tamoxifen treatment on polyps from postmenopausal women (92, 110). As with the previous two studies, these authors used different techniques for image analysis, however, they both observed that glandular ER expression was lower in tamoxifen polyps and concluded that this may be due to the weak estrogen-like effect of tamoxifen. Finally, Hachisuga studied only polyps from tamoxifen treated women and is the only study to examine the ER and PR receptor isoforms, ER-alpha, ER-beta PR-A and PR-B (84). The study sample comprised 10 postmenopausal polyps and 1 pre-menopausal polyp and concluded that the distribution of ER-alpha, PR-A and PR-B in all samples were consistent with the findings for hormone receptors in glandular epithelium of the proliferative endometrium. Unlike proliferative endometrium, however, they observed that ER-beta
expression was consistently lower than ER-alpha, and proposed that ER-alpha maybe a key receptor in the development of tamoxifen polyps.

Two studies have compared endometrium and polyps in pre-menopausal women. Firstly, Mittal used 14 curettage specimens that contained both polyp and endometrium within the same sample (111); 8 endometrial samples were proliferative and 8 secretory. However, only 5 of the 14 polyps showed a corresponding phasic response; the remainder were non-functional. Despite this, the authors found no significant differences in ER and PR expression between functional and non-functional polyps. They suggested that polyps may result from a decrease in ER and PR in stromal cells rendering these lesions relatively insensitive to cyclic hormonal changes causing decidualisation and shedding that affect the rest of the endometrium. Secondly, Taylor et al. observed significant differences in ER and PR expression in polyps compared with normal endometrium from the same menstrual cycle phase (91). In contrast to the previous study however, specimens were obtained from different patients and no distinction was made between functional and non-functional polyps. They concluded that significant differences exist between polyps and endometrium, and suggested that the reduced PR expression they observed in polyps could support the theory that polyps are relatively insensitive to the effects of progesterone.

Three studies have compared polyps and endometrium from postmenopausal women with no hormonal treatment. Two of these used polyp and endometrial tissue from the same patient (112, 113), whereas the third obtained samples from different patients (81). All found higher ER and PR expression in polyp glands and stroma compared to the endometrium and suggest that this increased sensitivity to steroid hormones is critical in the pathogenesis of polyps. Sant’Ana de Almeida et al. go on to suggest that high stromal ER expression in polyps supports trophic paracrine interaction between stromal and epithelial cells, with the mechanism of action for the induction of epithelial proliferation occurring via stromal ER (112). They also concluded that the higher steroid receptor expression seen in polyps is at odds with early events in endometrial carcinogenesis, where a loss in ER and PR expression occurs.
Lopes et al. are the only authors to have studied polyps and endometrium from both pre- and postmenopausal women (114). In all cases, polyp and endometrium was obtained from the same patient. In contrast to Gul et al. and McGurgan et al., they found that ER and PR expression in polyps and endometrium was similar in pre- and postmenopausal women. In keeping with Sant’Ana de Almeida et al., Belisario et al. and Inceboz et al., they found that glandular ER and PR expression was greater in polyps compared to endometrium (81, 112, 113).

Three studies have investigated differences in ER and PR expression in endometrium and polyps from postmenopausal women treated with tamoxifen (115-117). The tamoxifen polyps examined by Schwartz et al. included 4 polyps with adenocarcinoma, carcinoma in-situ, increased mitoses or atypia. However, despite this heterogeneity, they reported a pattern of reduced stromal ER expression and increased glandular PR expression that was a consistent with tamoxifen used regardless of the histological diagnosis. Dibi et al. found that tamoxifen polyps were more likely to express ER and PR than tamoxifen endometrium and concluded that this increased sensitivity to steroid hormones may play a role in polyp pathogenesis, as other authors have done (81, 112, 113). Leao et al. are the only authors to have studied ER and PR expression in polyps using tissue microarray (117). They compared tamoxifen polyps to postmenopausal endometrium from women with no hormonal treatment and found higher ER and PR expression in the polyps. Because of the nature of this comparison, it is not possible to conclude whether this is a tamoxifen effect, or a polyp effect. In addition, their findings of increased PR expression in the glands and stroma of tamoxifen polyps compared to non-tamoxifen polyps is in keeping with the observations of McGurgan et al. (92).

These studies demonstrate that there is altered ER and PR expression in polyps. However, only one study has examined ER-alpha, ER-beta PR-A and PR-B, and this was limited to a comparison between pre- and postmenopausal polyps from tamoxifen treated women. Given that these receptor isoforms have varying down-stream effects,
understanding their distribution within polyps compared with endometrium in postmenopausal women will give add to our understanding of how polyps develop.
Table 2.1

Studies examining ER and PR receptor expression in polyps and endometrium.

Abbreviations: PreM = premenopausal; PostM = postmenopausal; Tx = tamoxifen; NHT = no hormonal treatment; HRT = hormone replacement therapy; ER = estrogen receptor; PR = progesterone receptor; +ve = positive; EG = endometrial glands; ES = endometrial stroma; PG = polyp glands; PS = polyp stroma; E = endometrium; P = polyp

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<td>% +ve stained cells to nearest 5% and staining intensity graded 0 to 3. Values multiplied together to give score</td>
<td>ER and PR expression higher in PostM PG compared to PreM PG; ER expression higher in PostM PS compared to PreM PS; PR expression no different between PreM PS and PostM PS</td>
<td>Significant differences in ER and PR in PreM P and PostM P; P appear to be sensitive to their hormonal environment, exhibiting changes in ER and PR depending on menopausal status</td>
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<td>2009</td>
<td>Gul</td>
<td>25 polyps (PreM) All from proliferative phase 25 polyps (PostM, NHT)</td>
<td>Hysteroscopic polypectomy</td>
<td>ER PR</td>
<td>% +ve stained cells graded weak, moderate or strong</td>
<td>PR expression higher in PreM PS compared to PostM P; ER and PR expression higher in PostM PG compared to PostM PS</td>
<td>ER and PR expression patterns differ between PreM PG and PostM P, however this difference did not exist in PreM P</td>
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<td>Cohen</td>
<td>28 polyps (PostM, Tx) 14 polyps (PostM, NHT)</td>
<td>Hysteroscopy or hysterectomy</td>
<td>ER PR</td>
<td>Staining intensity graded from 0 to 3+</td>
<td>ER expression lower in Tx PG and Tx PS compared to NHT PG and NHT PS; No differences PR expression between Tx PG and Tx PS compared to NHT PG and NHT PS</td>
<td>Higher ER expression in NHT PostM PG compared with Tx PG supports Tx’s weakly estrogenic effect; Higher ER expression in NHT PostM PS compared with Tx PS suggests a unique Tx effect</td>
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<tr>
<td>Year</td>
<td>Author</td>
<td>Samples</td>
<td>Tissue retrieval</td>
<td>Receptors</td>
<td>Image analysis method</td>
<td>Key findings</td>
<td>Authors’ Conclusions</td>
<td>Ref</td>
</tr>
<tr>
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</tr>
<tr>
<td>2006</td>
<td>McGurgan</td>
<td>9 polyps (PostM, Tx) 16 polyps (PostM, NHT)</td>
<td>Polypectomy</td>
<td>ER PR</td>
<td>% +ve stained cells to nearest 5%, and staining intensity graded 0 to 3. Values multiplied together to give score</td>
<td>ER expression lower in Tx PG compared to NHT PG; PR expression higher in stroma of Tx PS compared to NHT PS</td>
<td>The contrasting effect of Tx on ER and PR may be due to its partial estrogen antagonist properties</td>
<td>(92)</td>
</tr>
<tr>
<td>2003</td>
<td>Hachisuga</td>
<td>1 polyp (PreM Tx) 10 polyps (PostM, Tx)</td>
<td>Hysteroscopic polypectomy</td>
<td>ER-alpha ER-beta, PR-A, PR-B</td>
<td>Staining intensity graded -ve, +, or ++</td>
<td>ER-alpha, ER-beta, PR-A and PR-B expression higher in PG compared to PS; ER beta expression lower than ER-alpha in PG</td>
<td>Findings of ER-alpha, PR-A and PR-B are similar to those reported in glandular epithelium of proliferative phase E in other studies; As ER-beta expression consistently lower than ER-alpha, ER-alpha may be a key receptor in the development of Tx P</td>
<td>(84)</td>
</tr>
<tr>
<td>1996</td>
<td>Mittal</td>
<td>14 polyp (PreM) 3 proliferative, 2 secretory, 9 non-functional 14 endometrium (PreM) 8 proliferative, 10 secretory 4 basalis endometrium (PreM)</td>
<td>Curettage specimens with endometrium and polyp in same sample</td>
<td>ER PR</td>
<td>% +ve stained cells to nearest 5% and staining intensity graded 1+ to 4+.</td>
<td>No difference in ER or PR expression in PG compared with EG; ER and PR expression lower in PS compared with ES; PR staining intensity lower in PS compared with ES; ER and PR in functional polyps similar to cycling endometrium; ER and PR higher in functional PS compared to non-functional PS</td>
<td>P may result from a decrease in ER and PR in stromal cells preventing it from undergoing decidual change and menstrual shedding; Functional portions of a P may represent overlying or entrapped E</td>
<td>(111)</td>
</tr>
<tr>
<td>Year</td>
<td>Author</td>
<td>Samples</td>
<td>Tissue retrieval</td>
<td>Receptors</td>
<td>Image analysis method</td>
<td>Key findings</td>
<td>Authors’ Conclusions</td>
<td>Ref</td>
</tr>
<tr>
<td>------</td>
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<td>-----</td>
</tr>
<tr>
<td>2003</td>
<td>Taylor</td>
<td>20 polyps (PreM) 10 secretory 10 proliferative 38 endometrium (PreM) 30 secretory 8 proliferative</td>
<td>Endometrial biopsies, no detail on how polyps obtained</td>
<td>ER PR</td>
<td>% +ve stained cells graded +, ++, ++++, ++++</td>
<td>PR expression lower in proliferative PG and PS compared to proliferative EG and ES; ER expression higher in secretory PG compared to secretory EG</td>
<td>Significant differences in ER and PR expression between P and E from same menstrual phase; Reduced PR expression in P could point to an insensitivity to progesterone</td>
<td>(91)</td>
</tr>
<tr>
<td>2004</td>
<td>Sant’Ana de Almeida</td>
<td>44 polyps (PostM, NHT) 44 Endometrium (PostM, NHT)</td>
<td>Hysteroscopic polypectomy and endometrial sample from same patient</td>
<td>ER PR</td>
<td>% +ve stained cells graded 1 to 4 and staining intensity graded 1 to 3. Final score was sum of the 2 grades</td>
<td>ER expression higher in PG and PS compared to EG and ES; PR expression higher in PS compared to ES</td>
<td>PG more sensitive to steroid hormones, and so can develop without high local estrogen levels; High ER expression in PS supports trophic paracrine interaction between stromal and epithelial cells; High ER and PR in P is at odds with the loss of ER and PR in endometrial carcinogenesis</td>
<td>(112)</td>
</tr>
<tr>
<td>2006</td>
<td>Belisario</td>
<td>35 polyps (PostM, NHT) 35 endometrium (PostM, NHT)</td>
<td>Hysteroscopic polypectomy and endometrial sample from same patient</td>
<td>ER PR</td>
<td>% +ve stained cells graded 0 to 4</td>
<td>ER and PR expression higher in PG and EG compared to PS and ES; ER expression higher in PG and PS compared to EG and ES</td>
<td>More intense expression of ER in P compared to E; P represent a local growth of E and arise due to overexpression of ER, with or without a reduced expression of PR</td>
<td>(113)</td>
</tr>
<tr>
<td>2006</td>
<td>Inceboz</td>
<td>36 polyps (PostM, NHT) 16 endometrium (PostM, NHT)</td>
<td>Polypectomy hysterectomy</td>
<td>ER PR</td>
<td>ER and PR expression higher in PG and PS compared to EG and ES</td>
<td>Estrogen may have a role in the development of PostM P</td>
<td></td>
<td>(81)</td>
</tr>
<tr>
<td>Year</td>
<td>Author</td>
<td>Samples</td>
<td>Tissue retrieval</td>
<td>Receptors</td>
<td>Image analysis method</td>
<td>Key findings</td>
<td>Authors’ Conclusions</td>
<td>Ref</td>
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<td>-----</td>
</tr>
<tr>
<td>1997</td>
<td>Schwartz</td>
<td>15 polyps (PostM, Tx)</td>
<td>Pipelle, curettage and hysterectomy</td>
<td>ER PR</td>
<td>% +ve stained cells to nearest 5% and staining intensity 1+ to 4+. Values multiplied to give score</td>
<td>PR expression higher in Tx EG compared with NHT EG; ER expression lower in Tx ES compared with NHT ES; ER expression lower in Tx PS compared with NHT PS</td>
<td>High levels E PR expression consistent with an estrogenic Tx effect. But reduced ER expression in Tx PS compared with NHT PS is a distinct Tx effect, independent of histological diagnosis</td>
<td>(115)</td>
</tr>
<tr>
<td>2009</td>
<td>Dibi</td>
<td>6 polyps (PostM, Tx)</td>
<td>Hysteroscopic polypectomy or curettage endometrial biopsy</td>
<td>ER PR</td>
<td>Receptor staining either +ve or -ve</td>
<td>ER and PR expression higher in P compared with E</td>
<td>High expression of ER and PR in PG suggests an increased sensitivity to steroid hormones (although results for glands not reported separately)</td>
<td>(116)</td>
</tr>
<tr>
<td>2013</td>
<td>Leao</td>
<td>84 polyps (PostM, Tx)</td>
<td>Tissue microarray</td>
<td>ER PR</td>
<td>% +ve cells graded 0 to 5 and staining intensity graded from 0-3. Values added together to give a final score</td>
<td>ER expression higher in Tx PG and Tx PS compared to NHT EG and EHT ES; PR expression higher in Tx PG compared to NHT EG; PR expression higher in Tx PG and Tx PS compared with NHT PG and NHT PS; ER expression lower in Tx PG and Tx PS compared with PostM polyps NHT PG and NHT PS</td>
<td>Tx P have higher ER and PR expression compared to NHT E. ER expression being lower in Tx P compared to NHT P in this study is at odds with other studies</td>
<td>(117)</td>
</tr>
</tbody>
</table>
2.2 Aim

The aim of this study was to characterise and compare ER-alpha, ER-beta, PR-A and PR-B distribution in polyps and endometrium from postmenopausal women treated with tamoxifen as adjuvant therapy for breast cancer using immunohistochemistry.
2.3 Materials and Method

2.3.1 Tissue collection

All samples were collected at Leicester Royal Infirmary, with approval from the Leicestershire Health Authority Ethics Committee (Ethics committee reference number REC 6498) after obtaining written consent. Study participants were all postmenopausal women, whose last menstrual period was at least 12 months prior to specimen collection.

Endometrial polyps were obtained from postmenopausal women who had no history of recent exogenous hormone exposure \((n=10)\) or from women who had been taking tamoxifen as adjuvant hormonal therapy for breast cancer for a minimum of 6 months \((n=9)\). All polyps were diagnosed hysteroscopically and removed by hysteroscopic laser polypectomy. After removal, each polyp was cut along its longitudinal axis. Half of the specimen was used for immunohistochemistry, and half was examined by a consultant histopathologist. All polyps were confirmed to be benign, showing no evidence of hyperplasia or malignancy.

Control tissue was obtained from uterine samples, comprising myometrium and endometrium measuring approximately 2cm by 2cm. Samples were obtained from the fundal region of the uteri of postmenopausal women with no recent exogenous hormone exposure \((n=8)\) or from women taking tamoxifen as adjuvant hormonal therapy for breast cancer for a minimum of 6 months \((n=25)\). Women were undergoing either abdominal hysterectomy for benign ovarian cysts or vaginal hysterectomy for prolapse. Using full thickness uterine sections for examination rather than endometrial sampling using curettage allowed for a more reliable examination of the endometrium. After the uterine section for immunohistochemistry was excised, the remainder was examined by a consultant histopathologist. All pathology was confirmed to be benign, with no evidence of cervical intra-epithelial neoplasia, endometrial hyperplasia or malignancy.
2.3.2 Immunohistochemistry

Tissue samples were fixed in 10% formalin at room temperature for 2 days before being processed and embedded into paraffin blocks. Sections (5 µm-thick) were cut from each block and mounted onto silane-coated glass slides for the purposes of immunohistochemistry.

Each antibody experiment contained positive control tissue where the presence of the receptor isoform had previously been confirmed to validate the assay. Negative control slides were run in parallel by using iso-type IgG at the same concentration and dilutions as the respective primary antibody counterparts. Details of the primary and secondary antibodies used, their diluents and the control tissue used for each experiment is summarised in Table 2.2 below.

Table 2.2

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer [IgG]</th>
<th>Dilution</th>
<th>Diluent</th>
<th>Control tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-alpha</td>
<td>Novocastra [60µg/ml]</td>
<td>1:50 [1.2µg/ml]</td>
<td>10% NRS in TBS 0.1% BSA</td>
<td>Proliferative endometrium</td>
</tr>
<tr>
<td>ER-beta</td>
<td>Genetex [1mg/ml]</td>
<td>1:500 [2µg/ml]</td>
<td>10% NRS in TBS 0.1% BSA</td>
<td>Vulval skin</td>
</tr>
<tr>
<td>PR-A</td>
<td>Novocastra [360µg/ml]</td>
<td>1:200 [1.8µg/ml]</td>
<td>10% NRS in TBS 0.1% BSA</td>
<td>Secretory endometrium</td>
</tr>
<tr>
<td>PR-B</td>
<td>Novocastra [40µg/ml]</td>
<td>1:240 [0.16µg/ml]</td>
<td>10% NRS in PBS</td>
<td>Secretory endometrium</td>
</tr>
<tr>
<td>Negative control</td>
<td>Mouse IgG</td>
<td>Vector [1mg/0.5ml]</td>
<td>As per primary antibody</td>
<td>As per primary antibody</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Biotinylated rabbit (Fab’): anti mouse</td>
<td>Dako</td>
<td>1:400</td>
<td>TBS 0.1% BSA</td>
</tr>
</tbody>
</table>
Slides were firstly dewaxed by incubating in xylene for 3 periods of 3 minutes and then rehydrated by incubating in 99% IMS for 3 minutes, 99% IMS for 3 minutes, 95% IMS for 3 minutes and deionised water for 3 minutes. Antigen retrieval was carried out in all cases by microwaving the slides in 10 mM citric acid buffer (pH 6.0) for 30 minutes at 800 Watts before being cooling and washing in running tap water for 5 minutes. Endogenous peroxidase activity was suppressed by incubating slides in 6% hydrogen peroxide for 10 minutes, after which slides were again washed in running tap water for 5 minutes.

In order to block non-specific binding sites, for ER-alpha and ER-beta and PR-A, slides were then immersed in TBS containing 0.1% bovine serum albumin (BSA, Sigma) (v/v; TBA) for 5 minutes, and normal rabbit serum (Dako) diluted 1:10 in TBA was applied. For PR-B, slides were washed in PBS containing 0.05% Tween20 (v/v) for 5 minutes, and normal rabbit serum diluted 1:10 in PBS was applied. All slides were incubated at room temperature for 30 minutes.

Endogenous avidin- and biotin-binding sites were blocked for ER-alpha, ER-beta and PR-A immunohistochemistry by applying Avidin blocking solution to all slides at a concentration of 4 drops Avidin per ml 10% normal rabbit serum in TBA. Slides were incubated at room temperature and then washed for 5 minutes in TBA. Biotin blocking solution was then added to all slides at a concentration of 4 drops Biotin per ml 10% normal rabbit serum in TBA and incubated at room temperature for 15 minutes. For PR-B immunohistochemistry, Avidin blocking solution was applied to all slides at a concentration of 4 drops Avidin per ml of 10% normal rabbit serum in PBS. Slides were incubated at room temperature and then washed for 5 minutes in PBS containing 0.05% Tween20. Biotin blocking solution was then added to all slides at a concentration of 4 drops biotin per ml in 10% normal rabbit serum PBS and incubated at room temperature for 15 minutes.

In all cases, the primary antibody, diluted in the same blocking solution supplemented with 10% normal rabbit serum was then applied to the test slides and positive control.
Mouse IgG (Vector) at a corresponding dilution was applied to the negative control slide. Slides were incubated at 4°C overnight in a humid chamber.

For ER-alpha, ER-beta and PR-A, slides were removed from the humid chamber and washed in TBA for 20 minutes. For PR-B, slides were washed in PBS Tween20.

Immunoreactive antigen-antibody complexes were then detected using a secondary biotinylated rabbit (Fab’)2 anti-mouse antibody (Dako). For ER-alpha and ER-beta studies this was diluted (1:400) in TBA, for PR-A in TBS, and PR-B in PBS. All slides were incubated for 30 minutes at room temperature. ER-alpha, ER-beta and PR-A stained slides were then washed in TBA and PR-B stained slides were washed in PBS Tween20 for 20 minutes.

ABC Elite immunoperoxidase system (Vector) was made up in PBS for ER-alpha and PR-B, and in TBS for ER-beta and P-RA and applied to all slides for 30 minutes. ER-alpha, ER-beta and PR-A stained slides were then washed in TBA for 20 minutes and PR-B stained slides were washed in PBS Tween20. Diaminobenzidine (DAB) substrate was used as the chromogen. This was made up according to the manufacturer’s protocol and applied to all slides for 5 minutes. Unreacted substrate was washed off in deionised water.

Slides were briefly counterstained with Mayer’s haematoxylin (Sigma), washed in tap water and then dehydrated with 95% IMS and twice with 99% IMS for 3 minutes each before being cleared in xylene for 3 minutes. Slides were permanently mounted (DPX mountant) with glass coverslips and allowed to dry before image analysis was undertaken.

2.3.3 Image Analysis

Image capture and analysis was performed using Axioplan 2 light microscope (Carl Zeiss) and colour camera (Sony DXC-151P). Digital image analysis was performed using Axiovision image analysis software (version 4.0, Carl Zeiss). Illumination settings were standardised throughout the experiment. Glandular and stromal compartments were
examined separately. For each specimen, ten randomly selected fields were captured at X400 magnification. The number of positive (brown stained) and negative (blue stained) glandular and stromal cells in randomly captured fields were recorded and the percentage of positively stained cells per field calculated.

2.3.4 Statistical Methods

The data were tested for normality using the D’Agostino-Pearson omnibus normality test and were found to have been sampled from a Gaussian distribution. Data are presented as the mean percentage of positively stained cells and standard error for each of the 8 groups (postmenopausal endometrium glands, postmenopausal endometrium stroma, postmenopausal polyp glands, postmenopausal polyp stroma, tamoxifen endometrium glands, tamoxifen endometrium stroma, tamoxifen polyp glands and tamoxifen polyp stroma. Differences between means were compared using a one-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons. Differences were considered statistically significant if $P < 0.05$. All analysis was carried out using Graphpad Prism (version 6.07).
2.4 Results

2.4.1 ER-alpha

ER-alpha immunostaining was detected in the glandular epithelium and stroma of the endometrium and polyp of both the postmenopausal and tamoxifen treated groups. The results are summarised in Figure 2.1 and Figure 2.2, and Table 2.3.

Figure 2.1

Photomicrographs showing ER-alpha distribution in endometrial polyps and endometrium from tamoxifen treated and postmenopausal women with no recent prior hormone exposure taken at X400 magnification.

Plate A = postmenopausal polyp; plate B = tamoxifen polyp; plate C = postmenopausal endometrium; plate D = tamoxifen endometrium
ER-alpha immunoreactivity was significantly lower in the stroma compared with the glands of the postmenopausal polyp (73.58% ± 3.98 vs 99.10% ± 0.39; mean ± SEM, p = 0.001), the tamoxifen polyp (68.58% ± 5.54 vs 98.96% ± 0.58 p = 0.001) and the tamoxifen endometrium (76.20% ± 5.65 vs 98.97% ± 0.27, p < 0.0001). There was no significant difference between stroma and glands in the postmenopausal endometrium, (91.81% ± 2.91 vs 92.64% ± 2.22).

When the ER-alpha immunoreactivity in the glandular compartments of the polyp were compared with endometrium, there was no significant difference seen in either the postmenopausal (99.10% ± 0.39 vs 92.64% ± 2.22) or tamoxifen groups (98.96% ± 0.58 vs 98.97% ± 0.27). Equally so in the stroma, ER-alpha immunoreactivity was not significantly different between the polyp and endometrium in either the postmenopausal (73.58% ± 3.98 vs 91.81% ± 2.91) or tamoxifen groups (68.58% ± 5.45 vs 76.20% ± 5.65).

Considering the effect of tamoxifen on ER-alpha immunoreactivity in the glands, no significant difference was seen in either the endometrium (92.64% ± 2.22 vs 98.97% ± 0.27) or the polyp (99.10% ± 0.39 vs 98.96% ± 0.58). Tamoxifen exposure did also not significantly affect ER-alpha immunoreactivity in the stroma of either the endometrium (91.81% ± 2.91 vs 76.20% ± 5.65) or the polyp in this cohort (73.58% ± 3.98 vs 68.58% ± 5.45).
Table 2.3  

ER-alpha immunostaining in glands and stroma of postmenopausal and tamoxifen endometrium and polyp. Values are the mean percentage of positively stained cells per 10 randomly selected high powered fields per specimen ± SEM

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Glands</th>
<th></th>
<th></th>
<th>Stroma</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Postmenopausal</td>
<td>Tamoxifen</td>
<td>Postmenopausal</td>
<td>Tamoxifen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>Mean % positively stained cells (± SEM)</td>
<td>N</td>
<td>Mean % positively stained cells (± SEM)</td>
<td>N</td>
<td>Mean % positively stained cells (± SEM)</td>
</tr>
<tr>
<td>ER-alpha</td>
<td>Endometrium</td>
<td>8</td>
<td>92.64 ± 2.22</td>
<td>18</td>
<td>98.97 i ± 0.27</td>
<td>8</td>
</tr>
<tr>
<td>Polyp</td>
<td>10</td>
<td>99.10 ii ± 0.39</td>
<td>9</td>
<td>98.96 iii ± 0.58</td>
<td>10</td>
<td>73.58 ii ± 3.98</td>
</tr>
</tbody>
</table>

One-way ANOVA with Tukey's honestly significant difference post-test for multiple comparisons

i p < 0.001
ii P = 0.001
iii P = 0.001
Graphs of ER-alpha immunoreactivity in the glands and stroma of endometrium and polyps from postmenopausal and tamoxifen treated women. Data are presented as the mean ± SEM of percentage of positively stained cells.

Abbreviations: e = endometrium; p = polyp; pm = postmenopausal; tx = tamoxifen; g = glands; s = stroma; * = groups with $p < 0.05$ when compared using one-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons.
2.4.2 ER-beta

ER-beta immunostaining, like that of ER-alpha was detected in both glandular epithelium and stromal compartments of the endometrium and polyp in both the postmenopausal and tamoxifen treated groups. The results are summarised in Figure 2.3 and 2.4, and Table 2.4.

**Figure 2.3**

Photomicrographs showing ER-beta distribution in endometrial polyps and endometrium from tamoxifen treated and postmenopausal women with no recent prior hormone exposure taken at X400 magnification.

Plate A = postmenopausal polyp; plate B = tamoxifen polyp; plate C = postmenopausal endometrium; plate D = tamoxifen endometrium
As with ER-alpha, ER-beta immunoreactivity was significantly lower in the stroma compared with that in the glands of both the postmenopausal polyp (18.77% ± 3.81 vs 97.04% ± 1.43, p < 0.0001) and the tamoxifen polyp (2.89% ± 0.85 vs 90.93% ± 2.53, p < 0.0001). However, unlike ER-alpha, ER-beta was also significantly lower in stroma compared with glands of tamoxifen endometrium (21.25% ± 1.54 vs 97.28% ± 0.59, p < 0.0001) and the postmenopausal endometrium (36.30% ± 4.99 vs 82.90% ± 4.59, p < 0.0001).

When the ER-beta immunoreactivity in the glandular compartment of the polyp were compared with endometrium, there was a significant difference seen in the postmenopausal group (82.90% ± 4.59 vs 97.04% ± 1.43, p = 0.001) but not the tamoxifen groups (97.28% ± 0.59 vs 90.93% ± 2.53). In the stroma, ER-beta immunoreactivity was significantly lower in the polyp compared with the endometrium in both the postmenopausal (18.77% ± 3.81 vs 36.30% ± 4.99, p = 0.005) and tamoxifen groups (2.89% ± 0.85 vs 21.25% ± 1.54, p < 0.0001).

Tamoxifen produced a significant increase in ER-beta immunoreactivity in the glands of the endometrium (82.90% ± 4.59 vs 97.28% ± 0.59, p = 0.002) but a significant reduction in ER-beta immunoreactivity in the stroma of the endometrium (36.30% ± 4.99 vs 21.25% ± 1.54, p = 0.001). In the polyp, tamoxifen exposure did not significantly alter ER-beta immunoreactivity in either the glands (97.04% ± 1.43 vs 90.93% ± 2.53) or the stroma (18.77% ± 3.81 vs 2.89% ± 0.85).
Table 2.4

ER-beta immunostaining in glands and stroma of postmenopausal and tamoxifen endometrium and polyp. Values are the mean percentage of positively stained cells per 10 randomly selected high powered fields per specimen ± SEM

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Tissue</th>
<th>Glands</th>
<th></th>
<th>Stroma</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Postmenopausal</td>
<td>Tamoxifen</td>
<td>Postmenopausal</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean % positively stained cells (± SEM)</td>
<td>Mean % positively stained cells (± SEM)</td>
<td>Mean % positively stained cells (± SEM)</td>
<td>Mean % positively stained cells (± SEM)</td>
</tr>
<tr>
<td>ER-beta</td>
<td>Endometrium</td>
<td>8</td>
<td>82.90 (i, v, viii) ± 4.59</td>
<td>18</td>
<td>97.28 (i, viii) ± 0.59</td>
</tr>
<tr>
<td>ER-beta</td>
<td>Polyp</td>
<td>10</td>
<td>97.04 (iii, v) ± 1.43</td>
<td>8</td>
<td>90.93 (iv) ± 2.53</td>
</tr>
</tbody>
</table>

One-way ANOVA with Tukey's honestly significant difference post-test for multiple comparisons

\[i \quad P < 0.0001\]
\[ii \quad P < 0.0001\]
\[iii \quad P < 0.0001\]
\[iv \quad P < 0.0001\]
\[v \quad P = 0.01\]
\[vi \quad P = 0.005\]
\[vii \quad P < 0.0001\]
\[viii \quad P = 0.002\]
\[ix \quad P = 0.001\]
Figure 2.4

Graph of ER-beta immunoreactivity in the glands and stroma of endometrium and polyps from postmenopausal and tamoxifen treated women. Data are presented as the mean ± SEM of percentage of positively stained cells.

Abbreviations: e = endometrium; p = polyp; pm = postmenopausal; tx = tamoxifen; g = glands; s = stroma; * = groups with $p < 0.05$ when compared using one-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons.
2.4.3 PR-A

As with both estrogen receptor isoforms, PR-A immunostaining was also detected in both the glands and stroma of the endometrium and polyp in both the postmenopausal and tamoxifen treated women. The results are summarised in Figure 2.5, Figure 2.6 and Figure 2.6.

**Figure 2.5**

*Photomicrographs showing PR-A distribution in endometrial polyps and endometrium from tamoxifen treated and postmenopausal women with no recent prior hormone exposure taken at X400 magnification.*

Plate A = postmenopausal polyp; plate B = tamoxifen polyp; plate C = postmenopausal endometrium; plate D = tamoxifen endometrium.

PR-A immunoreactivity was significantly lower in the stroma compared with the glands of the postmenopausal endometrium (80.44% ± 3.31 vs 99.28 ± 0.41, p = 0.002), postmenopausal polyp (72.72% ± 4.11 vs 98.44% ± 0.92, p < 0.0001), tamoxifen
endometrium (80.31% ± 3.68 vs 98.98% ± 0.03, p < 0.0001) and tamoxifen polyp (76.46% ± 2.88 vs 98.36% ± 0.81, p < 0.0001).

No significant difference was seen in PR-A immunoreactivity in the glandular compartment of the polyp were compared with endometrium, in either the postmenopausal (98.44% ± 0.92 vs 99.28% ± 0.41) or tamoxifen groups (98.36% ± 0.81 vs 98.98 ± 0.03). Similarly, in the stroma, PR-A immunoreactivity was not significantly different between the polyp and endometrium in either the postmenopausal (72.72% ± 4.11 vs 80.44% ± 3.31) or tamoxifen groups (76.46% ± 2.88 vs 80.31 ± 3.68).

PR-A immunoreactivity in the glands was not influenced by tamoxifen in either the endometrium (99.28% ± 0.41 vs 98.98% ± 0.3) or the polyp (98.44% ± 0.92 vs 98.36% ± 0.81). Tamoxifen treatment also did not appear to modulate stromal PR-A immunoreactivity in either the endometrium (80.44% ± 3.31 vs 80.31% ± 3.68) or the polyp (72.72% ± 4.11 vs 76.46% ± 2.88).
Table 2.5

PR-A immunostaining in glands and stroma of postmenopausal and tamoxifen endometrium and polyp. Values are the mean percentage of positively stained cells per 10 randomly selected high powered fields per specimen ± SEM.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Tissue</th>
<th>Glands</th>
<th></th>
<th></th>
<th>Stroma</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Postmenopausal</td>
<td>Tamoxifen</td>
<td>Postmenopausal</td>
<td>Tamoxifen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>Mean % positively stained cells (± SEM)</td>
<td>n</td>
<td>Mean % positively stained cells (± SEM)</td>
<td>n</td>
<td>Mean % positively stained cells (± SEM)</td>
</tr>
<tr>
<td>PR-A</td>
<td>Endometrium</td>
<td>8</td>
<td>99.28 i</td>
<td>± 0.41</td>
<td>18</td>
<td>98.98 ii</td>
<td>± 0.03</td>
</tr>
<tr>
<td></td>
<td>Polyp</td>
<td>9</td>
<td>98.44 iii</td>
<td>± 0.92</td>
<td>10</td>
<td>98.36 iv</td>
<td>± 0.81</td>
</tr>
</tbody>
</table>

One-way ANOVA with Tukey’s honestly significant difference post-test for multiple corrections

i P = 0.002
ii P < 0.0001
iii P < 0.0001
iv P < 0.0001
Figure 2.6

Graphs of PR-A immunoreactivity in the glands and stroma of endometrium and polyps from postmenopausal and tamoxifen treated women. Data are presented as the mean ± SEM of the percentage of positively stained cells.

Abbreviations: e = endometrium; p = polyp; pm = postmenopausal; tx = tamoxifen; g = glands; s = stroma; * = groups with p < 0.05 when compared using one-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons
2.4.4 **PR-B**

PR-B immunostaining was also detected in both the glands and stroma of the endometrium and polyp in both postmenopausal and tamoxifen treated women. The results are summarised in Figure 2.7 and 2.8 and Table 2.6.

**Figure 2.7**

Photomicrographs showing PR-B distribution in endometrial polyps and endometrium from tamoxifen treated and postmenopausal women with no recent prior hormone exposure taken at X400 magnification.

*Plate 1 = postmenopausal polyp; plate 2 = tamoxifen polyp; plate 3 = postmenopausal endometrium; plate 4 = tamoxifen endometrium*

<table>
<thead>
<tr>
<th>PR-B</th>
<th>Postmenopausal polyp</th>
<th>Tamoxifen polyp</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Postmenopausal endometrium</th>
<th>Tamoxifen endometrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>D</td>
</tr>
</tbody>
</table>

PR-B immunoreactivity, was significantly lower in the stroma compared with the glands of the postmenopausal endometrium (79.67% ± 1.83 vs 99.83% ± 0.03, p = 0.015), tamoxifen endometrium (80.58% ± 2.89 vs 98.14 ± 0.34, p = 0.0004) and tamoxifen polyp...
(53.22% ± 9.54 vs 96.94 ± 1.84, p < 0.0001). In contrast, there was no significant difference seen in PR-B immunoreactivity between the stroma and glands of postmenopausal polyps (93.91% ± 3.45 vs 98.64% ± 0.32).

Comparing the polyp and endometrium, PR-B immunoreactivity like that of PR-A immunoreactivity in the glandular compartment was not significantly different in either the postmenopausal (98.64% ± 0.32 vs 99.83% ± 0.03) or tamoxifen groups (96.94% ± 1.84 vs 98.14% ± 0.34). PR-B stromal immunoreactivity also like that of PR-A immunoreactivity, was not significantly different between the polyp and endometrium in either the postmenopausal (93.91 % ± 3.45 vs 79.67% ± 1.83) or tamoxifen groups (53.22% ± 9.54 vs 80.58% ± 2.89). However unlike with PR-A, PR-B immunoreactivity was higher in postmenopausal polyp stroma compared to postmenopausal endometrial stroma, but lower in tamoxifen polyp stroma compared to tamoxifen endometrial stroma.

PR-B immunoreactivity in the glands was not influenced by tamoxifen in either the endometrium (99.83% ± 0.03 vs 98.14% ± 0.34) or the polyp (98.64% ± 0.32 vs 96.94% ± 1.84). Tamoxifen treatment also did not significantly influence stromal PR-B immunoreactivity in the endometrium (79.67% ± 1.83 vs 80.58% ± 2.89) or the polyp (93.91% ± 3.45 vs 53.22% ± 9.54). However, there was a notable trend towards reduced expression in tamoxifen polyp stroma compared with postmenopausal polyp stroma.
Table 2.6

PR-B immunostaining in glands and stroma of postmenopausal and tamoxifen endometrium and polyp. Values are the mean percentage of positively stained cells per 10 randomly selected high powered fields per specimen ± SEM.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Tissue</th>
<th>Glands</th>
<th></th>
<th>Stroma</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Postmenopausal</td>
<td>Tamoxifen</td>
<td>Postmenopausal</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean % positively stained cells (± SEM)</td>
<td>Mean % positively stained cells (± SEM)</td>
<td>Mean % positively stained cells (± SEM)</td>
<td>Mean % positively stained cells (± SEM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>18</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>PR-B</td>
<td>Endometrium</td>
<td>99.83 ± 0.03</td>
<td>98.14 ± 0.34</td>
<td>79.67 ± 1.83</td>
<td>80.58 ± 2.89</td>
</tr>
<tr>
<td></td>
<td>Polyp</td>
<td>98.64 ± 0.32</td>
<td>96.94 ± 1.84</td>
<td>93.91 ± 3.45</td>
<td>53.22 ± 9.54</td>
</tr>
</tbody>
</table>

One-way ANOVA with Tukey’s honestly significant difference post-test for multiple corrections

\( ^1 P = 0.015 \)
\( ^{ii} P = 0.0004 \)
\( ^{iii} P < 0.0001 \)
**Figure 2.8**

Graphs of PR-B immunoreactivity in the glands and stroma of endometrium and polyps from postmenopausal and tamoxifen treated women. Data are presented as the mean ± SEM of the percentage of positively stained cells.

Abbreviations: e = endometrium; p = polyp; pm = postmenopausal; tx = tamoxifen; g = glands; s = stroma; * = groups with $p < 0.05$ when compared using one-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons.
2.5 Discussion

This is the first study to examine ER and PR subtype distribution in endometrial polyps using immunohistochemistry techniques. The results demonstrate that all endometrial polyps studied here express the ER and PR subtypes. This suggests that like their surrounding endometrium, polyps are hormonally sensitive, and respond to estrogen and progesterone via receptor dependent pathways. The results also show that there are not only differences but also similarities between polyps and endometrium, which suggest their role in mechanisms for polyp pathogenesis.

ER-alpha expression was significantly reduced in the stroma compared to glands in both postmenopausal polyps and tamoxifen polyps. Similar stromal-glandular differences in polyps have been reported by other authors (84, 109, 111, 113), albeit with ER rather than receptor subtypes. This has been seen in other pathological states in the endometrium. Kreizman-Shefer et al. found ER-alpha expression was also significantly reduced in the stroma compared to glands in endometrial adenocarcinoma. Glandular and stromal compartments within the endometrium do not function in isolation, but maintain uterine physiology and function through complex interactions, mediated in part through steroid receptor activation. For example, evidence from studies in mice have provided evidence that estrogen driven epithelial proliferation is driven by stromal ER-alpha rather than epithelial ER-alpha (118). Also, estrogen mediated down-regulation of PR in the uterine epithelium has been shown to be dependent on stromal but not epithelial ER-alpha (119). The altered ratio of glandular to stromal of ER expression seen in polyps may represent dysregulation, and is likely to be important in polyp pathogenesis.

Stromal ER-beta expression was significantly lower in both postmenopausal polyps compared to postmenopausal endometrium and tamoxifen polyps compared to tamoxifen endometrium. However, there were no significant differences in ER-alpha expression when polyp and endometrium were compared directly. Mittal et al. and Taylor et al. observed similar changes in polyp stroma compared with endometrial
stroma, although both studies examined tissue from premenopausal women and neither looked at receptor subtypes (91, 111). Reduced ER beta expression has also been shown to occur in atypical hyperplasia of the endometrium, breast, ovarian and endometrial cancer (120, 121).

The two estrogen receptor subtypes ER-alpha and ER-beta are nuclear receptors and are products of separate genes ESR1 and ESR2, located on different chromosomes (122). Binding of a ligand to the estrogen receptor triggers conformational changes in the receptor which leads to changes in the rate of transcription of estrogen-regulated genes, via a number of events including receptor dimerization, receptor-DNA interaction, recruitment of and interaction with coactivators and other transcription factors, and formation of a preinitiation complex (123, 124). The ligand-binding domain of the ER subtypes alpha and beta are structurally similar, but despite these similarities, ER-beta has been shown to restrict the pro-proliferative functions of ER-alpha in response to estrogen activation (100, 125). Some have hypothesised ER-beta has a tumour suppressor role (126). Therefore, a reduction of ER-beta expression in polyps could result in a preponderance of ER-alpha induced effects including proliferation and the secretion of vascular endothelial growth factors to stimulate angiogenesis and the growth of the vascular core around which stroma and glands condense to form a polyp.

Finally, whilst not statistically significant, stromal PR-B expression was higher in postmenopausal polyp compared with the postmenopausal endometrium. Sant’Ana de Almeida et al. reported the same finding in their study of postmenopausal polyps and endometrium (112). The opposite effect was seen in tamoxifen polyps however, where stroma showed decreased PR-B expression relative to tamoxifen endometrial stroma. The biological response to progesterone, a highly effective antagonist of estrogen-induced endometrial proliferation, is mediated by two forms of the intracellular progesterone receptor; PR-A and PR-B. These two isoforms are transcribed from distinct, estrogen-inducible promoters within a single-copy progesterone receptor gene (127). Although the isoforms have indistinguishable hormone binding affinities, like ER, they too have different downstream effects. In an experiment to study functional
differences between the progesterone receptor isoforms in the uterus, Mulac-Jericevic et al. found that selective ablation of PR-A in a mouse model, resulted in exclusive production of PR-B, and revealed that PR-B contributes to, rather than inhibits, epithelial cell proliferation both in response to estrogen alone and in the presence of progesterone and estrogen. Therefore, in the uterus, the PR-A isoform is necessary to oppose estrogen-induced proliferation as well as PR-B-dependent proliferation (128). Highly malignant forms of endometrial, cervical and ovarian cancers have been correlated with overexpression of PR-B (129). The change in relative expression levels of PR-A and PR-B in polyps may therefore be a stimulus for glandular and stromal proliferation and polyp formation.

The observation in this study that stromal PR-B expression was reduced in tamoxifen polyps raises the possibility that these lesions become relatively insensitive to progesterone and therefore are more vulnerable to the proliferative and estrogenic effect of tamoxifen, which increases their potential for malignant transformation.

Progestin therapy is widely used in the management of endometrial cancer where treatment has been shown to markedly affect the histopathological characteristics of endometrial malignancies (130). In addition, intrauterine progestin delivered via the LNG-IUS has been shown to prevent the growth of endometrial polyps in tamoxifen treated women (95, 96). The similarities observed in PR-A and PR-B expression in postmenopausal and tamoxifen-exposed endometrium suggests that progestins exert this protective effect via similar mechanisms.

As shown in Table 2.1 above, the studies examining ER and PR receptor expression in endometrial polyps using immunohistochemistry have used a variety of image analysis techniques to assess the degree of receptor staining. Dibi et al. were the only authors to use a simplistic ‘positive or negative’ method where receptor staining was positive if at least 1% of nuclei were stained, without considering the glands and stroma separately (116). Five authors reported the intensity of receptor staining as either ‘weak, moderate or strong’ (109), graded from 0 to 3 (110), as ‘negative positive or intensely positive’
(84), as ‘+, ++, +++ or ++++’ (91), or from 0 to 4 (113). In each case, the degree of staining intensity was correlated with a percentage of positively stained nuclei. The remaining authors used a score derived from either multiplication of the percentage of positively stained cells assessed subjectively by the intensity of staining, or addition of these two values (92, 108, 111, 112, 114, 115, 117). Whilst there is validity in each of these methods, the range of techniques used makes comparisons between results of these studies difficult.

In this study, a quantitative method of manually counting the number of positively stained (brown) nuclei as a percentage of all nuclei per high-powered field. If cells contained any brown staining they were counted as positively stained. Ten randomly selected high power fields were examined in each sample. This methodology, as described by Hamilton (131) has the advantage of being reproducible and objective, and takes into account the non-stoichiometric nature of DAB chromogen binding in immunohistochemistry reactions. It has been validated within our laboratory (97, 104, 132).

In contrast to a number of other studies summarised in Table 2.1, tissue samples were not obtained from the same patient. The fact that Mittal et al., Sant’Ana de Almeida et al., Belisario et al. and Lopes et al. all studied endometrium and polyp tissue from the same patient could affect the validity of their results by under-reporting differences (111-114). Pal et al. studied aromatase gene expression in endometrial samples obtained under direct vision in the same uterus from a polyp, from endometrium 2cm away from the polyp, and from remote endometrium on an opposing wall (68). They found marked differential expression between the polyp and remote sites. They conclude that not only is there a focal over-expression of aromatase within polyps, but that there may also be a gradation in gene expression within the endometrium. The advantage therefore of this study, which used endometrium sampled from consistent locations in non-polyp bearing uteri for comparison, is that it eliminates the possibility of sampling endometrium close to a polyp that may be part of the field change.
This study describes similarities and differences in ER and PR receptor subtype distribution in endometrial polyps compared with endometrium in postmenopausal women receiving no hormonal treatment, and in women on tamoxifen, which are likely to play a role in polyp pathogenesis. The genetic pathways that may underlie polyp pathogenesis will be studied in the following chapter.
3 A Cross-Platform Microarray of Endometrial Polyps and Endometrium from Postmenopausal Women Treated With and Without Tamoxifen

3.1 Background

Endometrial polyps are common lesions and are frequently observed in women with abnormal uterine bleeding after the menopause. Aside from the bothersome symptoms they are associated with, endometrial polyps also pose a risk for developing endometrial cancer. The mainstay of treatment where endometrial polyps are diagnosed, is to remove them based on the assumption that this best removes the potential malignant focus.

Theories of endometrial carcinogenesis suggest that the hormonal environment of the endometrium may serve as a ‘trigger’ or ‘promoter’ for the molecular change(s) that triggers malignant transformation and progression from occult to clinically detectable malignancy (133). In support of this notion, is the evidence that tamoxifen, known to be associated with an increased risk of endometrial cancer with an over-representation of high grade, non-endometroid histological subtypes, induces changes in the gene expression profile of the endometrium (134, 135). However, it is not clear whether the early changes induced by tamoxifen persist once malignancy ensues, as a published microarray study of tamoxifen-associated endometrial cancers and matched cases not associated with this exposure, found no evidence to support the hypothesis that tamoxifen induces a distinct gene expression profile (136).

In the previous chapter, where estrogen and progesterone receptor subtype expression (ER-alpha, ER-beta, PR-A and PR-B) was compared in the glandular and stromal compartments of endometrium and polyps from postmenopausal women with and without tamoxifen treatment, a clear ‘polyp’ specific effect was noted; ER-beta expression was reduced in polyps relative to endometrium in the stroma. Because this was seen in both the tamoxifen treated and postmenopausal groups, it can be more confidently asserted that this is a true ‘polyp’ effect.
This evidence, suggests that there is a distinct polyp pathway that occurs within the endometrium that culminates in the macroscopic and molecular changes that distinguish an endometrial polyp from its surrounding endometrium. Given that this pathway is likely to originate at a genetic and gene expression level, and appears to be triggered by alterations in the hormonal environment, identifying the pathways involved in the genesis of polyps may further our understanding of their pathogenesis and help in developing more sophisticated therapeutic targets.
3.2 Aim

In this study, data sets from two microarray experiments were combined in order to identify genes specific to the endometrial polyp.

The first two data sets were obtained from a microarray carried out using the Illumina platform of polyp tissue obtained from postmenopausal women with no exogenous hormone exposure and women receiving tamoxifen treatment for breast cancer. Analysing these data sets alone would provide information about the genes that are differentially expressed in tamoxifen polyps compared with postmenopausal polyps, but would not answer questions about how gene expression in polyps differs from endometrium. The second two data sets were obtained by Dr Taylor and Dr Panchal using the Affymetrix microarray platform on postmenopausal endometrium and tamoxifen endometrium. These provided a comparative group for each polyp data set. The four data sets were combined to identify differentially expressed genes between the postmenopausal endometrium and the postmenopausal polyp (group A) and the tamoxifen endometrium and the tamoxifen polyp (group B). Given that the genes in group A are likely to represent ‘postmenopausal polyp genes’, and the genes in group B are likely to represent ‘tamoxifen polyp genes’, the genes that are common to these two groups (i.e. the AB intersect) represent essential polyp genes. The experimental design is as follows, and is summarised in Figure 3.1.

3.3 Experimental Design

Firstly, gene expression data was obtained from postmenopausal polyps and tamoxifen polyps using an Illumina microarray. This generated a ‘postmenopausal polyp’ expression value and a ‘tamoxifen polyp’ expression value for each gene on the Illumina Illumina HT-12 v3 Expression BeadChip. This data was used for analysis alongside gene expression data obtained from postmenopausal endometrium and tamoxifen endometrium. This microarray had previously been carried out by Dr Taylor and Dr Panchal using the Affymetrix platform and had generated ‘postmenopausal
endometrium’ expression value and a ‘tamoxifen endometrium’ expression value for each gene expressed on the Affymetrix human HU133_plus 2 microarray chip.

In order that the gene expression data generated from the Illumina and Affymetrix microarray platforms could be directly compared, the data were normalised. Following this process, fold changes were calculated for the genes common to both arrays in postmenopausal polyps compared to postmenopausal endometrium, and in tamoxifen polyps compared to tamoxifen endometrium. The most biologically relevant genes were identified by eliminating those genes with a fold change between +2 and -2.

The two gene-fold change lists (postmenopausal endometrium vs postmenopausal polyp is list A and tamoxifen endometrium vs tamoxifen polyp is list B) were compared in order to identify the genes that are upregulated and downregulated in both groups. This list, the ‘AB intersect’ as shown in Figure 3.1 is a list of polyp genes that were further examined using pathway analysis software to identify which genetic pathways are most significantly modulated in polyps.
Figure 3.1

Flow diagram summarising experimental design for cross-platform analysis of microarray data

- Postmenopausal polyp Genes (Illumina)
- Tamoxifen polyp Genes (Illumina)
- Postmenopausal endometrium Genes (Affymetrix)
- Tamoxifen endometrium genes (Affymetrix)

Data normalisation

Eliminate genes with fold change between +2 and -2

List A
Gene-fold change (Postmenopausal polyp vs postmenopausal endometrium)

List B
Gene-fold change (Tamoxifen polyp vs tamoxifen endometrium)

Compare List A and List B to identify genes which are upregulated and downregulated in both lists

List A
List B
AB intersect
Pathway analysis
3.4 Materials and Methods

3.4.1 Specimens

All samples were collected at Leicester Royal Infirmary, according to Leicestershire Health Authority Ethics Committee (Ethics committee reference number REC 6498). Written consent was obtained from all women prior to collecting samples.

Twelve endometrial polyps were retrieved by hysteroscopic polypectomy from postmenopausal women receiving no exogenous hormonal treatment (n=6) and from postmenopausal women receiving tamoxifen as adjuvant treatment of breast cancer (n=6). Half of the extracted polyp tissue was ‘snap-frozen’ in liquid nitrogen and stored at -80°C until ready for use. The remainder was sent for routine histological assessment. A further 12 endometrial samples were collected from postmenopausal women with no recent history of hormonal treatment (n=6) and from postmenopausal women receiving tamoxifen as adjuvant therapy for breast cancer. All were undergoing either vaginal hysterectomy for prolapse, or abdominal hysterectomy for benign ovarian cysts. At hysterectomy, once the specimen was removed a sagittal incision from fundus to isthmus of the uterus was made and endometrium was removed by sharp curette into a sterile tube, ‘snap-frozen’ in liquid nitrogen and stored at -80°C for RNA extraction. The remaining uterine specimen was sent for routine histological assessment. None of the specimens used in this study contained complex or atypical hyperplasia, malignancy, or cervical intraepithelial neoplasia.

3.4.2 RNA extraction

Polyp and endometrial tissue was thawed to room temperature on ice in Tri Reagent® (Sigma-Aldrich) using 1ml of reagent per 100 mg of tissue and then homogenised using an Ultra-turrax homogeniser (IKA). Samples were allowed to stand at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes.

Two hundred µL of chloroform was added per ml to each sample, and samples were then shaken vigorously for 15 seconds. After standing at room temperature for 3
minutes, the samples were centrifuged at 7500 RPM at 4°C for 15 minutes. The aqueous phase from each sample was transferred to a fresh tube and 500 µL of isopropanol added. This was left to stand at room temperature for 10 minutes and then centrifuged at 13000 RPM at 4°C for 10 minutes. The supernatant was discarded and the remaining RNA pellet was washed with 1 ml of 75% ethanol in DEPC treated water. This was again centrifuged at 13000 RPM at 4°C for 5 minutes and the supernatant discarded. The pellet was air dried, re-suspended in 100 µL of DEPC water and heated to 55°C for 5 minutes.

3.4.3 RNA Purification

Each RNA sample, suspended in 100 µL of DEPC water, was cleaned to remove contaminating DNA and protein using the ‘RNeasy Mini Protocol for RNA Cleanup’ (Qiagen). Aqueous total RNA was mixed with ethanol, applied to the RNeasy mini columns (Qiagen) purified and concentrated through ethanol precipitation, according to the manufacturer’s instructions.

3.4.4 RNA Quantification and Quality Assessment

The total RNA concentration obtained from each tissue sample was determined using a NanoDrop™ 8000 spectrophotometer (ThermoFisher Scientific). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) and a ‘RIN’ number assigned. This number is generated by Agilent software and provides an independent assessment of RNA integrity that is determined not by the ratio of ribosomal bands but by the entire electrophoretic trace including the presence or absence of degradation products. The RNA samples were stored at -80°C until ready for use.

3.4.5 Polyp Microarray

Samples were prepared for the microarray using the Illumina TotalPrep RNA Amplification Kit (Ambion). Firstly, the RNA samples were thawed and adjusted to contain 250 ng of RNA in 11 µL of nuclease-free water. A ‘Reverse Transcription Master Mix’ was prepared containing a T7 Oligo (dT) Primer to synthesise cDNA containing a T7 promoter sequence. The Master Mix was added to each RNA sample and incubated at
42°C for 2 hours. Second strand master mix containing DNA polymerase and RNase H was prepared and added to each sample in order to convert the single stranded cDNA into double-stranded DNA and to simultaneously degrade the RNA. Samples were incubated at 16°C for 2 hours.

A cDNA Binding Buffer was added to each sample and then each sample was passed through a cDNA filter cartridge to remove RNA, primers, enzymes and salts. In-vitro transcription (IVT) was then undertaken to generate multiple copies of biotinylated cRNA from the double-stranded cDNA templates by adding an IVT ‘Master Mix’ to each sample and then incubated for 14 hours at 37°C.

After stopping the IVT reaction, the resulting cRNA was purified to remove enzymes, salts and un-incorporated nucleotides by using a cRNA Filter Cartridge. The concentration of cRNA was assessed using the NanoDrop™ 8000 spectrophotometer and its quality assessed using the Agilent 2100 Bioanalyzer.

The microarray was then carried out with 750 ng of cRNA being dissolved to a volume of 5µl with RNAse free water. The Illumina HT-12 v3 Expression BeadChip (Illumina) was used, which contains 12 microarrays and therefore allows for parallel processing of all 12 samples. Each microarray on the BeadChip contains about 48,000 ‘50-mer’ probes that target more than 25,000 annotated genes. The 12 cRNA samples were dispensed onto the BeadChip and incubated for 16 hours to allow hybridisation to occur. The BeadChip was then washed and stained with streptavidin-Cy3. The BeadChip was scanned using the Illumina Beadstation 500 (Illumina) to record relative fluorescence values for each probe. The scanned images were saved, and the raw data exported directly to Illumina BeadStudio (version 3) for preliminary analysis involving grouping, differential expression analysis and quality control.

3.4.6 Polyp microarray gene expression analysis

Raw probe level fluorescence signal intensity expression data was converted to gene-level data after background subtraction and subtraction of unexpressed probes. Cubic
spline normalization was carried out in order to remove systematic variation of non-biological origin.

### 3.4.7 Endometrium Microarray

This was carried out by my colleagues, Dr Taylor and Dr Panchal. Endometrial RNA obtained from each of the 2 patient groups (n=6) was pooled and samples submitted to the MRC GeneService (Cambridge) who prepared biotinylated cRNA with a total of 10 \( \mu \text{g} \) of total cellular RNA according to Affymetrix protocols. The integrity of the labelled cRNA and fragmentation products were assessed on the Agilent 2100 Bioanalyzer. Fifteen \( \mu \text{g} \) of biotinylated cRNA fragments were then hybridized to humanHU133_plus 2 microarray chips (Affymetrix) overnight and then washed, stained and scanned according to Affymetrix protocols.

### 3.4.8 Endometrium microarray gene expression analysis

Fluorescence data were corrected by background subtraction and normalized to housekeeping genes on the U133_plus 2 arrays. Further analysis was undertaken using dChip Analyzer software version 1.4.

### 3.4.9 Cross Platform Analysis

In order that fluorescence gene expression data from the Illumina and Affymetrix platforms could be directly compared as outlined in Figure 3.1 above (postmenopausal polyp with postmenoausal endometrium, and tamoxifen polyp with tamoxifen endometrium), the values were normalised to compensate for systematic technical differences between chips. Average signal intensity values for each gene for the 6 postmenopausal polyp (PP) samples, and the 6 tamoxifen polyp (TP) samples were tabulated against signal intensity values for each gene for the single postmenopausal endometrial (PE) sample and the single tamoxifen endometrium (TE) sample. Firstly, a ‘global’ method was used, applying a constant scaling factor to every gene fluorescence measurement was performed so that each group had the same median intensity making Illumina and Affymetrix data directly comparable. Applying a constant factor means that the relative intensity between spots in each microarray remains unchanged. Secondly,
a quantile method of normalisation was applied in order that data from Affymetrix and Illumina followed the same distribution - on the assumption that the distribution of gene abundances is nearly the same in all samples. The distribution of genes in each group was demonstrated, and then groups were normalised by calculating, for each intensity value, the quantile of that value in the distribution of gene intensities and transforming the original value to that quantile's value in the reference group.

Fold-changes based on the normalised signal intensity values were then calculated for gene expression in the polyp relative to the endometrium in the postmenopausal group (Figure 3.1, List A) and the tamoxifen group (Figure 3.1, List B). Genes with a fold-change of between -2 and +2 were excluded as these genes were assumed not to be biologically significant. Finally, genes that were either upregulated or downregulated in both groups were identified as the ‘AB intersect’ shown in Figure 3.1.

### 3.4.10 Pathway analysis

Pathway analysis was carried out on the resulting list of polyp genes in this ‘AB intersect’ using PathVisio version 1.1., which uses pathway content from WikiPathways. A chi-squared test was applied to identify whether the number of genes mapped to each pathway was significantly different to the number that would be expected by chance. A p value of <0.05 was taken as significant. The significantly regulated pathways were then categorised according to the RGD pathway portal headings.

### 3.4.11 Preparation of cDNA for Quantitative RT-PCR

One µg of RNA from each of the 12 samples used in the Illumina microarray and from 10 of the 12 samples used in the ‘pooled’ Affymetrix array was thawed on ice and reverse transcribed into cDNA using AMV-RT (Promega) according to the manufacturer’s protocols using 5X AMV buffer, RNasin (Promega), Anchored oligo(dT)23 (Sigma) and dNTPs (Promega) in DEPC water. Samples were incubated at 42°C for 1 hour and then 95°C for 1 minute to denature all enzymes. Samples were stored at 4°C until ready for use.
3.4.12 Quantitative RT-PCR

One µL of cDNA was combined with both sets of gene specific primers at a concentration of 200pm/µL in a SYBR green mix. The forward and reverse primer sequences, along with the PCR thermal and cycling conditions for denaturation annealing and primer extension are outlined in Table 3.1. Human GAPDH expression was used as an internal reference. The listed thermal and cycling conditions were applied in the Roche Lightcycler 1.2. Standard curves were constructed using primer dilutions of ‘neat’, 1/5, 1/10, 1/50, 1/100 and water for each gene target and the relative expression of each gene in the tamoxifen polyp relative to the postmenopausal polyp was calculated using the 2^ΔΔCt method after normalisation to GAPDH levels (137).

**Table 3.1**

*Primer Sequences and Thermal Conditions*

<table>
<thead>
<tr>
<th>Gene (Ref)</th>
<th>Forward sequence</th>
<th>Reverse Sequence</th>
<th>Activation</th>
<th>Denaturation, Annealing, Amplification</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt 5a (138)</td>
<td>5’ GGG AGG TTG GCT TGA ACA TA 3’</td>
<td>5’ GAA TGG CAC GCA ATT ACC TT 3’</td>
<td>95°C 10 mins</td>
<td>95°C 30 s, 60°C 1 min, 72°C 30 s</td>
<td>40</td>
</tr>
<tr>
<td>Beta-catenin (138)</td>
<td>5’ TGC AGT TCG CCT TCA CTA TG 3’</td>
<td>5’ ACT AGT CGT GGA ATG GCA CC 3’</td>
<td>95°C 10 mins</td>
<td>95°C 30 s, 60°C 1 min, 72°C 30 s</td>
<td>40</td>
</tr>
<tr>
<td>Notch 2 (139)</td>
<td>5’ TGA GTA GGC TCC ATC CAG TC 3’</td>
<td>5’ TGG TGT AG GTA GGG ATG CT 3’</td>
<td>94°C 30 s</td>
<td>58°C 1 min, 72°C 1 min, 72°C 5 mins</td>
<td>24</td>
</tr>
</tbody>
</table>
3.5 Results

3.5.1 Cross Platform Analysis

There were 16,594 genes identified as being common across the Illumina and Affymetrix platforms following the normalisation steps outlined above. Excluding those with a fold change between 2 and -2, this left 4127 genes that were common to the postmenopausal polyp and postmenopausal endometrium and 4539 genes that were common to the tamoxifen polyp and tamoxifen endometrium. A total of 3289 genes were found to be common to both groups, and of these, 3272 genes were either upregulated or downregulated in both the postmenopausal polyp compared to postmenopausal endometrium, and in the tamoxifen polyp compared to the tamoxifen endometrium. This group of genes are therefore ‘polyp genes’ and represent the AB intersect as outlined in Figure 3.1 above. Of these polyp genes, 1659 genes were upregulated in polyps relative to the endometrium, and 1613 genes down regulated in polyps relative to the endometrium. These results are summarised in Figure 3.2 below. The 20 most significantly upregulated genes in the latter group are shown in Table 3.2and the 20 most significantly downregulated genes are shown in Table 3.3.
Figure 3.2

Results of the cross-platform analysis showing the numbers of genes that were identified at each stage

[Diagram showing the flow of data normalization, elimination of genes with fold change, and comparison of lists to identify upregulated and downregulated genes]

List A
Gene fold change
(Postmenopausal polyp vs postmenopausal endometrium)

List B
Gene fold change
(Tamoxifen polyp vs tamoxifen endometrium)

Compare List A and List B to identify genes which are upregulated and downregulated in both lists

List A
1659 up
1613 down
AB intersect

List B

Pathway analysis
Table 3.2

The 20 most significantly up regulated genes in polyps relative to endometrium with their fold changes, description and Gene Ontology classifications

Abbreviations: PmP = postmenopausal polyp; PmE = postmenopausal endometrium; TxP = tamoxifen polyp; TxE = tamoxifen endometrium

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Gene symbol</th>
<th>Fold change</th>
<th>Description</th>
<th>Gene Ontology: Molecular function</th>
<th>Gene Ontology: Cellular component</th>
<th>Gene Ontology: Biological process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PmP/PmE</td>
<td>TxP/TxE</td>
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<tr>
<td>AI734124</td>
<td>RPS14</td>
<td>150</td>
<td>196</td>
<td>Ribosomal Protein S14</td>
<td>poly(A) RNA binding, translational regulator activity</td>
<td>Nucleoplasm, nucleolus, cytoplasm, mitochondrion</td>
</tr>
<tr>
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<tr>
<td>NM_024318</td>
<td>LILRB3</td>
<td>83</td>
<td>89</td>
<td>Leukocyte Immunoglobulin Like Receptor B3</td>
<td>Transmembrane receptor signalling activity, protein binding</td>
<td>Integral component of plasma membrane</td>
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<tr>
<td>AF109683</td>
<td>LAIR1</td>
<td>81</td>
<td>81</td>
<td>Leukocyte Associated Immunoglobulin Like Receptor 1</td>
<td>Protein binding</td>
<td>Plasma membrane, integral component of membrane, extracellular exosome</td>
</tr>
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<tr>
<td>NM_004367</td>
<td>CCR6</td>
<td>74</td>
<td>94</td>
<td>C-C motif chemokine receptor 6</td>
<td>Chemokine receptor activity, protein binding</td>
<td>Cytosol, plasma membrane. Cell surface</td>
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<tr>
<td>NM_005989</td>
<td>AKR1D1</td>
<td>73</td>
<td>82</td>
<td>Aldo-Keto-Reductase Family 1 Member D1</td>
<td>Steroid binding, delta 4-3-oxosteroid 5 beta reductase activity</td>
<td>Cytosol, extracellular exosome</td>
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<td></td>
</tr>
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<td>Accession number</td>
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<td>Fold change</td>
<td>Description</td>
<td>Gene Ontology: Molecular function</td>
<td>Gene Ontology: Cellular component</td>
<td>Gene Ontology: Biological process</td>
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<td>NM_052852</td>
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<td>71/93</td>
<td>Zinc finger protein 486</td>
<td>Nucleic acid binding</td>
<td>Nucleus, extracellular exosome</td>
<td>catabolic process, digestion</td>
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<tr>
<td>NM_001562</td>
<td>IL18</td>
<td>71/116</td>
<td>Interleukin 18</td>
<td>Cytokine activity, protein binding</td>
<td>Extracellular space, cytosol</td>
<td>MAPK cascade, angiogenesis, inflammatory response,</td>
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<tr>
<td>NM_002439</td>
<td>MSH3</td>
<td>67/83</td>
<td>MutS homologue 3</td>
<td>Damaged DNA binding</td>
<td>Nuclear chromosome</td>
<td>Meiotic mismatch repair</td>
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<td>AI793222</td>
<td>LOC399900</td>
<td>63/72</td>
<td>Uncharacterised RNA gene</td>
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<td>No information</td>
<td>No information</td>
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<td>AI951640</td>
<td>PDCD7</td>
<td>60/66</td>
<td>Programmed Cell death 7</td>
<td>No information</td>
<td>Nucleoplasm</td>
<td>mRNA splicing, apoptotic process</td>
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<tr>
<td>NM_005497</td>
<td>GJC1</td>
<td>56/17</td>
<td>Gap junction protein gamma 1</td>
<td>Ion channel activity, gap junction channel activity, protein binding</td>
<td>Endoplasmic reticulum membrane, gap junction, connexion complex</td>
<td>Vasculogenesis, transport, cell-cell signalling, cell-cell junction assembly</td>
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<tr>
<td>AF269088</td>
<td>ANKRD30B</td>
<td>41/46</td>
<td>Ankyrin repeat domain 30 b</td>
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<td>No information available</td>
<td>No information available</td>
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<td>N21682</td>
<td>XPNPEP3</td>
<td>39/64</td>
<td>X-Prolyl Aminopeptidase 3</td>
<td>Aminopeptidase activity, metallopeptidase activity</td>
<td>Mitochondrion, extracellular exosome</td>
<td>Glomerular filtration, protein processing</td>
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<tr>
<td>BC018086</td>
<td>CDKN2AIPNL</td>
<td>36/53</td>
<td>CDKN2A Interacting Protein N-Terminal Like</td>
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<td>No information</td>
<td>No information</td>
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<td>SHCBP1</td>
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<td>SHC Binding and Spindle Associated 1</td>
<td>Protein binding</td>
<td>No information</td>
<td>Fibroblast growth factor receptor signalling pathway, regulation of neural precursor cell proliferation</td>
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<tr>
<td>Accession number</td>
<td>Gene symbol</td>
<td>Fold change</td>
<td>Description</td>
<td>Gene Ontology: Molecular function</td>
<td>Gene Ontology: Cellular component</td>
<td>Gene Ontology: Biological process</td>
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<tr>
<td>87931</td>
<td>ROCK2</td>
<td>32P/PmE 16TxE</td>
<td>Rho associated coiled-coil containing protein kinase 2</td>
<td>Protein serine/threonine kinase activity, structural molecule activity, protein binding</td>
<td>Nucleus, centrosome, cytosol</td>
<td>Cytokinesis, positive regulation of protein phosphorylation, smooth muscle contraction</td>
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<tr>
<td>NM_006669</td>
<td>LILRB1</td>
<td>30P/PmE 37TxE</td>
<td>Leukocyte Immunoglobulin Like Receptor B1</td>
<td>Protein phosphatase 1 binding, MHC class I receptor activity, SH2 domain binding</td>
<td>Extracellular region, cytoplasm, plasma membrane</td>
<td>negative regulation of T cell mediated cytotoxicity, negative regulation of cytokine secretion involved in immune response</td>
</tr>
<tr>
<td>AI951606</td>
<td>CDAN1</td>
<td>30P/PmE 31TxE</td>
<td>Codanin 1</td>
<td>Protein binding</td>
<td>Nucleus, cytoplasm, plasma membrane</td>
<td>Chromatin organisation, protein localisation, negative regulation of DNA replication</td>
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<tr>
<td>AI268054</td>
<td>MCART1</td>
<td>27P/PmE 35TxE</td>
<td>Mitochondrial carrier triple repeat protein 1</td>
<td>Structural constituent of ribosome</td>
<td>Mitochondrial inner membrane</td>
<td>Translation, transport</td>
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<tr>
<td>BE042939</td>
<td>DDX51</td>
<td>27P/PmE 42TxE</td>
<td>DEAD-Box Helicase S1</td>
<td>ATP-dependent RNA helicase activity, ATP binding, Poly(A) RNA binding</td>
<td>nucleolus</td>
<td>rRNA processing, RNA secondary structure unwinding</td>
</tr>
</tbody>
</table>
Table 3.3

The 20 most significantly down regulated genes in polyps relative to endometrium with their fold changes, description and Gene Ontology classifications

Abbreviations: PmP = postmenopausal polyp; PmE=postmenopausal endometrium; TxP = tamoxifen polyp; TxE = tamoxifen endometrium

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Gene symbol</th>
<th>Fold change</th>
<th>Description</th>
<th>Gene Ontology: Molecular function</th>
<th>Gene Ontology: Cellular component</th>
<th>Gene Ontology: Biological process</th>
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<tr>
<td>NM_021029</td>
<td>RPL36A</td>
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<td>Ribosomal Protein L36A</td>
<td>Poly(RNA) binding</td>
<td>Cytosol, ribosome</td>
<td>RNA processing</td>
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<td>NM_018304</td>
<td>PRR11</td>
<td>-98</td>
<td>Proline rich 11</td>
<td>No information</td>
<td>Nucleus, cytoplasm, membrane</td>
<td>Regulation of cell cycle</td>
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<td>AW574664</td>
<td>LOC388344</td>
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<td>Uncharacterised gene</td>
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<td>No information</td>
<td>No information</td>
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<td>NM_004907</td>
<td>IER2</td>
<td>-79</td>
<td>Immediate Early Response 2</td>
<td>DNA binding</td>
<td>Nucleus, nucleoplasm, cytoplasm</td>
<td>Neuron differentiation, cell motility, response to fibroblast growth factor</td>
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<tr>
<td>NM_030979</td>
<td>PABPC3</td>
<td>-77</td>
<td>Poly(A) Binding Protein Cytoplasmic 3</td>
<td>Nucleotide binding</td>
<td>Cytoplasm, extracellular exosome</td>
<td>mRNA metabolic process</td>
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<tr>
<td>AA191576</td>
<td>NPM1</td>
<td>-67</td>
<td>Nucleophosmin</td>
<td>Transcription co-activator, RNA binding, protein kinase co-activator activity</td>
<td>Nucleus, nucleoplasm, nucleolus</td>
<td>DNA repair, nucleosome assembly, intracellular protein transport</td>
</tr>
<tr>
<td>AF315951</td>
<td>NACAP1</td>
<td>-66</td>
<td>Nascent Polypeptide Associated Complex Alpha Subunit Pseudogene 1</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
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<tr>
<td>Accession number</td>
<td>Gene symbol</td>
<td>Fold change</td>
<td>Description</td>
<td>Gene Ontology: Molecular function</td>
<td>Gene Ontology: Cellular component</td>
<td>Gene Ontology: Biological process</td>
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<tr>
<td>AI982754</td>
<td>CLU</td>
<td>-62</td>
<td>-38</td>
<td>Clusterin</td>
<td>Protein binding</td>
<td>Extracellular region, nucleus, cytoplasm, mitochondrion Cell morphogenesis, microglial cell activation, release of cytochrome c from mitochondria, platelet degranulation</td>
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<tr>
<td>NM_006103</td>
<td>WFDC2</td>
<td>-56</td>
<td>-49</td>
<td>WAP Four-Disulfide Core Domain 2</td>
<td>Endopeptidase inhibitor activity</td>
<td>Extracellular space Proteolysis, spermatogenesis, negative regulation of endopeptidase activity</td>
</tr>
<tr>
<td>U62824</td>
<td>HLA-C</td>
<td>-54</td>
<td>-33</td>
<td>Major Histocompatibility Complex, Class I, C</td>
<td>Antigen binding</td>
<td>Golgi membrane, endoplasmic reticulum Antigen processing and presentation via of peptide antigen via MHC class 1</td>
</tr>
<tr>
<td>BF683426</td>
<td>RPL29</td>
<td>-49</td>
<td>-50</td>
<td>Ribosomal Protein L29</td>
<td>Poly(RNA) binding, cadherin binding involved in cell-cell adhesion</td>
<td>Cytosol, cell-cell adherens junction RNA processing</td>
</tr>
<tr>
<td>NM_025029</td>
<td>FAM128B</td>
<td>-49</td>
<td>-42</td>
<td>Mitotic Spindle Organizing Protein 2B</td>
<td>Protein binding</td>
<td>Centrosome, spindle No information available</td>
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<tr>
<td>AI800419</td>
<td>NBPF12</td>
<td>-47</td>
<td>-39</td>
<td>Neuroblastoma Breakpoint Family Member 12</td>
<td>No information</td>
<td>Cytoplasm No information available</td>
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<td>AF274949</td>
<td>HMGN3</td>
<td>-43</td>
<td>-39</td>
<td>High Mobility Group Nucleosomal Binding Domain 3</td>
<td>Chromatin binding</td>
<td>Chromatin, nucleus Regulation of transcription from RNA polymerase II promoter</td>
</tr>
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<td>AK022174</td>
<td>FBXW12</td>
<td>-42</td>
<td>-29</td>
<td>F-Box And WD Repeat Domain Containing 12</td>
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<td>No information No information</td>
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<tr>
<td>AW057781</td>
<td>RPL10</td>
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<td>-58</td>
<td>Ribosomal Protein L10</td>
<td>Poly(RNA) binding</td>
<td>Endoplasmic reticulum Translation, RNA processing</td>
</tr>
<tr>
<td>Accession number</td>
<td>Gene symbol</td>
<td>Fold change</td>
<td>Description</td>
<td>Gene Ontology: Molecular function</td>
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<td>Gene Ontology: Biological process</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>PmP/PmE</td>
<td>TxP/TxE</td>
<td></td>
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<tr>
<td>X79536</td>
<td>HNRNPA1</td>
<td>-41</td>
<td>-38</td>
<td>Heterogeneous Nuclear Ribonucleoprotein A1</td>
<td>RNA binding</td>
<td>nucleus</td>
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<tr>
<td>NM_002547</td>
<td>OPHN1</td>
<td>-40</td>
<td>-33</td>
<td>Oligophrenin 1</td>
<td>Actin binding, GTPase activator activity,</td>
<td>Cytoplasm, cytosol, actin skeleton</td>
</tr>
<tr>
<td>NM_014827</td>
<td>ZC3H11A</td>
<td>-40</td>
<td>-37</td>
<td>Zinc Finger CCCH-Type Containing 11A</td>
<td>Protein binding, poly(A) binding</td>
<td>Transcription export complex, nucleoplasm</td>
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<td>NM_004547</td>
<td>NDUFB4</td>
<td>-39</td>
<td>-57</td>
<td>NADH:Ubiquinone Oxidoreductase Subunit B4</td>
<td>NADH dehydrogenase (ubiquinone) activity</td>
<td>Nucleoplasm, mitochonodrion, mitochondrial inner membrane</td>
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</table>
3.5.2 Pathway Analysis

Pathway analysis of the list of 3272 polyp genes facilitated a more biologically relevant understanding of these genes by mapping them onto well-recognised pathways. One hundred and twenty-eight pathways were found to be significantly regulated. The 20 most significantly regulated pathways, as calculated, are shown in Table 3.4 and are defined according to the RGD pathway portal. This portal uses data derived largely from published scientific reviews, from the Pathway Interaction Database, and from the Kyoto encyclopedia of genes and genomes (KEGG) database. The ontology comprises five nodes; classic metabolic, regulatory, signalling, drug and disease pathways.

Amongst the pathways that were most significantly regulated, 7 were regulatory pathways, and include ‘translation factors’, ‘proteasome degradation’, ‘ribosome biogenesis’, ‘mRNA processing’, ‘androgen receptor signalling’, ‘inhibition of DNA binding signalling’ and ‘insulin signalling’. Ten were signalling pathways and include ‘Notch signalling’, ‘TGF beta signalling’, ‘alpha-6-beta-4 integrin signalling’, ‘interleukin 4 signalling’, ‘interleukin 2 signalling’, ‘interleukin 5 signalling’, ‘epidermal growth factor 1 signalling’, ‘cell adhesion signalling’, ‘interleukin 6 signalling’ and wnt signalling’. Finally, 3 were disease pathways and include ‘colorectal cancer’, ‘chronic myeloid leukaemia’ and ‘Huntington’s disease’.
Table 3.4

20 most significantly regulated gene pathways in endometrial polyps identified using Pathvisio version 1.1, and mapped to headings using the RGC pathway portal.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Number of Genes measured</th>
<th>Number of genes in pathway</th>
<th>% of genes in pathway</th>
<th>( \chi^2 )</th>
<th>P value</th>
<th>Pathway ontology 1</th>
<th>Pathway ontology 2</th>
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<td>Translation Factors</td>
<td>24</td>
<td>50</td>
<td>48.0</td>
<td>12.63</td>
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<td>Regulatory</td>
<td>pertinent to DNA replication and repair, cell cycle, maintenance of genomic integrity, RNA and protein biosynthesis; Translation</td>
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<td>Notch Signalling pathway</td>
<td>21</td>
<td>49</td>
<td>42.9</td>
<td>8.38</td>
<td>0.004</td>
<td>Signalling</td>
<td>Signalling pathway pertinent to development; cell-cell signalling</td>
</tr>
<tr>
<td>Proteasome Degradation</td>
<td>27</td>
<td>66</td>
<td>40.9</td>
<td>9.811</td>
<td>0.002</td>
<td>Regulatory</td>
<td>pertinent to protein folding, sorting, modification, translocation and degradation</td>
</tr>
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<td>Ribosome Biogenesis pathway</td>
<td>35</td>
<td>88</td>
<td>39.8</td>
<td>12.05</td>
<td>0.005</td>
<td>Regulatory</td>
<td>pertinent to DNA replication and repair, cell cycle, maintenance of genomic integrity, RNA and protein biosynthesis; translation</td>
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<tr>
<td>mRNA processing</td>
<td>51</td>
<td>131</td>
<td>38.9</td>
<td>16.56</td>
<td>&lt;0.001</td>
<td>Regulatory</td>
<td>pathway pertinent to DNA replication and repair, cell cycle, maintenance of genomic integrity, RNA and protein biosynthesis</td>
</tr>
<tr>
<td>Androgen Receptor Signalling Pathway</td>
<td>43</td>
<td>114</td>
<td>37.7</td>
<td>12.65</td>
<td>&lt;0.001</td>
<td>Regulatory</td>
<td>pathway pertinent to DNA replication and repair, cell cycle, maintenance of genomic integrity, RNA and protein biosynthesis; transcription</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>31</td>
<td>84</td>
<td>36.9</td>
<td>8.36</td>
<td>0.004</td>
<td>Disease</td>
<td>Cancer pathway</td>
</tr>
<tr>
<td>Inhibitor of DNA binding Signalling Pathway</td>
<td>19</td>
<td>52</td>
<td>36.5</td>
<td>4.71</td>
<td>0.030</td>
<td>Regulatory</td>
<td>pathway pertinent to DNA replication and repair, cell cycle, maintenance of genomic integrity, RNA and protein biosynthesis; transcription</td>
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<td>TGF-beta Signalling Pathway</td>
<td>55</td>
<td>152</td>
<td>36.2</td>
<td>14.38</td>
<td>&lt;0.001</td>
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<td>Growth factor signalling</td>
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<tr>
<td>Pathway</td>
<td>Number of Genes measured</td>
<td>Number of genes in pathway</td>
<td>% of genes in pathway</td>
<td>( \chi^2 )</td>
<td>P value</td>
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<td>Pathway ontology 2</td>
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<td>--------------------------------------------------------</td>
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<td>Alpha6-Beta4 Integrin Signalling Pathway</td>
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<td>67</td>
<td>35.8</td>
<td>5.73</td>
<td>0.017</td>
<td>Signalling</td>
<td>Cell adhesion signalling</td>
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<tr>
<td>Chronic myeloid leukaemia</td>
<td>27</td>
<td>76</td>
<td>35.5</td>
<td>6.36</td>
<td>0.012</td>
<td>Disease</td>
<td>Cancer pathway</td>
</tr>
<tr>
<td>Interleukin-4 Signalling Pathway</td>
<td>22</td>
<td>63</td>
<td>34.9</td>
<td>4.77</td>
<td>0.029</td>
<td>Signalling</td>
<td>Signalling pathway pertinent to immunity; Cytokine mediated signalling pathway</td>
</tr>
<tr>
<td>Interleukin-2 Signalling Pathway</td>
<td>26</td>
<td>76</td>
<td>34.2</td>
<td>5.35</td>
<td>0.021</td>
<td>Signalling</td>
<td>Signalling pathway pertinent to immunity; Cytokine mediated signalling pathway</td>
</tr>
<tr>
<td>Insulin Signalling</td>
<td>54</td>
<td>160</td>
<td>33.8</td>
<td>11.13</td>
<td>&lt;0.001</td>
<td>Regulatory</td>
<td>Energy homeostasis</td>
</tr>
<tr>
<td>Interleukin-5 Signalling Pathway</td>
<td>23</td>
<td>69</td>
<td>33.3</td>
<td>4.23</td>
<td>0.040</td>
<td>Signalling</td>
<td>Signalling pathway pertinent to immunity; Cytokine mediated signalling pathway</td>
</tr>
<tr>
<td>Epidermal growth factor 1 Signalling Pathway</td>
<td>57</td>
<td>177</td>
<td>32.2</td>
<td>15.08</td>
<td>&lt;0.001</td>
<td>Signalling</td>
<td>Growth factor signalling</td>
</tr>
<tr>
<td>Cell adhesion signalling pathway</td>
<td>25</td>
<td>78</td>
<td>32.1</td>
<td>3.971</td>
<td>0.046</td>
<td>Signalling</td>
<td></td>
</tr>
<tr>
<td>Interleukin-6 Signalling Pathway</td>
<td>32</td>
<td>100</td>
<td>32.0</td>
<td>5.19</td>
<td>0.023</td>
<td>Signalling</td>
<td>Signalling pathway pertinent to immunity; Cytokine mediated signalling pathway</td>
</tr>
<tr>
<td>Wnt signalling pathway</td>
<td>35</td>
<td>110</td>
<td>31.8</td>
<td>5.58</td>
<td>0.012</td>
<td>Signalling</td>
<td>Signalling pathway pertinent to development</td>
</tr>
<tr>
<td>Huntington’s Disease</td>
<td>58</td>
<td>192</td>
<td>30.2</td>
<td>7.60</td>
<td>0.006</td>
<td>Disease</td>
<td>Neurodegenerative disease pathway</td>
</tr>
</tbody>
</table>
3.5.3 **Quantitative RT-PCR**

Quantitative RT-PCR analyses of Beta-catenin (CTNNB1), Notch 2 and Wnt5a expression in tamoxifen and postmenopausal polyp RNA are summarised in Table 3.5 and Figure 3.3, and confirm the findings from the Illumina microarray. Expression of CTNNB1, Notch-2 and Wnt 5a were downregulated in tamoxifen polyps compared to postmenopausal polyps.

**Table 3.5**

*Expression of CTNNB1, Notch 2 and Wnt5a in the Illumina microarray and RT-PCR in tamoxifen polyps (n=6) compared with postmenopausal polyps (n=6). The microarray fold change was calculated by dividing the mean array expression values for tamoxifen polyps by the mean array expression value for postmenopausal polyps. The RT-PCR fold change was calculated using the $2^{\Delta\Delta CT}$ method as described by Livak and Schmittgen (137)*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Microarray fold change</th>
<th>RT-PCR fold change (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTNNB1</td>
<td>-1.4</td>
<td>-1.51 (0.20)</td>
</tr>
<tr>
<td>Notch 2</td>
<td>-1.1</td>
<td>-2.12 (0.12)</td>
</tr>
<tr>
<td>Wnt5a</td>
<td>-1.3</td>
<td>-1.67 (0.24)</td>
</tr>
</tbody>
</table>
Figure 3.3

Graph of Gene fold change demonstrated on the Illumina Microarray and by RT-PCR (with genes normalised to GAPDH)
3.6 Discussion

There are a number of platforms available for use to generate microarray data. These platforms differ in the kind of probes used, the hybridization technique the labelling method and the production method (140). In this study, data from Illumina and Affymetrix platforms were combined for pathway analysis. These platforms have a number of key differences. Firstly, Affymetrix arrays are produced by in situ synthesis of 25-mer oligonucleotides, while the Illumina process involves using standard oligonucleotide synthesis methods. The oligonucleotides are attached to microbeads, which are then put onto microarrays using a random self-assembly mechanism. Secondly, Affymetrix uses multiple probes for each gene along with one-base mismatch probes intended as controls for nonspecific hybridization. In contrast, the randomly generated Illumina arrays yield approximately 30 copies of the same oligonucleotide on the array, which provide an internal technical replication. Thirdly, Affymetrix arrays are constructed in a specific layout, with each probe being synthesized at a predefined location, while individual Illumina arrays must undergo a ‘decoding’ step in which the locations of each probe on the array are determined using a ‘molecular address’. Finally, multiple Illumina arrays are placed on the same physical substrate, meaning that hybridization and other steps are performed in a parallel manner, while Affymetrix arrays are processed separately. These are shown in in Figure 3.4 below.

Figure 3.4

Pictures of the Illumina HT-12 beadchip (A) and Affymetrix HU133 chip (B) used in this study.
The validity of combining these 2 microarray platforms to draw conclusions has previously been demonstrated (140). Barnes et al. found there to be very high agreement, particularly for genes that are predicted to be differentially expressed when Illumina and Affymetrix arrays were directly compared. Further studies have confirmed high inter-platform concordance between Affymetrix and Illumina for thousands of genes known to be differentially expressed between 2 reference RNA samples under a range of experimental conditions (141, 142). In addition, these 2 platforms have been shown to demonstrate similar performance in their ability to differentially expressed genes (143).

This study has drawn upon the illumina-affymetrix inter-platform concordance in order to compare gene expression data generated in both platforms. Validated microarray data on human postmenopausal endometrium and tamoxifen endometrium had already been generated within our research group by Dr Taylor and Dr Panchal (unpublished work) and was therefore suitable control material with which to compare postmenopausal and tamoxifen polyp gene expression in order to identify the differences that exist between polyp and endometrium in the postmenopausal and tamoxifen treated states. A number of factors led to the use of an alternative platform to obtain polyp gene expression data. Firstly, the cost implications favoured the use of Illumina. Secondly, the Genetics Department at the University of Leicester possessed an Illumina Beadchip scanner, which meant the polyp microarray experiment could be carried out on-site (whereas the Affymetrix microarray had been performed off-site).

Microarray analysis of human endometrium has previously been reported, but this is the first to examine the differences between endometrium and polyps in women with and without tamoxifen treatment. Post-array analysis has identified a subset of 3272 genes that are significantly regulated in endometrial polyps, and the top 20 up and down regulated are summarised in Table 3.2 and Table 3.3.

Amongst the 20 most upregulated polyp genes is MSH3, upregulated 67 fold in postmenopausal polyps compared to postmenopausal endometrium, and 83 fold in
tamoxifen polyps compared to tamoxifen endometrium. The protein encoded by the MSH3 gene forms a heterodimer with MSH2 to form MutS beta, part of the post-replicative DNA mismatch repair system. Hereditary defects in DNA mismatch repair genes, predominantly MSH2 and MLH1, are responsible for Lynch syndrome, an autosomal dominant condition associated with an increased risk of colorectal cancer and cancers of the endometrium, ovary, stomach, small intestine, hepatobiliary tract, urinary tract, brain, and skin. As a result of defects in the DNA mismatch repair system, tumours that arise in individuals with Lynch syndrome characteristically demonstrate microsatellite instability (144). Although no germ line mutations in MSH3 have been associated with Lynch Syndrome, it is known that mutations in MSH3 lead to a partial defect in mismatch repair, and to some microsatellite instability suggesting a role for MSH3 in the resultant carcinogenesis (145, 146).

Microsatellite instability has been shown to be important in the molecular pathogenesis of endometrial cancer (147), and has been studied in many conditions characterized by polyps, including intestinal, nasal and gastric polyps (148-150). Rios et al. studied microsatellite instability in 109 endometria polyps from pre- and postmenopausal women, and found that it was evident in 6.4% of cases, and associated with multiple polyps rather than single lesions (151). Although microsatellite instability was an infrequent observation in this study, the study population was heterogeneous, and only a small number of microsatellite instability markers were used. In addition, the study did not include polyps from any women taking tamoxifen.

The results from this microarray study, which demonstrated significant alterations on MSH3 expression in polyps, supports a role for an attenuated DNA mismatch repair process and microsatellite instability as a pathogenic mechanism in endometrial polyps.

Amongst the most down regulated genes in this microarray study is whey-acidic-protein four-disulfide core domain protein 2 (WFDC2). It is also known as human epididymis protein 4 (HE4). This gene was down regulated 56 fold in postmenopausal polyps relative to postmenopausal endometrium, and 49 fold in tamoxifen polyps relative to tamoxifen
endometrium. The gene and protein it encodes are highly overexpressed in epithelial ovarian cancer compared to normal ovarian tissue. Galgano et al. also demonstrated that it was over-expressed in endometroid endometrial carcinoma compared with normal endometrium (152). These findings were supported by Deng et al., who also demonstrated that increased expression levels of HE4 protein was associated with malignant biological behaviour of endometrial carcinoma (153). In contrast, the benign polyps in used in this study appear to demonstrate reduced expression of this gene, which supports the low oncogenic potential of the endometrial polyps. Chen et al. have recently demonstrated that WFDC2-knockdown resulted in upregulation of ERβ in human ovarian cell cancer lines, and an inhibition of cell growth and proliferation (154). In contrast, results shown in chapter 2 have already shown a reduced expression of ER-beta in endometrial polyp stroma. These contrasting findings suggest that reduced WFDC2 (HE4) expression may have different downstream effects in endometrial polyp stroma compared with epithelial ovarian cancer cell lines.

Whilst identification of differences in single gene expression is of value, this array has identified numerous genetic differences between polyps and endometrium and therefore infers that numerous gene and protein interactions are likely to result in the formation of endometrial polyps. For this reason, the genetic pathways that describe how multiple genes interact to regulate each other and alter biological processes within the tissue were studied.

Of the pathways identified, two signalling pathways were of particular interest. Firstly, the Notch pathway, which is a fundamental signalling pathway that controls a broad range of events including cell differentiation, proliferation and apoptosis as well as vascular development and angiogenesis (155-157). Notch is a transmembrane receptor and belongs to the growth factor family. It has four isoforms (Notch-1, Notch-2, Notch-3 and Notch-4), each comprising a heterodimer located on the cell surface. Ligands bind to extra-cellular domains. Notch ligands are classified into two structurally related groups: Delta-like ligands (DLL-1, DLL-2, DLL-3 and DLL-4) and Serrate-like ligands (Jagged-1 and Jagged-2). The pathway is activated when a signal-sending cell expressing
a Notch ligand physically interacts with a signal-receiving cell expressing a Notch receptor (158). Notch is cleaved releasing Notch intracellular domain (NICD) which translocates into the nucleus where it regulates the transcription of target genes (159).

Notch receptors are found throughout the endometrium, and these, along with their ligands, have been shown to be differentially expressed within the menstrual cycle with Notch-4 being predominantly expressed in the proliferative phase, Notch-1 and Jagged-1 being predominantly expressed in the secretory phase and the expression of both being decreased in postmenopausal endometrium (160). In addition, Cobellis et al. demonstrated that Notch receptor and ligand expression is dysregulated in endometrial pathologies, including polyps, hyperplasia and carcinoma (160). In support of these findings, this study has identified that the Notch signalling pathway is significantly regulated in endometrial polyps.

Notch signalling has been shown to affect normal and pathological vascular development, and in particular endothelial cell functions including growth, migration and lumen formation (161). There is also evidence that this pathway influences endothelial cell function via modulation of vascular endothelial growth factor (VEGF) signalling (162). Conversely, studies in various model systems have established that the VEGF pathway can regulate the expression of Notch signalling components (163). Erinanc et al. have recently demonstrated that VEGF is expressed more strongly in endometrial polyps than endometrium using immunohistochemistry (164), and suggest that increased blood vessel proliferation may be critical in endometrial polyp growth. These findings, along with those outlined above and the findings from this microarray support a potential role for disordered angiogenesis, triggered by altered Notch signalling and VEGF pathway cross-talk in endometrial polyp pathogenesis.

The second pathway of interest amongst those most significantly regulated in endometrial polyps is the Wnt pathway. Secreted Wnt proteins bind to cell surface receptors known as ‘Frizzled’, which then activate ‘Dishevelled’, which in turn causes inactivation of glycogen synthase kinase 3β. This results in cytoplasmic accumulation of
Beta-catenin molecules that enter the nucleus, to regulate gene transcription (138). This is summarised in Figure 3.5 below.

**Figure 3.5**

The Wnt signalling pathway before and after binding of Wnt, adapted from Wnt Homepage website (165). In the absence of Wnt, a destruction complex consisting of Axin, Adenomatous polyposis coli (APC) and glycogen synthase kinase-3 (GSK3) resides in the cytoplasm, where it binds to and phosphorylates β-catenin, which is then degraded. Disheveled (Dvl) is required for activating the pathway as well. In the nucleus, T cell factor (TCF) is in an inactive state as the consequence of binding to the repressor Groucho. Binding of Wnt to its receptors induces the association of Axin with phosphorylated lipoprotein receptor-related protein (LRP). The destruction complex falls apart, and β-catenin is stabilized, subsequently binding TCF in the nucleus to up-regulate target genes.

The Wnt signalling pathway is important in endometrial epithelial growth and regression (166), and abnormal activation of this pathway way is thought to play an important role in the onset of type 1, estrogen-dependent endometrial cancers via mutations in the
CTNNB1 gene, which codes for the protein β-catenin (167). Evidence from microarray studies confirm that Wnt genes are regulated in the endometrium during the menstrual cycle (168, 169), and in support, Nei et al. found nuclear β-catenin expression was enhanced during the proliferative phase, but mostly located in the cytoplasm and around the cell membrane during the secretory phase (170). These findings support those of Wang et al., who have suggested that prolonged estrogen induced-Wnt signalling triggers endometrial glandular hyperplasia, which may then develop into endometrial cancer (171).

Activation of the canonical Wnt pathway in the colonic epithelium appears to be one of the key events in the polyp initiation process (172), and similarly, this microarray has demonstrated that the Wnt signalling pathway is significantly regulated in endometrial polyps. However, the risk of malignant transformation in these lesions is lower than that associated with endometrial hyperplasia, and that seen in colonic polyps. This suggests that Wnt signalling in endometrial polyps may not trigger carcinogenesis but influence their development via an alternative mechanism. Kiewisz et al., have suggested that estrogen mediates angiogenesis within the endometrium via its effects on the Wnt signalling pathway (173). This could therefore be a possible mechanism for endometrial polyp pathogenesis.

The strength of this study is that endometrium and polyps from two groups of women were compared separately in order to identify genes that are specific to endometrial polyps. However, data from the Affymetrix microarray should be interpreted cautiously due to the lack of biological replicates. A single pooled endometrial RNA sample from tamoxifen and postmenopausal endometrium was used and was deemed necessary due to the small amounts of RNA obtained from atrophic endometrial tissue.

The aim of identifying polyp specific genes not only enables a better understanding of potential mechanisms for development, but also identifies potential therapeutic targets. There is already evidence from clinical trials that the LNG-IUS inhibits polyp formation in tamoxifen treated women, and it is possible that the decidualising action of
levonorgestrel via progesterone receptors in tamoxifen-primed endometrium, and via its influence on these key polyp genes and pathways may play a role. This will be further elucidated in the following chapters.
4 Stromal Decidualisation and IGFBP-1 expression in Endometrium and Serum and of Tamoxifen Treated Women Exposed to Intrauterine Levonorgestrel via the LNG-IUS

4.1 Background

4.1.1 Decidualisation

Decidualisation is a highly regulated process of transformation that occurs in estrogen primed endometrial tissue, under the influence of progesterone, in order that it should become receptive to pregnancy (174). Decidual stromal cells regulate trophoblast invasion, resist inflammatory and oxidative insults, and diminish local maternal immune responses to support pregnancy (175).

The histological changes that occur in the endometrium throughout the menstrual cycle, and that characterise decidualisation were well described by Noyes et al. in 1950 and have been since used to define criteria for endometrial dating (176). They observed that after ovulation, at around day 18 of the menstrual cycle, there was progressive stromal oedema; stromal cells appeared as ‘dense naked nuclei separated by extracellular fluid’ (176). From day 23, the stromal cells were observed to swell, to become ‘pre-decidual’ in type, earlier termed as such because the stromal cells were indistinguishable from decidual cells of pregnancy (177). It has since been observed that whilst large, mature decidual cells can measure over 25 μm in diameter are observed throughout pregnancy, pre-decidual cells seen in the late secretory endometrium are smaller in size, and represent cells at an earlier stage of the decidualisation process (178).

Endometrial stromal cells undergo morphological changes during decidualisation from elongated fibroblast-like cells to enlarged round shaped cells, with polyploid nuclei, increased amount of polyribosomes and rough endoplasmic reticulum (179). This process of stromal cell differentiation occurs firstly near terminal spiral arteries, and then eventually spreads so that pre-decidual cells replace the whole superficial endometrial layer (176). Once decidualised, the endometrium requires progesterone
signalling to continue in order to maintain its function (180), therefore, if implantation of a blastocyst occurs, and progesterone levels rise, then decidualisation continues. However, if implantation does not occur, the resultant fall in progesterone levels trigger a cascade of events that results in apoptosis, proteolytic breakdown of superficial endometrium and menstrual shedding (180, 181).

Microarray analyses of endometrial stromal cells in vitro have demonstrated that significant changes in gene expression profile occur (182, 183), and the downstream results are of a unique proteomic and secretory profile. Decidualised stromal cells differentially express proteins involved in actin cytoskeleton regulation, cell adhesion, protein folding and degradation, redox homeostasis, and signal transduction in comparison with non-decidualised stromal cells (184).

### 4.1.2 Markers of Decidualisation

Along with the characteristic morphological changes to endometrial stromal fibroblasts that occur during decidualisation, insulin-like growth factor binding protein-1 (IGFBP-1) and prolactin are widely used as markers of decidualisation. IGFBP-1 is one of a family of structurally related soluble proteins that modulates the bioavailability of insulin-like growth factor I (IGF-1) – which in turn mediates its action via the type 1 IGF receptor. It has important downstream effects on growth, apoptosis, metabolism and development (185). Endometrial IGFBP-1 expression is modulated throughout the menstrual cycle, being predominantly found in stromal rather than epithelial cells of the secretory endometrium (186). IGFBP-1 mRNA and the protein itself is also highly expressed in the decidua during pregnancy with levels peaking around 16 weeks of gestation (187, 188). One of the key functions of IGFBP-1 is thought to be the stimulation of trophoblastic invasion (189). Dysregulation of the IGF/IGFBP-1 system has been implicated in pregnancy complications such as preeclampsia and fetal growth restriction (185).

Prolactin is also synthesised and secreted during decidualisation of the human endometrial stroma (190). In the non-pregnant uterus, prolactin synthesis occurs in the late secretory phase of the menstrual cycle, and coincides with the first histological signs
of decidualisation. In the event of pregnancy, decidual prolactin secretion increases steadily after implantation as the size and number of decidual cells increase, reaching a peak at about weeks 20–25 of pregnancy. Levels then decline towards term (191). Decidual prolactin has been suggested to stimulate trophoblast growth and invasion, to promote angiogenesis, to modulate uterine natural killer cell survival, to prevent immune rejection, and to regulate water transport across the amnion toward the maternal compartment (192, 193). In the presence of progesterone, the decidualised endometrium secretes prolactin at increasing concentrations and in its absence, prolactin secretion ceases within 2–3 days (194).

### 4.1.3 Levonorgestrel

Levonorgestrel is a synthetic progestin derived from 19-nor testosterone that has a range of therapeutic uses. It is used in a single oral dose of 1.5 mg for emergency contraception and prevents fertilisation by inhibiting ovulation (195). Levonorgestrel is also used orally at a lower dose (30 µg) as a ‘progestrone only’ contraceptive where its effects are primarily brought about through changes in cervical mucus and to a lesser degree, the suppression of ovulation (196, 197). It is used in conjunction with ethinyl estradiol at doses ranging from 90 µg – 250 µg in the combined oral contraceptive pill, which prevents pregnancy primarily through its interaction with the hypothalamo-pituitary-ovarian axis to inhibit ovulation (198). Levonorgestrel can be administered transdermally at a dose of 10 µg per 24 hours together with ethinyl estradiol as hormone replacement therapy, where it counters the proliferative effects of unopposed estrogen that could lead to hyperplasia (199).

Intrauterine levonorgestrel can be delivered via an intra-uterine system (IUS) using the Mirena® device. The Mirena® comprises a plastic T-shaped frame with a reservoir containing 52 mg of levonorgestrel that is released into the uterine cavity at a rate of 20 µg per 24 hours. Maximum serum levels of levonorgestrel have been shown to be reached within a few hours of insertion, and to be maintained at 150-200 pg per ml (200). It is licensed for use as a contraceptive for five years from insertion. The Mirena® is also licensed for the treatment of menorrhagia, and for preventing endometrial
hyperplasia with estrogen replacement therapy (201). In addition to these uses, Mirena® has also been shown to be effective at treating pelvic pain associated with endometriosis and adenomyosis, as well as in treating atypical endometrial hyperplasia (202, 203).

4.1.4 **Endometrial Effects of Levonorgestrel**

Intrauterine administration of levonorgestrel results in profound effects on endometrial morphology and function and in premenopausal women; these effects are similar regardless of the phase of menstrual cycle. Critchley *et al.* demonstrated that histological changes following insertion of the LNG-IUS are not limited to the contact site (204). Within a month of intrauterine levonorgestrel exposure, endometrial glands have been shown to become largely atrophic (204-206). Hejmadi *et al.* studied endometrial biopsies of 106 women who had been fitted with Mirena® for between 4 and 70 months (207). Although the indications for Mirena® insertion and menopausal status were not described in the study, all patients were required to undergo sequential endometrial sampling. They found that with regard to the glands, 80% of specimens had purely atrophic glands, 17% of specimens retained some glands with secretory morphology amongst the atrophic glands, whereas 2% exhibited predominantly secretory glands with sparse atrophic glands. Although this spectrum of change is likely to be related to duration of exposure, it was not correlated. That study also confirmed 1 case of simple hyperplasia, 3 cases of complex hyperplasia without atypia and 1 case of complex hyperplasia with atypia. However, given that the endometrial status prior to Mirena® insertion was not known in these cases, it is not possible to say whether these histological appearances represent a regression or progression of pathology.

Under the influence of intrauterine levonorgestrel, stromal cells take on the decidual morphology, forming ‘sheets of plump, polygonal pseudodecidualised cells’ (207). However, this may not necessarily occur throughout the endometrium. Critchley *et al.* found that stromal cells nearest to the LNG-IUS remained more typically rounded, whereas stromal cells sampled from locations further away from the LNG-IUS were spindle-shaped. The authors suggest that the more remote decidualisation was
‘diminished in intensity’ (204). Although these changes take place within a month of exposure to LNG-IUS, there is evidence of a more prominent stromal decidual change with a longer duration of exposure (205). In addition to the characteristic stromal and glandular changes seen with the LNG-IUS, stromal leukocytic infiltrates with foci of necrosis and scarring may be observed particularly after 3 years of use, along with altered spiral artery formation (205, 208).

The local administration of levonorgestrel via an IUS also has important effects on the biochemistry of the endometrium. In keeping with the decidual phenotype, prolactin and IGFBP-1 are strongly expressed in endometrial tissue (208, 209), and there is a significant reduction in the expression of both estrogen and progesterone receptor subtypes in both glands and stroma following LNG-IUS insertion (206).

### 4.1.5 The Effect of Levonorgestrel on Tamoxifen Treated Endometrium

As already discussed, tamoxifen is a selective estrogen receptor modulator that has been widely used as adjuvant therapy for breast cancer. It acts as a competitive estrogen receptor antagonist in breast tissue and through this action, significantly improves overall survival in women with estrogen receptor positive disease (33). However, tamoxifen exerts a proliferative, estrogen-like effect on the endometrium, increasing the risk of developing endometrial polyps, hyperplasia and endometrial cancer.

A recent Cochrane review (published in 2015) concluded that the LNG-IUS in tamoxifen users led to a significant reduction in the incidence of endometrial polyps over both a 12-month period (OR 0.22, 95% CI 0.08 to 0.64) and over a long-term follow-up period of 24 to 60 months (OR 0.22, 95% CI 0.13 to 0.39) (96). In addition, the authors found that the LNG-IUS in tamoxifen users led to a reduction in the incidence of endometrial hyperplasia over a long-term follow-up period (OR 0.13, 95% CI 0.03 to 0.6) (96). The mechanisms by which levonorgestrel exerts this protective endometrial effect are uncertain but may be linked to stromal decidualisation.
4.2 Aims

The aims of this study were to confirm the process of stromal decidualisation in tamoxifen treated postmenopausal women following LNG-IUS insertion by endometrial histology and immunohistochemistry for IGFBP-1 expression. The systemic effect induced by the LNG-IUS in this time frame was assessed by measuring serum IGFBP-1 concentrations using an ELISA technique.
4.3 Materials and Methods

All samples were collected at Leicester Royal Infirmary, according to Leicestershire Health Authority Ethics Committee approval (Ethics committee reference number REC 6498). Written consent was obtained from all women prior to collecting samples.

4.3.1 Tissue and Serum Samples

Endometrial pipelle biopsies and serum samples were obtained from 20 postmenopausal women with breast cancer who required adjuvant treatment with tamoxifen at a dose of 20 mg daily. None had received progestin therapy since their diagnosis of breast cancer. Ten women were randomly allocated to receive the LNG-IUS, which was inserted at the start of tamoxifen treatment, and 10 women were randomly allocated to the control group. All women had endometrial pipelle biopsies and venous blood samples obtained at the start of treatment, and again after 12 months. The samples obtained from the control group at entry are denoted C-0 and after 12 months, C-12. Samples obtained from the LNG-IUS group at entry are denoted LNG-0 and after 12 months, LNG-12.

Endometrial pipelle samples were fixed in formalin at room temperature for 2 days before being processed and embedded in paraffin blocks. Five µm-thick sections were cut from each block and mounted on silane-coated glass slides for immunohistochemistry. A further 5 µm-thick section was mounted on a glass slide and stained with haematoxylin and eosin for light microscopy for histological diagnosis. Histological reporting was carried out by a consultant histopathologist at Leicester Royal Infirmary.

Venous blood (5 ml) was collected using serum-separating vacutainers from the participants’ antecubital fossae using an aseptic technique. Samples were transported to the laboratory at room temperature and centrifuged at 1200g for 30 minutes. Two ml of supernatant comprising serum was taken from each specimen and stored at -20°C until analysis.
### 4.3.2 Immunohistochemistry

#### Table 4.1

*Primary and secondary antibody manufacturers, antibody concentrations, dilutions and diluents along with positive control tissue used*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer [IgG]</th>
<th>Dilution [IgG]</th>
<th>Diluent</th>
<th>Control tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Antibody</td>
<td>Anti human IGFBP-1 (H-3)</td>
<td>1:125</td>
<td>10% NRS in PBS 3% BSA</td>
<td>Trophoblast from 1st trimester pregnancy</td>
</tr>
<tr>
<td>Negative control</td>
<td>Mouse IgG</td>
<td>As per primary antibody</td>
<td>As per primary antibody</td>
<td></td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Biotinylated rabbit (Fab')2 anti mouse</td>
<td>1:400</td>
<td>PBS</td>
<td></td>
</tr>
</tbody>
</table>

Standard immunohistochemistry protocols were used to identify the presence and distribution of IGFBP-1 in the endometrial pipelle samples using the primary and secondary antibodies summarised in Table 4.1 above. The methodology has been described in detail in chapter 2. Briefly, after dewaxing and rehydration, antigen retrieval was carried out by microwaving the slides in 10 mM citric acid buffer (pH 6.0) for 20 minutes at 800 Watts. Endogenous peroxidise was suppressed by incubating slides in 6% hydrogen peroxide. Normal rabbit serum was applied to avoid non-specific antibody binding. Endogenous avidin and biotin sites were blocked using Avidin-Biotin blocking solutions and the test slides and positive control tissues incubated with primary antibody, anti-human IGFBP-1 (Santa Cruz) at a concentration of 1:125. Mouse IgG (Vector) at a corresponding dilution was applied to the negative control slide. After incubation overnight in humid chambers and careful washing, the secondary antibody biotinylated rabbit anti-mouse IgG (Dako) diluted to 1:400 was applied to all slides. Finally, HRP-conjugated avidin-biotin complexes (Vector) was used to identify biotinylated secondary antibodies, with DAB substrate used as the chromogen. After counterstaining with haematoxylin, slides were finally dehydrated through graded alcohol and mounted in XAM ready for microscopy.
4.3.3 ELISA

Serum IGFBP-1 concentrations were measured using the DSL-10-7800 ACTIVE® Total IGFBP-1 ELISA Kit according to the manufacturer’s instructions. Alongside the 7 Standards and 2 Control samples with known IGFBP-1 concentrations, were the 40 study samples and 3 serum samples obtained from women in the 1st trimester of pregnancy, which were used as positive controls. As reported by the manufacturer the assay has a minimum detection limit of 0.25 ng/ml. The intra-assay coefficient of variation is 1.7–4.6% and the inter-assay coefficient of variation is 6.2–7.6%.

Study samples, manufacturer’s standards and controls were thawed, and along with all reagents brought to room temperature. Twenty five µL of the manufacturer’s standards and controls and study samples was added in duplicate to appropriate microtitration wells coated with anti-IGFBP-1 antibody. After the addition of 50 µL of assay buffer, wells were incubated at room temperature on an orbital shaker at 750 rpm for 1 hour. Non-bound reagent in the wells were then aspirated and the contents washed 5 times using the pre-prepared Wash solution.

One hundred µL of Antibody-Enzyme conjugate was then added to each well, after which wells were again incubated at room temperature on an orbital microplate shaker at 750 rpm for 30 minutes, the unreacted conjugate aspirated and the contents washed 5 times with the wash solution. Tetramethylbenzidine (TMB) chromagen solution (100 µL) was then added to each well and incubated at room temperature on an orbital microplate shaker at 750 rpm for 10 minutes and then 100 µL of acidic stopping solution was added. Absorbance of the solutions in each well was read using a Multiskan Ascent ELISA plate reader (Labsystems), with the detection filter set at 450 nm and the reference set at 620 nm. Background absorbance (620 nm) was subtracted from the detected 450 nm values.

The mean absorbance for each standard, control and study sample was calculated from the duplicate values. A standard curve of absorbance versus IGFBP-1 concentration was generated using the standard control samples supplied. IGFBP-1 concentrations in the
study serum samples were calculated by interpolation from the standard curve. Normality testing carried out using the D’Agostino-Pearson omnibus normality test confirmed that the data were sampled from a Gaussian distribution.

The mean and standard error of IGFBP-1 concentrations at entry in both the control group (C-0) and the LNG-IUS group (LNG-0) and at 12 months in both groups (C-12, LNG-12) were calculated and compared using a one-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons; p < 0.05 was considered significant. Data were analysed using Graphpad Prism (version 6.07).
4.4 Results

The mean duration of tamoxifen treatment when the 1st endometrial pipelle biopsy and blood samples were obtained was 34.4 days (SD 25 days) and was not significantly different between the control and intervention groups.

4.4.1 Immunohistochemistry

At entry (C-0 and LNG-0), 7 out of 10 samples in each group were reported as either ‘atrophic’ or ‘inactive’. Atrophic endometrium arises in response to the diminished stimulatory effect of estrogen and is a normal finding in postmenopausal women. Epithelial glands are typically small, narrow, tubular and are lined with ‘inactive’ cubo-columnar epithelium showing neither secretory nor proliferative activity with no mitoses. Stroma is typically compact, with scanty cytoplasm (210). Three out of 10 samples in each group at entry contained insufficient tissue for histological diagnosis.

After 12 months of tamoxifen treatment, 5 out 10 samples in the control group (C-12) had atrophic endometrium, whilst the remainder contained insufficient tissue for histological analysis. By contrast, in the treatment group (LNG-12) after 12 months of concurrent oral tamoxifen and intrauterine levonorgestrel treatment, 9 out of 10 samples showed evidence of stromal decidualisation not seen in any of the other groups. Histological findings of the endometrial pipelle biopsies are summarised in Table 4.2.

Immunohistochemistry staining for IGFBP-1 expression was performed only on the samples where there was adequate tissue for histological reporting (7 samples in the C-0 group, 7 samples in the LNG-0 group, 5 samples in the C-12 group and 9 samples in the LNG-12 group). IGFBP-1 staining was not present in any of the C-0, C-12 or LNG-0 samples, but was present in stromal cells of all of the LNG-12 samples where decidualisation had been confirmed. The photomicrographs in Figure 4.1 show IGFBP-1 staining in the decidualised stromal cells of endometrial biopsy samples obtained after 12 months of concurrent oral tamoxifen and intrauterine levonorgestrel treatment.
Table 4.2

Key histology findings of endometrial pipelle samples in the control and levonorgestrel treated groups at entry (C-0, LNG-0) and after 12 months (C-12 and LNG-12).

<table>
<thead>
<tr>
<th>Histology reporting</th>
<th>Control group (n=10)</th>
<th>LNG-IUS group (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-0</td>
<td>C-12</td>
</tr>
<tr>
<td>Inadequate for analysis</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Inactive glands or Atrophic</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Stromal decidualisation</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 4.1

IGFBP-1 staining in endometrial biopsy samples from tamoxifen treated women at entry and after 12 months with and without the LNG-IUS (200 x magnification).

Plate A Tamoxifen treated at entry (C-0); Plate B Tamoxifen treated after 12 months (C-12); plate C Tamoxifen + LNG-IUS at entry (LNG-0); Plate D Tamoxifen + LNG-IUS after 12 months (LNG-12) showing positive IGFBP-1 staining in stromal cells; Plate E 1st trimester decidua showing positive IGFBP-1 staining (positive control). The size bar = 50 µm.
4.4.2 ELISA

Serum from all study participants at entry and after 12 months was obtained and their IGFBP-1 concentrations ascertained using an ELISA kit. The standard curve generated from duplicates of the standard control samples provided with known IGFBP-1 concentrations is shown in Figure 4.2. This was used to interpolate experimental values.

Figure 4.2

Graph of the mean absorbance of duplicates of the 7 standard control samples with known IGFBP-1 concentrations.

The mean serum IGFBP-1 concentration at entry was 17.41 ng/ml (SEM 3.68) in the control group, and 22.32 ng/ml (SEM 5.33) in the LNG-IUS group. These values were not significantly different ($p = 0.97$). The mean IGFBP-1 concentration in the positive control serum samples obtained from women in the first trimester of pregnancy was 43.71 ng/ml (SEM 4.73). Although this was approximately double the concentrations found in the two entry groups, the differences were not statistically significant ($p = 0.19$ when compared with the control group at entry, and $p = 0.38$ when compared with the LNG-IUS group at entry). After 12 months, the mean serum IGFBP-1 concentration in the
control group had doubled to 34.72 ng/ml (SEM 9.34), but this difference was not statistically significant ($p = 0.22$). In the LNG-IUS group, the mean serum IGFBP-1 concentration had remained virtually static, measuring 20.68 ng/ml (SEM 2.49). This difference was also not statistically significant ($p = 0.99$). These results are summarised in Table 4.3 and Figure 4.3.

**Table 4.3**

*Serum IGFBP-1 concentrations in control and LNG-IUS groups at entry and 12 months using ELISA. The mean serum IGFBP-1 concentration in the positive control samples obtained from women in the 1st trimester of pregnancy was 43.71 ng/ml, (SEM 4.73).*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control C-0 [IGFBP-1] ng/ml</th>
<th>Control C-12 [IGFBP-1] ng/ml</th>
<th>LNG-IUS LNG-0 [IGFBP-1] ng/ml</th>
<th>LNG-IUS LNG-12 [IGFBP-1] ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.97</td>
<td>7.64</td>
<td>11</td>
<td>10.84</td>
</tr>
<tr>
<td>2</td>
<td>12.22</td>
<td>21.60</td>
<td>12</td>
<td>16.18</td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
<td>16.89</td>
<td>38.50</td>
<td>14</td>
<td>15.80</td>
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<tr>
<td>5</td>
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<td>78.05</td>
<td>15</td>
<td>54.83</td>
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<tr>
<td>6</td>
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<td>18</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.49</td>
</tr>
</tbody>
</table>
**Figure 4.3**

Graph of serum IGFBP-1 concentrations in control and LNG-IUS treated patients at entry and 12 months. Values plotted are mean and SEM. Differences between groups was tested using one-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons. None of the differences were found to be statistically significant.
4.5 Discussion

The endometrial effects of concurrent tamoxifen and either oral or intra-uterine progestin treatment have been previously reported, however, the occurrence of stromal decidualisation in response to this treatment does not appear to be a universal finding. The earliest study to report stromal decidualisation in association with tamoxifen and progestin was by Cohen et al. in 1996 (211). In their study, 12 postmenopausal women with breast cancer who were taking tamoxifen (20-30 mg daily) were studied. All were prescribed high dose progestins, either oral megestrol 40 mg four times daily, or medroxyprogesterone acetate 100 mg four times daily, for metastatic disease. Endometrial biopsies confirmed stromal decidualisation occurred in 11 out of 12 patients. The mean duration of combined tamoxifen and progestagen therapy was 5.6 months. In contrast, Powles et al. studied 51 postmenopausal women at high risk of developing breast cancer who were using tamoxifen (20 mg daily) as chemoprevention and were found to have an endometrial thickness of more than 8 mm on transvaginal ultrasound (212). All were given 3 months of cyclical oral norethisterone 2.5 mg for 21 days out of 28. After progestin treatment, although the authors found that pipelle biopsies retrieved insufficient endometrial tissue for analysis from 78% of patients, they did not report any cases of stromal decidualisation.

More recently, 4 randomised trials have been carried out to investigate the endometrial protective effect associated with the LNG-IUS in postmenopausal women taking tamoxifen for breast cancer. Firstly, Gardner et al. randomised 122 postmenopausal women who had been taking tamoxifen (20 mg daily) for a year or more either to receive a LNG-IUS along with endometrial surveillance (treatment group), or endometrial surveillance alone (control group) (94). After 12 months of follow-up, all those from the treatment group from whom adequate endometrial samples were obtained showed decidual changes, compared with none in the control group. In 2009, when the 5-year follow-up was reported, decidualised endometrium was found in those from the treatment group who completed follow-up and still had the LNG-IUS in situ (95). No participants in the control group showed decidualised endometrium.
Secondly, Chan et al. conducted a randomised trial, where 129 women with early stage breast cancer taking tamoxifen (dose not specified) as adjuvant therapy were randomised to either a similar treatment group (LNG-IUS with endometrial surveillance) at the start of tamoxifen treatment, or a control group (endometrial surveillance alone) (213). After 12 months, 91% of those in the treatment group who completed follow-up were reported to have ‘atrophic or inactive endometrium’, compared to 33% in control group (p < 0.001 using Chi-squared test). There were no reported cases of stromal decidualisation. Wong et al. reported findings after 5 years of follow-up of the original trial cohort (214). They reported evidence of an unspecified ‘progestagen effect’ in just 2 out of the 46 women (4.3%) randomised to the treatment group after 5 years of follow-up. The remainder had persistently ‘inactive or atrophic’ endometrium. The fact that intrauterine levonorgestrel was administered at the start of tamoxifen treatment may have affected its ability to induce such widespread stromal decidualisation as seen in Gardner’s study cohort.

Thirdly, Kesin et al. randomised 148 postmenopausal breast cancer patients taking tamoxifen (5 mg daily) for more than 12 months to similar treatment and control groups (215). After 36 months of follow-up, they reported stromal decidualisation in 43% of participants in the treatment group, compared with none in the control group. The lower dose of tamoxifen used in this study may have offered less estrogenic priming of the endometrium thereby making levonorgestrel less able to induce a strong stromal decidual response.

Finally, and most recently Omar et al. randomised 150 pre- and postmenopausal women with early stage breast cancer taking tamoxifen (dose not specified) as adjuvant therapy to similar treatment and control groups (216). The LNG-IUS was inserted before tamoxifen was commenced. After 2 years of follow-up, none of the participants in the treatment group showed evidence of stromal decidualisation. These findings support those of Chan et al., and raise the possibility that the timing of intra-uterine levonorgestrel treatment influences the ability of the endometrial stroma to undergo a decidual change.
In support of Gardner et al., yet in conflict with the findings of Chan et al. and Omar et al., the study presented in this chapter has demonstrated histological evidence of stromal decidualisation in postmenopausal women who received the LNG-IUS at the onset of tamoxifen treatment (95, 213). In addition, it goes further than other studies by demonstrating histological changes were associated with the expression of IGFBP-1, a key marker of decidualisation. It is likely therefore that stromal decidualisation is a central mechanism for levonorgestrel’s ‘progestagenic antagonism’ of tamoxifen’s effects on the endometrium (95).

The time frame for the appearance of this change (i.e. 12 months) is beyond that of Cohen’s study where high dose oral progestin treatment was used, but in keeping with the findings of Gardner et al. (94) who reported decidualisation after 12 months of LNG-IUS treatment. The use of a 12 month time frame for assessing a response to treatment also facilitated a direct comparison with Chan et al., who reported their findings at this point (213).

Serum levels of IGFBP-1 in women treated with the LNG-IUS did not significantly alter after 12 months. This was to be expected, as it has previously been shown that the changes in endometrial IGFBP-1 that occur during the normal menstrual cycle are not reflected in serum levels (217). In addition, Pakarinen et al. found no change in serum IGFBP-1 levels in a cohort of pre-menopausal women after 3 months of treatment with Mirena® (218). This finding is in contrast to that found in pregnancy, where the decidua is the primary source of IGFBP-1 in maternal serum (219). These findings suggest that the decidualisation induced by LNG-IUS treatment is different to the true decidua of pregnancy. Although both circumstances produce decidualised cells, in the case of LNG-IUS, the decidual layer is stable, whereas the function of decidua in pregnancy is to facilitate trophoblast invasion, with the resultant tissue damage possibly leading to the release of decidual IGFBP-1 into the circulation. The increase in serum IGFBP-1 seen in the control group after 12 months of tamoxifen treatment, although not statistically significant, is also in keeping the findings of other studies. Tamoxifen has been shown to supress plasma levels of IGF-1, an important
mediator of estrogen action, most of which circulates bound to IGF binding proteins (220). Lonning et al. confirmed that in addition to its effect on IGF-1, tamoxifen treatment increased plasma IGFBP-I concentration by a mean value of 78% in postmenopausal breast cancer patients, supporting the theory of its ‘estrogen agonistic’ effects (221).

The mechanisms whereby stromal decidualisation, produced by levonorgestrel and tamoxifen treatment, modulate endometrial polyp formation will be studied in the following chapter.
5 Effect of decidualisation on Beta-catenin and Notch-2 Expression in Tamoxifen and Estrogen-Primed Endometrial and Polyp Stromal Cells

5.1 Background

The tissue microarray described in chapter 3 revealed a group of genes whose expression is significantly altered in endometrial polyps compared with endometrium. After mapping these polyp genes onto known biological pathways, the Wnt and Notch signalling pathways were found to be amongst those most significantly regulated suggesting that these pathways play a key role in the development of endometrial polyps. The possible mechanisms whereby Wnt and Notch signalling pathways could influence polyp formation have been discussed previously, and include modulation of VEGF signalling, disordered angiogenesis, and an association with pathways leading to endometrial carcinogenesis. However, this was a tissue-wide microarray examining gene expression across all cell types within the samples used, and was not specific to stromal cells.

There is an increasing body of evidence to support intrauterine levonorgestrel’s ability to protect the endometrium and prevent polyps from forming in tamoxifen treated women (96), although its ability to induce decidualisation in this context has been variably reported (95, 213, 215, 216). In the previous chapter, the decidualising effect of intrauterine levonorgestrel on postmenopausal tamoxifen-treated endometrium was confirmed both by demonstrating the appearance of decidualised stroma histologically, and by the expression if IGFBP-1 in these stromal cells using immunohistochemistry. This therefore raises the possibility that levonorgestrel prevents polyp formation via decidualisation and the subsequent inhibition of key genetic pathways, including the Wnt and Notch pathways.

Although there is evidence for epithelial and stromal cell cross talk in the process of decidualisation, the cellular changes that occur during this process are confined to the stromal compartment. Using an *in-vitro* model of endometrial and polyp stromal cells
will allow for further study of the effects of decidualisation on the genes involved in these 2 polyp pathways.

**Figure 5.1**

Pictomicrographs of decidualised human endometrial stromal cells from published literature. **Plates A and B** are endometrial stromal cells as shown by Gellersen et al. grown to confluence, showing an undifferentiated, spindle shaped phenotype (plate A) and then following treated with either 8-br-cAMP alone or in combination with a progestin showing an altered, more rounded decidual cell phenotype (Plate B) (222).

**Plates C and D** endometrial stromal cells as shown by Matsumotu et al. grown to confluence showing a spindle-shaped phenotype (plate C) and then treated with estradiol (10^8 M) and medroxyprogesterone acetate (10^6 M) for 28 days showing a decidual phenotype (plate D) – albeit less pronounced than that seen in plate B (223).

Synthetic progestins are used in clinical practice to mimic the actions of endogenous progesterone. They are either derived from testosterone via 19-nortestosterone, from progesterone via 17-hydroxyprogesterone and 19-norprogesterone or from spironolactone (224, 225). Although their biological potency varies depending on the
end point measured, the so called’ second and third generation compounds’ are the most active producing ovulation inhibition and endometrial transformation at much lower doses (225). Synthetic progestins exhibit varying pharmacokinetic properties and have different binding affinities towards serum globulin proteins and intracellular steroid receptors, which influences not only their free unbound concentrations, but also that of endogenous steroids which compete and bind with the same proteins (225, 226).

Medroxyprogesterone acetate, widely used as an oral or injectable contraceptive, as the progestin component of HRT, as well as treatment for endometriosis. It is derived from 17-hydroxyprogesterone and acts as an agonist at the progesterone, androgen and glucocorticoid receptors. However, it has an insignificant affinity for mineralocorticoid and estrogen receptors (227). By contrast, levonorgestrel is a second-generation progestin but despite having a similar range of clinical uses, unlike medroxyprogesterone acetate, is derived from 19-nortestosterone. Compared with medroxyprogesterone acetate, it has a stronger affinity for the progesterone, androgen and mineralocorticoid receptors, but a lower glucocorticoid receptor affinity. Desogestrel is a third-generation progestin also derived from 19-nortestosterone. It is used as an oral contraceptive and its active metabolite is 3-ketogestrel, also known as etonogestrel, used in the subdermal contraceptive implant and intra-vaginal contraceptive ring. Desogestrel and etonogestrel are highly selective for the progesterone receptor and have a low binding affinity for the androgen receptor (226).

Although the actions of these compounds are similar in terms of their progestagenic effects, progestins have been shown to produce marked intracellular differences. Kayisli et al. (228) compared changes in global gene expression profile in human endometrial stromal cells in culture in response progesterone and progestins (medroxyprogesterone acetate and etonogestrel) using Illumina’s Human HT–12 v4 Expression BeadChip. They identified common and unique alterations in gene expression triggered by each progestin. Although they did not specifically use decidualisation as an endpoint, their findings support the importance of considering the effects of progestins individually rather than as a class.
Reports from case series and randomised controlled trials have demonstrated that decidualisation in tamoxifen treated endometrium occurs after treatment with oral megestrol, oral medroxyprogesterone acetate and intrauterine levonorgestrel, although the effect on polyp formation has only been reported with intrauterine levonorgestrel (94, 95, 211). The ability of other progestins to produce this polyp inhibitory effect in tamoxifen treated women remains unknown.
5.2 Aim

The aim of this study was to establish an *in-vitro* model for decidualisation of human endometrial and polyp stromal cells with 3 clinically relevant progestins in conjunction with 8-br-cAMP and either 17β-estradiol or tamoxifen. Firstly, the effect of 17β-estradiol and tamoxifen treatment in conjunction with progestins on the IGFBP-1 concentration in cell culture supernatant were tested using ELISA. Secondly, using quantitative RT-PCR, the effect of decidualisation on the expression of 2 genes from the canonical Wnt and Notch signalling pathways shown to be significantly regulated in endometrial polyps, Beta-catenin and Notch-2 expression, were evaluated.
5.3 Materials and Method

5.3.1 Specimens

All samples were collected at Leicester Royal Infirmary, with approval from Leicestershire Health Authority Ethics Committee (Ethics committee reference number REC 6498). Written consent was obtained from all women prior to collecting samples.

Normal endometrial stromal cells were cultured from 3 pre-menopausal uteri removed by hysterectomy for benign indications (menorrhagia, multiple fibroids and dysmenorrhoea; histological examination by a consultant histopathologist in all cases concluded none had evidence of CIN or endometrial polyps or hyperplasia). The endometrium of all uteri was classified as secretory according to histological criteria (229). Prior to formalin fixing, the anterior wall of the uterus was incised from the fundus to the cervix to expose the endometrial cavity and endometrial tissue was collected using a curette. Stromal cells were also cultured from 3 benign endometrial polyps collected by hysteroscopic laser polypectomy from women presenting with postmenopausal bleeding. All polyps were confirmed by a consultant histopathologist to be benign with no features of hyperplasia, atypia or malignancy. Following polypectomy, the sample was divided in order that part could be formalin fixed for histology and the remainder for cell culture.

5.3.2 Preparation and Culture of Stromal Cells

Tissue collected immediately after the surgical specimens were removed, were transported to the laboratory in 25ml of ice-cold PBS (GIBCO™, ThermoFisher Scientific) and macerated into 1mm³ pieces with sterile scalpels and added to 25ml of DMEM:F12 culture media (GIBCO™, ThermoFisher Scientific) containing 10% FBS (Sigma-Aldrich), 1% of an antibiotic and antifungal mix containing 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone® Antimycotic (GIBCO™, ThermoFisher Scientific). At this point, 50 mg of collagenase (Sigma-Aldrich) was added and the mixture shaken in a water bath at 37°C for 2 hours and 30 minutes.
The suspension was passed through a 70-micron filter to remove mucus and undigested tissue matter and then a 40-micron filter to remove endometrial glands. The remaining cell mixture was centrifuged at 1000g for 5 minutes and the pellet re-suspended in a further 20 ml of DMEM:F12 media containing 10% FBS, 1% penicillin-streptomycin and fungizone®. After repeating the centrifugation and resuspension process twice more, cells were transferred to a sterile 75cm² vented flask in 30 ml of DMEM:F12 media containing 10% FBS, 1% penicillin-streptomycin and fungizone® and incubated at 37°C with 5% CO₂ in air. Cells were grown to confluence with a change of media every 48 hours. Once confluent, culture media was removed, cells washed with 10 ml of sterile PBS and then incubated with 2.5ml of trypsin/EDTA solution at 37°C for 3 minutes in order to re-suspend them into solution. The trypsinisation process was stopped by adding 10 ml of DMEM:F12 media containing 10% FBS, 1% penicillin-streptomycin and fungizone®. Cells were then re-seeded into 3 x 12-well plates (36 wells) at a density of 10⁵ cells per well with 2ml of DMEM:F12 media containing 10% FBS, 1% penicillin-streptomycin and fungizone® per well and again grown to confluence. Media was changed every 48 hours.

Once cells reached confluence in the 12-well plate, the culture media was changed for phenol-free DMEM:F12 (GIBCO™, ThermoFisher Scientific) with 2% charcoal striped FBS (GIBCO™ ThermoFisher Scientific) and 1% penicillin-streptomycin and fungizone®. Cells were incubated for a further 24 hours before exposing them to range of hormonal treatment regimens. The treatments are summarised in Table 5.1. The 17β-estradiol (Sigma-Aldrich), tamoxifen (Sigma-Aldrich), medroxyprogesterone acetate (Sigma-Aldrich), levonorgestrel (Sigma-Aldrich) and desogestrel (Sigma-Aldrich) were all dissolved in 0.1% ethanol, whilst 8-bromo adenosine-3’, 5’-cyclic monophosphate (8-br-cAMP) (Sigma-Aldrich) was dissolved in culture media.

Cells were maintained in culture for 21 days to allow decidualisation to occur, with experimental media being changed every 48 hours and with cells being examined under a light microscope for evidence of the presence of decidual cell morphology.
Hormonal treatments applied to stromal cells derived from 3 uteri and 3 polyps cultured in 12-well plates. Each treatment was repeated in triplicate.

Abbreviations: M = phenol-free DMEM:F12 with 2% charcoal striped FBS, 1% penicillin-streptomycin and fungizone®; cAMP = 0.5mM 8-bromo adenosine 3',5'-cyclic monophosphate; E2 = 17β-estradiol; TX = tamoxifen; MPA = medroxyprogesterone acetate; LNG = levonorgestrel; DES = desogestrel

<table>
<thead>
<tr>
<th>Plate</th>
<th>Well</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
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<td>M + 0.5mM 8-br-cAMP + 0.1% ethanol</td>
</tr>
<tr>
<td></td>
<td>4, 5, 6</td>
<td>M + 0.5mM 8-br-cAMP + 10^6 M E2</td>
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<tr>
<td></td>
<td>7, 8, 9</td>
<td>M + 0.5mM 8-br-cAMP + 10^6 M Tx</td>
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<tr>
<td></td>
<td>10, 11, 12</td>
<td>M + 0.5mM 8-br-cAMP + 10^6 M MPA</td>
</tr>
<tr>
<td>B</td>
<td>13, 14, 15</td>
<td>M + 0.5mM 8-br-cAMP + 10^6 M LNG</td>
</tr>
<tr>
<td></td>
<td>16, 17, 18</td>
<td>M + 0.5mM 8-br-cAMP + 10^6 M DES</td>
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<td>34, 35, 36</td>
<td>M + 0.5mM 8-br-cAMP + 10^6 M Tx + 10^6 M DES</td>
</tr>
</tbody>
</table>

5.3.3 ELISA

On day 21, media from triplicates of the control group and 5 out of the 11 treatment groups (17β-estradiol, tamoxifen, levonorgestrel, 17β-estradiol + levonorgestrel and tamoxifen + levonorgestrel) from a single uterine sample were collected in order to measure the IGFBP-1 concentration using the DSL-10-7800 ACTIVE® Total IGFBP-1 ELISA Kit according to the manufacturer’s instructions (as described in chapter 4).

In addition to the 7 Standards and 2 Control samples with known IGFBP-1 concentrations, 18 cell culture media samples were assayed. Each was sampled in
duplicate and the mean absorbance for each standard, control and study sample was calculated from the duplicate values. A standard curve of absorbance versus IGFBP-1 concentration was generated using the standard control samples supplied and IGFBP-1 concentrations in the cell culture media samples were calculated by interpolation from the standard curve. Normality testing was carried out using the Shapiro-Wilk normality test. This confirmed that the data were sampled from a Gaussian distribution. The mean IGFBP-1 concentration and standard error was calculated for each of the five treatment groups. The differences between groups was tested using a one-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons; p < 0.05 was considered as being statistically significant. Analysis was performed using GraphPad Prism (version 6.07).

5.3.4 RNA Extraction

On day 21, culture media was removed and 1ml of TRI Reagent® (Sigma-Aldrich) was added to each well and left at room temperature for 10 minutes in preparation for RNA extraction from cells. The cells were released from the well into the TRI Reagent® using a plastic long handled scraper. The mixture was transferred to an RNAse free microfuge tube and stored at -80°C until the experiment had been repeated in all tissue replicates.

The TRI Reagent and cell mixture samples were thawed, and 200μL of chloroform was added per ml. Samples were then shaken vigorously for 15 seconds and after standing at room temperature for 3 minutes, were centrifuged at 7500 RPM at 4°C for 15 minutes. The aqueous phase from each sample was transferred to fresh RNAse free microfuge tubes and 500μL of isopropanol added. After standing at room temperature for 10 minutes, samples were centrifuged at 13000 RPM at 4°C for 10 minutes. The supernatant from each sample was discarded and the remaining RNA pellets were washed with 1ml of 75% ethanol in DEPC-treated water. After a further centrifugation step at 13000 RPM at 4°C for 5 minutes, supernatants were discarded and the remaining RNA pellet were air dried, re-suspended in 100μL of DEPC-treated water and heated at 55°C for 5 minutes.
5.3.5 RNA purification

Aqueous total RNA samples were then purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA was mixed with ethanol, applied to the RNeasy mini columns purified and concentrated through ethanol precipitation.

5.3.6 RNA Quantification and Quality Assessment

The total RNA concentration obtained was determined using a NanoDrop™ 8000 spectrophotometer (ThermoFisher Scientific) and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA samples were then stored at -80°C until ready for use.

5.3.7 Preparation of cDNA for Quantitative RT-PCR

One µg of RNA was reverse transcribed into cDNA using AVM-RT (Promega) according to the manufacturer’s protocol. A master mix of 9.62µL for each RNA sample was prepared comprising 5X AMV buffer, RNasin, Anchored oligo(dT)23, 10 mM dNTPs and AMV RT enzyme and added to each RNA sample (1µg of RNA in 15.38µL of DEPC treated deionised water). Samples were centrifuged at 13000 rpm for 10 seconds and transferred to the thermal cycler where they were incubated at 42°C for 1 hour and then 95°C for 1 minute to denature all enzymes. Samples were stored at 4°C until ready for use.

5.3.8 Quantitative RT-PCR

One µL of cDNA was combined with both sets of gene specific primers at a concentration of 200 pmol/µL in a SYBR green 1 mix. The forward and reverse primer sequences, along with the PCR thermal and cycling conditions for denaturation annealing and primer extension are outlined in Table 5.2. Human GAPDH expression was used as a reference. Thermal and cycling conditions were applied in the Roche Lightcycler 1.2 (as described in detail in Chapter 4).
### Table 5.2

Details of the primers, thermal cycler conditions and cycle numbers used for the detection of Beta-catenin and Notch 2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse Sequence</th>
<th>Activation</th>
<th>Denaturation Annealing Amplification</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-catenin (138)</td>
<td>5’ TGC AGT TCG CCT TCA CTA TG 3’</td>
<td>5’ ACT AGT CGT GGA ATG GCA CC 3’</td>
<td>95 °C, 10 min</td>
<td>95°C 30 sec 60°C 1 min 72°C 30 sec 58°C 1 min 72°C 1 min 72°C 5 min</td>
<td>40</td>
</tr>
<tr>
<td>Notch-2 (139)</td>
<td>5’ TGA GTA GGC TCC ATC CAG TC 3’</td>
<td>5’ TGG TGT AG GTA GGG ATG CT 3’</td>
<td>94°C, 30 sec</td>
<td>95°C 30 sec 60°C 1 min 72°C 30 sec 58°C 1 min 72°C 1 min 72°C 5 min</td>
<td>24</td>
</tr>
</tbody>
</table>

Standard curves were constructed using primer dilutions of ‘neat’, 1/5, 1/10, 1/50, 1/100 and water for each gene target and the relative expression of each gene to the control group was calculated using the $2^{-\Delta\Delta C_t}$ method after normalisation to the amount of GAPDH transcript (137). The mean mRNA expression value was calculated for the triplicates of each of the 11 treatments in the 6 tissue groups (n=3 normal uterus stromal cell and n=3 uterine polyp stromal cell). Normality testing was carried out using the Shapiro-Wilk normality test, which confirmed that the data were sampled from a Gaussian distribution. The mean fold-change and standard error for beta catenin and Notch 2 with each treatment in the two tissue groups was then calculated. The effect of each treatment on the relative expression of each gene within each tissue group, and between tissue groups was analysed using a two-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons with $p < 0.05$ being considered statistically significant. Analysis was performed using GraphPad Prism (version 6.07).
5.4 Results

5.4.1 IGFBP-1 ELISA

As shown in Table 5.3 and the graph in Figure 5.2, the IGFBP-1 concentrations in endometrial stromal cell culture supernatants significantly increased in all 5 treatment groups compared to that of the control cells after 21 days in culture. The mean IGFBP-1 concentration (± SEM) in the control group was 201.13 ± 48.8 ng/ml. With estradiol only, the concentration increased to 356.42 ± 4.91 ng/ml. With tamoxifen only, it increased to 365.71 ± 2.66 ng/ml, with levonorgestrel only it increased to 323.60 ± 39.99 ng/ml. The greatest increases were seen with estradiol + levonorgestrel (372.82 ± 6.56 ng/ml) and with tamoxifen + levonorgestrel (385 ± 6.56 ng/ml). There was no significant difference in the IGFBP-1 concentrations between the estradiol + levonorgestrel group and the tamoxifen + levonorgestrel group.
Table 5.3

IGFBP-1 concentration in cell culture supernatant after 21 days in culture with treatments of 17β-estradiol (E2), tamoxifen (TX), and levonorgestrel (LNG), alone and in combination from a single endometrial stromal sample used in this study. All treatments were repeated in triplicate. Data are expressed as mean from triplicate wells ± SEM. Statistical significance was determined using a one-way ANOVA with Tukey’s honestly significant difference post-test for multiple comparisons with P < 0.05 being considered as statistically significant.

Abbreviations: E2 = 17β-estradiol; TX = tamoxifen; LNG = levonorgestrel

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Mean [IGFBP-1] (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>201.13 ± 48.33</td>
</tr>
<tr>
<td>E2</td>
<td>3</td>
<td>356.42 ± 4.91</td>
</tr>
<tr>
<td>TX</td>
<td>3</td>
<td>365.71 ± 2.66</td>
</tr>
<tr>
<td>LNG</td>
<td>3</td>
<td>323.60 ± 39.99</td>
</tr>
<tr>
<td>E2 + LNG</td>
<td>3</td>
<td>372.82 ± 6.56</td>
</tr>
<tr>
<td>TX + LNG</td>
<td>3</td>
<td>385.63 ± 1.58</td>
</tr>
</tbody>
</table>

One-way ANOVA with Tukey’s honestly significant difference post test for multiple comparisons

\[ ^1 P = 0.011 \]
\[ ^{ii} P = 0.007 \]
\[ ^{iii} P = 0.05 \]
\[ ^{iv} P = 0.006 \]
\[ ^v P = 0.003 \]
Figure 5.2

Graph of IGFBP-1 concentration ± SEM in the supernatant of a single endometrial stromal cell culture supernatant after 21 days of exposure to 17β-estradiol, tamoxifen and levonorgestrel alone and in combination. 

Abbreviations: E2 = 17β-estradiol; TX = tamoxifen; LNG = levonorgestrel; * P < 0.05 with one-way ANOVA test with Tukey’s honestly significant difference post-test for multiple comparisons
5.4.2 RT-PCR

The photomicrographs in Figure 5.3 and Figure 5.4 show the typical appearances of stromal cells on day 0 before the addition of hormonal treatments, and after 21 days in culture with each of the 11 hormonal treatment and the control treatment regimens described in Table 5.1. Appearances of uterine and polyp stromal cells at confluence before being exposed to steroid hormones were indistinguishable on light microscopy. Cells were long and spindle shaped, displaying a characteristic fibroblast morphology. The high homogeneity indicates a high degree of purity of the population. After 21 days in culture, once cells with a decidual morphology were apparent, RNA was extracted from all wells.
**Figure 5.3**

Photomicrographs of normal uterine stromal cells taken on day 0 (D0) and day 21 (D21) of culture with the indicated hormonal treatments. Images are representative of untreated cells (C), or cells treated with 17β-estradiol (E2), tamoxifen (TX), medroxyprogesterone acetate (MPA), levonorgestrel (LNG) or desogestrel (DES) or in combination. Data are from 1 of 3 patients and were taken at X400 magnification. Arrows point to cells with a characteristic decidual-type morphology.
Figure 5.4

Photomicrographs of stromal cells isolated from uterine polyps taken on day 0 (D0) and day 21 (D21) of culture with the indicated hormonal treatments. Images are representative of untreated cells (C), or cells treated with 17β-estradiol (E2), tamoxifen (TX), medroxyprogesterone acetate (MPA), levonorgestrel (LNG) or desogestrel (DES) or in combination. Data are from 1 of 3 patients and were taken as X400 magnification.
The mean fold-change, calculated using the 2-ΔΔCT described by Livak and Schmittgen (137) with the standard error for Beta-catenin expression relative to the control group after each of the 11 hormone treatments in endometrial and polyp stromal cells after 21 days in culture is shown in Table 5.4, and the graphs in Figure 5.5 and Figure 5.6.

In endometrial stromal cells, Beta-catenin expression was upregulated in all groups. Differences between groups were not statistically significant. In polyp stromal cells however, 17β-estradiol and tamoxifen alone caused a down regulation of Beta-catenin, whereas progestin treatments alone caused upregulation of Beta-catenin. This was most marked with desogestrel treatment, which resulted in a mean fold-change of 11.75 (± SEM 2.97) in Beta-catenin expression. When 17β-estradiol was combined with each of the progestins to produce decidualisation, 17β-estradiol and tamoxifen in combination with medroxyprogesterone acetate caused downregulation of Beta-catenin, whilst in combination with levonorgestrel caused upregulation of beta catenin, and with desogestrel caused a marked upregulation of Beta-catenin. There was a statistically significant difference with tamoxifen and desogestrel treatment in combination (25.41 fold ± 6.58) compared with 17β-estradiol alone (-2.17 fold ± 1.87, p = 0.004), tamoxifen alone (-3.62 fold ± 4.30, p = 0.002), medroxyprogesterone acetate alone (0.33 fold ± 0.78, p = 0.015), levonorgestrel alone (0.45 fold ± 2.40, p = 0.016), 17β-estradiol and medroxyprogesterone acetate in combination (-0.31 fold ± 1.39, p = 0.011), tamoxifen and medroxyprogesterone acetate in combination (-0.07 fold ± 1.98, p = 0.013) and tamoxifen and levonorgestrel in combination (2.29 fold ± 1.81, p = 0.038).

The mean fold-change (± SEM) for Notch-2 expression relative to the control group after each of the 11 hormone treatments in endometrial and polyp stromal cells after 21 days in culture is shown in Table 5.4 and the graphs in Figure 5.7 and Figure 5.8.

In endometrial stromal cells, Notch-2 expression was upregulated with 17β-estradiol treatment, but downregulated with tamoxifen treatment. Notch-2 expression was upregulated by all three progestin treatments. 17β-estradiol and medroxyprogesterone acetate in combination, and 17β-estradiol with levonorgestrel in combination caused upregulation of Notch-2, but expression was downregulated by 17β-estradiol and
desogestrel in combination. Notch-2 was upregulated by tamoxifen in combination with
in all three progestins. None of the differences were statistically significant. In polyp
stromal cells Notch-2 was downregulated by 17β-estradiol alone, tamoxifen alone,
medroxyprogesterone acetate alone and levonorgestrel alone. Desogestrel alone
caused upregulation of Notch-2, whilst 17β-estradiol in combination with
medroxyprogesterone acetate and with desogestrel also caused upregulation of Notch-
2, but in combination with levonorgestrel caused downregulation. Tamoxifen in
combination with medroxyprogesterone acetate and with levonorgestrel caused
downregulation of Notch-2, but in combination with desogestrel caused upregulation of
Notch 2.
**Table 5.4**

Mean fold-change (± SEM) for Beta-catenin and Notch 2 expression in endometrial and polyp stromal cells after 21 days in culture with each of hormonal treatment. Expression levels for each gene were calculated using the $2^{\Delta\Delta CT}$ method, using GAPDH as the housekeeping gene. Fold-changes are expressed in relation to the mean expression of the control group which received no additional steroid hormone treatment as detailed in Table 5.1. Differences between means were compared using a one-way ANOVA test with Tukey’s Honestly Significant Difference post-test for multiple comparisons. $P < 0.05$ was taken to be statistically significant.

Abbreviations: $E2 = 17\beta$-estradiol; $TX = tamoxifen; MPA = medroxyprogesterone acetate; LNG = levonorgestrel; DES = desogestrel

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Beta-catenin</th>
<th>Notch-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endometrial stroma (n=3)</td>
<td>Polyp stroma (n=3)</td>
</tr>
<tr>
<td></td>
<td>Mean fold-change ± SEM</td>
<td>Mean fold-change ± SEM</td>
</tr>
<tr>
<td>E2</td>
<td>4.20 ± 1.34</td>
<td>-2.17±i</td>
</tr>
<tr>
<td>TX</td>
<td>2.72 ± 0.94</td>
<td>-3.62±ii</td>
</tr>
<tr>
<td>MPA</td>
<td>5.73 ± 2.42</td>
<td>0.33±iii</td>
</tr>
<tr>
<td>LNG</td>
<td>7.22 ± 4.15</td>
<td>0.45±iv</td>
</tr>
<tr>
<td>DES</td>
<td>4.06 ± 3.59</td>
<td>11.75±iii</td>
</tr>
<tr>
<td>E2 MPA</td>
<td>2.73 ± 0.52</td>
<td>-0.31±v</td>
</tr>
<tr>
<td>TX MPA</td>
<td>4.66 ± 1.57</td>
<td>-0.07±vi</td>
</tr>
<tr>
<td>E2 LNG</td>
<td>7.03 ± 4.11</td>
<td>0.26±vii</td>
</tr>
<tr>
<td>TX DES</td>
<td>10.37 ± 3.87</td>
<td>2.29±viii</td>
</tr>
<tr>
<td>E2 DES</td>
<td>3.30 ± 5.52</td>
<td>19.68±ix</td>
</tr>
<tr>
<td>TX DES</td>
<td>8.13 ± 9.01</td>
<td>25.41±ix</td>
</tr>
</tbody>
</table>

One-way ANOVA with Tukey’s honestly significant difference post test for multiple comparisons

i $P = 0.004$
ii $P = 0.002$
iii $P = 0.015$
iv $P = 0.016$
v $P = 0.011$
vi $P = 0.013$
vii $P = 0.038$
Figure 5.5

Graph of mean fold-change (± SEM) for Beta-catenin relative to the mean expression level of an untreated control group in endometrial stromal cells after 21 days in culture with 11 hormonal treatments.

Abbreviations: e = endometrial stromal cells; E2 = 17β-estradiol; TX = tamoxifen; MPA = medroxyprogesterone acetate; LNG = levonorgestrel; DES = desogestrel
**Figure 5.6**

Graph of mean fold-change (± SEM) for Beta-catenin relative to the mean expression level of an untreated control group in polyp stromal cells after 21 days in culture with 11 hormonal treatments

Abbreviations: p = polyp stromal cells; E2 = 17β-estradiol; TX = tamoxifen; MPA = medroxyprogesterone acetate; LNG = levonorgestrel; DES = desogestrel; * = P< 0.05 one-way ANOVA test with Tukey’s Honestly Significant Difference post-test for multiple comparisons
**Figure 5.7**

Graph of mean fold-change (± SEM) for Notch-2 relative to the mean expression level of an untreated control group in endometrial stromal cells after 21 days in culture with 11 hormonal treatments.

Abbreviations: *e* = endometrial stromal cells; *E2* = 17β-estradiol; *TX* = tamoxifen; *MPA* = medroxyprogesterone acetate; *LNG* = levonorgestrel; *DES* = desogestrel
Figure 5.8

Graph of mean fold-change (± SEM) for Notch-2 relative to the mean expression level of an untreated control group in polyp stromal cells after 21 days in culture with 11 hormonal treatments.

Abbreviations: p = polyp stromal cells; E2 = 17β-estradiol; TX = tamoxifen; MPA = medroxyprogesterone acetate; LNG = levonorgestrel; DES = desogestrel
5.5 Discussion

The first aim of this study was to establish a model for decidualisation of human uterine endometrial stromal cells with 3 clinically relevant and structurally distinct progestins: medroxyprogesterone acetate, levonorgestrel and desogestrel. As these compounds transform ‘estrogen-primed’ endometrium in-vivo, and because levonorgestrel produces decidualisation in ‘tamoxifen-primed’ endometrium in postmenopausal women, both 17β-estradiol and tamoxifen were used alongside each progestin. The model used in this study was adapted from that described by Ganef et al. who cultured endometrial stromal cells obtained by endometrial biopsies in the proliferative and secretory phases of the menstrual cycle (230). They induced decidualisation using 8-Br-cAMP and medroxyprogesterone acetate over 12 days and observed in an increase in IGFBP-1 mRNA levels and expression of IGFBP-1 protein in the culture supernatant. In contrast to Ganef et al. however, cultures in this study were maintained for 21 days because this was the time at which the decidual phenotype became apparent. It is not clear why the decidual phenotype took longer to appear, although the endometrial samples in this study were obtained from the secretory endometrium, may offer an explanation. Their responsiveness to changes in their external hormonal environment may have differed to those used by Ganef et al. This is supported by Evron et al., who observed that that effects of endometrial stromal cells on epithelial cell receptivity in-vitro using a co-culture model are dependent on the phase of the menstrual cycle from which they were sampled (231).

Just as the morphological changes associated with decidualisation in response to the rise in progesterone levels in-vivo appear around 10 days after ovulation, there is also a lag in-vitro when endometrial stromal cells are treated with progesterone or a progestin (232). The fact that progesterone and progestins alone therefore have been shown to be very weak inducers of the decidual phenotype in-vitro led Gellersen et al. to suggest that the expression of decidua-specific genes is unlikely to be under direct transcriptional control of the activated progesterone receptor (222). Initiation of stromal cell differentiation has been shown to be dependent on elevated levels of
intracellular cAMP, a second messenger molecule, which activates the protein kinase A pathway, which in turn sensitises cells to progesterone (222). Cyclic AMP binds to a cAMP response element binding protein (CREB) which acts as a transcription factor and co-regulator of other transcription factors including the activated PR and glucocorticoid receptor (GR). Once decidualised therefore, stromal cells become dependent on continuous cAMP and progesterone signalling for homeostasis (182, 222).

After 21 days in culture, the IGFBP-1 concentration in the endometrial stromal cell culture supernatant was tested using an ELISA technique to confirm that decidualisation had occurred. The IGFBP-1 concentration was significantly higher after treatment with 17β-estradiol alone, tamoxifen alone, levonorgestrel alone, with 17β-estradiol in combination with levonorgestrel, and with tamoxifen in combination with levonorgestrel compared to the control group. This is most likely due to the effects of 8-br-cAMP, which was added to all groups including the controls. In addition, the IGFBP-1 concentration in the control cells (after treatment with 8-br-cAMP alone) was raised compared to what would have been expected in cells sustained in culture media alone. Matsumoto et al. found an IGFBP-1 concentration measured using ELISA of just 3.2ng/ml ± 0.2 in cultured human endometrial stromal cells after 28 days in culture with media only (223). As shown in Table 5.3, the IGFBP-1 concentration after 21 days in culture with 8-br-cAMP alone in this study was 201.13ng/ml ± 48.33.

Although 8-br-cAMP alone induces some decidualisation effects in stromal cells, the transformation is more pronounced when stromal cells are treated with 8-br-cAMP in addition to a progestin. Gellerson et al. demonstrated that that human endometrial stromal cells treated with cAMP in combination with medroxyprogesterone acetate produces much higher prolactin concentrations per microgram of DNA than treatment with cAMP alone (222). The results from the ELISA study summarised in Table 5.3 and Figure 5.2 demonstrate a similar finding, i.e. that a marker of decidualisation (IGFBP-1 concentration was used here rather than prolactin) was significantly higher in the groups where the a progestin (levonorgestrel rather than medroxyprogesterone acetate) was used in addition to 8-br-cAMP.
The increased IGFBP-1 concentration in the groups treated with 17β-estradiol alone and tamoxifen alone mimics the ‘priming’ effect that these hormones have *in-vivo*, whereby estrogen causes upregulation of PR and supports the estrogenic role of tamoxifen. It also suggests that 17β-estradiol and tamoxifen may be potentiating the effects of cyclic AMP. Extrapolating these results to the rest of this cell culture model, which has used not only levonorgestrel but also medroxyprogesterone acetate and desogestrel as progestins, it is likely that the addition of cAMP to all groups will have induced IGFBP-1 expression, although further studies would be needed to confirm this.

One weakness of this study is that cells for the ELISA experiment to examine IGFBP-1 concentrations were derived from the endometrium of a single sample, and the effect of a single progestin (levonorgestrel) alone and in combination with 17β-estradiol and tamoxifen was studied. Resource limitations meant that it was not possible to repeat this experiment using the other progestins and a larger study population. The validity of the methodology used, and the fact that the results are supported in the wider literature however, does improve the strength of the conclusions.

The stromal cell culture model used in this study to induce decidualisation in endometrial stromal cells was also used to apply identical experimental conditions to stromal cells derived from postmenopausal endometrial polyps. This has not been previously reported. Also, the effect of either 17β-estradiol or tamoxifen priming with each progestin treatment on the expression of polyp genes (specifically Beta-catenin and Notch-2, members of the canonical Wnt and Notch pathways shown to be significantly modulated in polyps relative to endometrium in the earlier microarray study) in the context of the cAMP induced decidualisation in this model has never previously been reported.

The quantitative RT-PCR results suggest firstly that both beta catenin and Notch-2 expression differs in response to decidualisation in endometrial and polyp stromal cells. However, none of the differences between polyp and endometrium reached the threshold for statistical significance. This is likely to be a reflection of the small number
of samples studied (n=3 in each group). Secondly, the results highlight the fact that the progestins used here appear to induce different effects.

Beta-catenin, a key mediator of the Wnt signal, was significantly downregulated in endometrial polyps relative to endometrium in the earlier microarray study. In postmenopausal polyps, Beta-catenin was down regulated 19.21 fold relative to postmenopausal endometrium, and by 22.52 fold in tamoxifen polyps relative to tamoxifen endometrium. In this study, in endometrial stromal cells, treatment with all 3 progestins in conjunction with either tamoxifen or 17β-estradiol caused the opposite effect; that is upregulation of Beta-catenin. Zhang et al. have (233) investigated the role of Beta-catenin in progesterone signalling and female reproductive physiology in mice in whom the Beta-catenin gene was deleted in the endometrial mesenchymal compartment (i.e. stroma and myometrium but not epithelium). They found that deletion of the Beta-catenin gene resulted in infertility and complete failure of the endometrium to undergo decidualisation. When ovariectomised mutant female mice were treated with a combined estradiol and progesterone hormone regimen consistent with early pregnancy, some mice developed endometrial metaplasia. The authors concluded that mesenchymal Beta-catenin is essential for indirectly opposing estrogen-induced epithelial proliferation by progesterone and have suggested a link between the progesterone and Beta-catenin signalling pathways. This evidence supports the possibility that upregulation of stromal Beta-catenin associated with decidualisation is responsible for the polyp-preventing effects of intrauterine levonorgestrel in tamoxifen treated women.

Notch-2 expression was also downregulated in polyps relative to endometrium in the earlier microarray study. In postmenopausal polyps, Notch-2 was down regulated 5.59-fold relative to postmenopausal endometrium, and by 6.13-fold in tamoxifen polyps relative to tamoxifen endometrium. In this study, Notch-2 expression in endometrial stromal cells was increased by all decidualising treatments except for 17β-estradiol combined with desogestrel.
In polyp stromal cells, Notch-2 expression was reduced by decidualising treatment with levonorgestrel and either 17β-estradiol or tamoxifen, and medroxyprogesterone acetate with tamoxifen. However, with desogestrel in combination with either 17β-estradiol or tamoxifen, and medroxyprogesterone acetate in combination with 17β-estradiol, Notch-2 expression was increased. None of these differences were statistically significant. Canonical Notch signalling is critically involved in development and tissue homeostasis controlling diverse cellular processes such as stem cell maintenance, proliferation, differentiation or apoptosis (234). Otti et al. investigated the role of the Notch signalling pathway in human decidual stromal cells isolated from early pregnancy and found that decidualisation with cAMP, estradiol and progesterone treatment resulted in increased activity of the canonical Notch pathway (235). This study therefore support the role of decidualisation in reversing the ‘polyp effect’ of Notch-2 expression.
6 Discussion of Findings and Future Work

6.1 Discussion of Findings

Endometrial polyps are increasingly diagnosed in gynaecological practice due to the widespread use of transvaginal ultrasound and outpatient hysteroscopy. They can be asymptomatic and represent an incidental finding, or more commonly are associated with abnormal uterine bleeding. In a recent systematic review and meta-analysis of the oncogenic potential of polyps, Lee et al. found that the prevalence of premalignant or malignant polyps was 5.42% in postmenopausal women compared with 1.7% in premenopausal women (relative risk 3.86, 95% CI 2.92–5.11) (10). In their study, the prevalence of malignancy within polyps in both pre and postmenopausal women with symptomatic bleeding was 4.15% compared with 2.16% for those without bleeding (relative risk 1.97; 95% CI 1.24 –3.14). Among symptomatic postmenopausal women with endometrial polyps, the prevalence of malignancy was 4.47% in comparison to 1.51% asymptomatic postmenopausal women (relative risk 3.36; 95% CI 1.45–7.80) (10).

Although the majority of polyps within the endometrium are benign, and despite the fact that small polyps have been observed to spontaneously regress (28), these lesions present a challenge to clinicians. The mainstay of management of these lesions is polypectomy in order to manage abnormal uterine bleeding, and also due to concerns over the potential for malignant transformation. However, it is unlikely that this excisional approach fully addresses the underlying pathological process.

The risk factors for endometrial polyps include menopause, HRT use, obesity and hypertension (236). In addition, use of the selective estrogen receptor modulating drug tamoxifen widely used as adjuvant therapy for breast cancer is also a recognised risk factor for polyp formation. Tamoxifen-related polyps tend to be larger than those seen in healthy untreated postmenopausal women, are more often multiple with a translucent appearance and a higher fibrotic content (45, 102). Polyps from postmenopausal tamoxifen treated women with breast cancer have a higher rate of malignant transformation than polyps in the healthy untreated postmenopausal population (45).
A number of mechanisms to explain the pathogenesis of endometrial polyps have been proposed including altered bcl-2 mediated apoptosis, endometrial inflammation and cox-2 expression, disordered expression of growth factors and their receptors such as epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF), dysregulation of insulin growth factor-1 (IGF-1) and insulin growth factor binding protein 1 (IGFBP-1), altered aromatase expression, K-ras mutation and clonal chromosomal rearrangements (236). Although the pathogenic process underlying polyp formation, and underlying the particular differences seen in tamoxifen polyps is not fully understood, it is widely accepted that endometrial polyps develop because of an excess of estrogenic activity in the endometrium. As polyps arise as small, discrete lesions within an otherwise ‘normal’ endometrium, their altered sensitivity to estrogen and progesterone via intracellular receptor expression has been studied extensively using immunohistochemistry. However, variations in tissue sampling techniques, study populations and the different methods used for image analysis makes combined analysis of the results of these studies difficult.

Despite the numerous studies reporting ER and PR expression in endometrial polyps, differences in the receptor subtype expression – i.e. ER-alpha, ER-beta PR-A and PR-B expression between polyp and endometrium has not previously been described. ER and PR receptor subtypes have different tissue distributions and functions within normal endometrium. Taylor et al. demonstrated that glandular ER-alpha expression is highest in the proliferative, early and mid-secretory phases but declines during the late secretory and menstrual phases and in the stroma, expression remained unchanged throughout the menstrual cycle; in contrast, glandular and stromal ER-beta expression was highest during the early secretory phase (97). Thomas et al. describe the divergent responses of ER-alpha and ER-beta which can be seen in their differing affinities to binding ligands and their differing responses following binding to the same ligand (237). PR-A and PR-B are present in endometrial epithelium during the proliferative phase and increase concordantly with estrogen levels. Late in the secretory phase, PR-A levels decline, whereas PR-B levels remain constant in the epithelial cells. Stromal cells, in contrast, exhibit a predominance of PR-A throughout the menstrual cycle (238). Patel et
al. have shown that the tissue response to progesterone is determined by the combined actions of PR-A and PR-B, which upon ligand binding form either homodimers or heterodimers that have distinct transcriptional activities at specific sets of gene promoters (239).

In the first experimental study in chapter 2 of this thesis, immunohistochemistry was used to identify the distribution of ER-alpha, ER-beta, PR-A and PR-B in the endometrium and in polyps of postmenopausal women with and without tamoxifen treatment using a validated, quantitative method for image analysis not previously described in endometrial polyp immunohistochemistry. The results demonstrated several differences and similarities in ER and PR receptor subtype expression in the glandular and stromal compartments of each tissue group, which support possible mechanisms of pathogenesis.

ER-alpha expression was significantly reduced in stroma compared to glands in postmenopausal polyps, whereas expression levels were equivalent in the glands and stroma of postmenopausal endometrium. This altered distribution between the glandular and stromal compartments has been observed in other pathological states in the endometrium and is likely to be important in polyp pathogenesis (240). However, the fact that this dysregulation was not seen in tamoxifen polyps and tamoxifen endometrium – in which the patterns of glandular and stromal ER-alpha distribution were similar suggests this may not an important mechanism in tamoxifen polyp pathogenesis.

Stromal ER-beta expression in this study was significantly lower in both postmenopausal polyps compared to postmenopausal endometrium and tamoxifen polyps compared to tamoxifen endometrium. ER-beta is thought to play a role in the modulation of estrogenic activity (84), and so, the loss of its inhibitory effect within the stroma is likely to result in a preponderance of ER-alpha induced effects including proliferation and the secretion of vascular endothelial growth factors to stimulate angiogenesis and the growth of the vascular core around which stroma and glands condense to form a polyp.
In contrast, glandular ER-beta expression was significantly higher in postmenopausal polyps compared with postmenopausal endometrium. There was no significant difference seen when tamoxifen polyps and tamoxifen endometrium were compared, suggesting that this is not a mechanism for tamoxifen polyp pathogenesis.

Stromal PR-B expression was significantly lower in tamoxifen polyps compared with tamoxifen endometrium, a difference not seen when postmenopausal polyps were compared with postmenopausal endometrium. PR-A expression, however, was not significantly different in polyps and endometrium from the tamoxifen or postmenopausal groups. These findings suggest that an altered ratio of stromal PR-A to PR-B is involved in the pathogenesis of tamoxifen polyps.

These observations describe how polyps differ from the endometrium in which they arise, and has also demonstrated similarities and differences between postmenopausal and tamoxifen polyps. The similarities between these 2 groups of polyps however suggests that there is likely to be a distinct ‘polyp-pathway’ that occurs within the endometrium and culminates in the macroscopic and molecular changes that distinguish an endometrial polyp from its surrounding endometrium.

The microarray study presented in chapter 3 of this thesis sought to ascertain the genes that are differentially expressed in this distinct ‘polyp-pathway’. Differentially expressed genes in postmenopausal polyps compared with postmenopausal endometrium and tamoxifen polyps and tamoxifen endometrium were identified using a cross-platform protocol. The Affymetrix platform was used to generate a microarray of genes in postmenopausal endometrium and endometrium from postmenopausal women treated with tamoxifen. The Illumina platform was used to generate a microarray of genes in benign postmenopausal polyps and benign polyps from postmenopausal women treated with tamoxifen. After normalisation of these 4 data sets, they were combined in order to compare postmenopausal polyp with postmenopausal endometrium and tamoxifen polyp with tamoxifen endometrium. The genes that were either up or down-regulated in both groups represent common subset of ‘polyp genes’.
Despite the differences in these two platforms in the kind of probes used, the hybridisation technique the labelling method and the production method, there is evidence of high inter-platform concordance between Affymetrix and Illumina (141, 142). In addition, these 2 platforms have been shown to demonstrate similar performance in their ability to differentially expressed genes (143).

This study identified 3272 such ‘polyp genes’. Of these polyp genes, 1659 genes were upregulated in polyps relative to the endometrium, and 1613 genes down regulated in polyps relative to the endometrium. Amongst the 20 most up-regulated polyp genes was MSH3, a component gene in the DNA mismatch repair system. Mutations of MSH3 have been shown to cause microsatellite instability, where electrophoretic shifts in the allele sizes of microsatellite DNA sequences occur. Microsatellite instability is an important mechanism in the pathogenesis of endometrial cancer. Rios et al. are the only authors to have studied microsatellite instability in endometrial polyps, and whilst they demonstrated it to be present in a small number of cases, in particular cases of multiple polyps, their study did not examine tamoxifen polyps (151). The findings from this study where MSH3 was markedly upregulated in polyps support a role for attenuation of the DNA mismatch repair process and microsatellite instability as a pathogenic mechanism in endometrial polyps.

Evidence from studies on the mechanisms of colorectal cancer have demonstrated that ER-beta expression is reduced during colonic tumorigenesis compared with normal colonic tissue, as it was shown to be in endometrial polyps compared with normal endometrium in the immunohistochemistry study in chapter 2 of this thesis (237, 241). In addition, microsatellite instability has been shown to occur in a higher frequency of proximally sited colorectal tumours where a loss of ER-beta expression in tumours is also most likely to occur (242). Therefore, just as estrogen has been shown to regulate the expression of mismatch repair proteins in colorectal cells via ER-beta, so mechanisms involving a loss of estrogen signalling via reduced ER-beta expression in the endometrium may account for upregulation of MSH3 expression, disordered mismatch repair and microsatellite instability resulting in endometrial polyp formation (243).
Amongst the 20 most downregulated genes in endometrial polyps was WFDC2, also known as HE4. The HE4 protein is over-expressed in epithelial ovarian cancer compared to normal ovarian tissue, and as a result, is used as a novel biomarker in ovarian cancer diagnosis (154). Its expression is also increased in endometroid endometrial carcinoma compared to normal endometrial tissue (152). In contrast to these findings in malignant conditions however, WFDC2 was highly downregulated in endometrial polyps relative to endometrium. This difference may be accounted for by the benign nature of the endometrial polyps studied, and the fact that none of the samples demonstrated atypia or malignancy. It remains to be established whether an upregulation of HE4 would be observed in polyps with malignant potential, but the pattern of HE4’s expression in other gynaecological malignancies supports this possibility.

In order to ascertain a more biologically relevant understanding of how the 3272 polyp genes interact together, pathway analysis was undertaken in order to map these genes to established genetic pathways. One hundred and twenty-eight gene pathways were found to be significantly regulated. Amongst the top 20 most regulated pathways, were 10 ‘signalling pathways’ including the Notch and Wnt pathways, as well as 7 ‘regulatory pathways’, and 3 ‘disease pathways’.

Dysregulated Notch receptor and ligand expression has been demonstrated in endometrial pathologies, including polyp hyperplasia and cancer (160). This cross-platform microarray study confirms that the genetic pathway encoding expression of these receptors and ligands is also dysregulated in polyps when compared with normal endometrium. The mechanisms whereby dysregulation of Notch receptors and ligands results in endometrial polyp formation is not established, but there is evidence to suggest that Notch signalling regulates endothelial cell function via modulation of vascular endothelial growth factor (VEGF) signalling, and that increased VEGF expression causing disordered angiogenesis is critical in endometrial polyp growth (162). Wnt signalling was also amongst the most significantly regulated pathways in endometrial polyps in this study. Wnt activation has been shown to be a key initiator of
polyp formation and carcinogenesis in colonic epithelium. Although the mechanisms whereby dysregulation of Wnt signalling in the endometrium leads to benign polyp growth is unclear, the low rate of malignancy in polyps does not support it triggering a carcinogenesis cascade of events. Alternative mechanisms may include disordered angiogenesis via a direct effect on endothelial cells, or via interaction with VEGF or endothelin 1 signalling causing a vascular core to grow out of the endometrial layer (173).

Having presented evidence to support a range of theories for polyp pathogenesis, the latter part of this thesis has focussed on how endometrial polyps can be prevented. Evidence from randomised controlled trials, and a meta-analysis has shown that intrauterine levonorgestrel via the LNG-IUS in tamoxifen treated women prevents endometrial polyp formation (96). However, the mechanisms by which this occurs have not been demonstrated, and these studies present conflicting evidence about whether stromal decidualisation occurs with tamoxifen and LNG-IUS treatment. As with endometrial polyps, progestins including intrauterine levonorgestrel are also effective in promoting disease regression in endometrial hyperplasia with and without atypia (244, 245). Endometrial hyperplasia occurs most commonly in the presence of chronic exposure to estrogen unopposed by progesterone. It is characterised by non-invasive proliferation of the endometrium and an increased volume of endometrial tissue with alterations of glandular architecture and gland to stroma ratio of greater than 1:1 (246). Proposed mechanisms by which progestins cause regression of endometrial hyperplasia include inducing apoptosis and inhibiting angiogenesis via interaction with the PR-B receptor (247-249).

In chapter 4, the effects of 12 months of treatment with tamoxifen and the LNG-IUS versus tamoxifen alone in postmenopausal women was studied. Stromal decidualisation was demonstrated in all cases of tamoxifen and LNG-IUS treatment, confirming the findings of Gardner et al., but in conflict with the findings of Chan et al. and Omar et al. who reported no cases of stromal decidualisation after 12 and 24 months respectively of concurrent tamoxifen and LNG-IUS treatment. In addition, to
demonstrating the presence of stromal decidual cells histologically in the tamoxifen and LNG-IUS group, this study is the first to demonstrate that decidual cells under the influence of tamoxifen and LNG-IUS treatment express IGFBP-1, a marker of decidualisation. Serum IGFBP-1 levels did not reflect the increased stromal cell expression in keeping with the observations of others (217, 218) and support the formation of a stable decidual layer. These findings suggest that stromal decidualisation induced by the LNG-IUS and associated with stromal IGFBP-1 expression in tamoxifen-primed postmenopausal endometrium is an essential mechanism for polyp inhibition.

Decidualisation is initiated during the secretory phase of the menstrual cycle when stromal PR-A expression persists but PR-B expression is down-regulated. This suggests that PR-A is the predominant mediator of decidualisation (250). *In-vitro* studies have also demonstrated that PR-A is a stronger activator of IGFBP-1 than PR-B (251). The immunohistochemistry study in chapter 2 of this thesis found that stromal PR-A expression in tamoxifen polyps was reduced (albeit not to the point of statistical significance) compared with stromal PR-A expression in tamoxifen endometrium, which confirms that tamoxifen endometrium is indeed susceptible to decidualisation, and that tamoxifen polyps, once formed, become relatively resistant to the decidualising effects of progestins. A similar pattern of reduced stromal PR-A expression in postmenopausal polyps compared with stromal PR-A expression in postmenopausal endometrium – again not reaching the point of statistical significance. This suggests that LNG-IUS treatment could also be effective at inducing decidualisation and preventing polyp formation in high-risk postmenopausal women who do not take exogenous hormonal treatment.

The fact that high PR-B expression appears to predict responsiveness to and disease regression with progestin therapy in cases of atypical endometrial hyperplasia suggests that this endometrial protective effect does not occur *via* mechanisms involving stromal decidualisation, a process that predominantly occurs *via* PR-A. Endometrial hyperplasia and polyps are therefore likely to represent wholly separate pathological processes. Endometrial hyperplasia is a proliferation of endometrial glands or irregular size and
shape, whereas the molecular mechanisms demonstrated here, that lead to polyp formation, are predominantly focussed in the stroma.

Chapter 5 describes how a stromal cell-culture model for inducing decidualisation in conditions similar to those in women taking tamoxifen along with intrauterine levonorgestrel via the LNG-IUS was developed in order to explore the apparent polyp-inhibitory effect of decidualisation on 2 polyp genes (Beta-catenin and Notch-2) identified by the microarray and gene pathway analysis study presented in chapter 3. The process of physiological decidualisation is initiated in the secretory phase of the menstrual cycle after estrogen, the dominant hormone of the proliferative phase, primes the endometrium inducing the expression of glandular and stromal PR-A and PR-B. The post-ovulatory rise in progesterone then transforms fibroblastic stromal cells into large, epithelioid like decidual cells. This morphological transition of stromal cells is accompanied by a marked rearrangement of the intracellular architecture, accumulation of glycogen, and secretion of various proteins, such as prolactin and IGFBP-1 (252).

In conventional endometrial stromal cells culture models described in the literature, decidualisation is induced with a combination of cyclic AMP, 17β-estradiol and either progesterone or medroxyprogesterone acetate (193, 250). In this study, tamoxifen was also used as a ‘priming’ agent in order to mimic the hormonal environment of tamoxifen-treated postmenopausal women in whom levonorgestrel has been shown to induce decidualisation and inhibit polyp formation. After 21 days in culture, there was an increase in the IGFBP-1 concentration in the groups treated with estrogen alone and tamoxifen alone compared with the control group which suggests that both hormones are able to ‘prime’ endometrial stromal cells in order to undergo decidualisation. The IGFBP-1 concentration was lowest in the group treated with levonorgestrel alone (although not statistically significant), demonstrating the importance of an estrogenic priming stimulus. The ELISA study on stromal cell culture supernatant therefore confirmed that decidualisation can be induced in-vitro using 8-br-cAMP, tamoxifen and levonorgestrel treatment.
The effects of this decidualising treatment on Beta-catenin and Notch-2 expression in endometrial and polyp stromal cells was then examined using RT-PCR. Priming was carried out with either 17β-estradiol or tamoxifen, and the effect of progestins desogestrel and medroxyprogesterone acetate was studied in addition to levonorgestrel. Beta-catenin expression was upregulated by decidualisation with all 3 progestins in endometrial stromal cells. By contrast, the microarray study described in chapter 3 revealed that Beta-catenin was downregulated 19.21-fold in postmenopausal polyps compared to postmenopausal endometrium, and 22.52-fold in tamoxifen polyps compared to tamoxifen endometrium. Notch-2 expression was upregulated by decidualisation with all decidualising treatments except 17-β-estradiol with desogestrel. This gene was down-regulated 5.59-fold in postmenopausal polyps compared to postmenopausal endometrium, and 6.13-fold in tamoxifen polyps compared to tamoxifen endometrium in the microarray study presented in chapter 3. Ascertaining the full extent of the downstream effects of altered Beta-catenin and Notch-2 expression on Wnt and Notch signalling in endometrial stromal cells is beyond the scope of this project but the fact that decidualisation with not just levonorgestrel but also desogestrel and medroxyprogesterone acetate can reverse expression of a key polyp gene suggests that these progestins may be useful in polyp inhibition.

The effects of decidualisation on Beta-catenin and Notch-2 expression differed in stromal cells obtained from the endometrium and stromal cells obtained from polyps, which suggests that the sensitivity of these cells to progestins differs. In particular, Beta-catenin expression in polyp stromal cells was relatively unaffected by medroxyprogesterone acetate and levonorgestrel, but was significantly upregulated by desogestrel. Medroxyprogesterone acetate is a derivative of 17-hydroxyprogesterone, whereas levonorgestrel and desogestrel are both gonane progestins derived from 19-nortestosterone. Desogestrel differs from levonorgestrel in that is has a higher affinity for progesterone receptors, as well as, less androgenic activity and a higher potency at the endometrium (225). Stromal PR-A and PR-B receptor expression was shown to differ in polyps compared to endometrium, and this study raises the possibility that
desogestrel’s pronounced effect on Beta-catenin expression in polyp stromal cells may be due to its interaction with PR-A and PR-B.

In conclusion therefore, this thesis has demonstrated a number of pathological mechanisms that arise in endometrial polyps. Firstly, that stromal ER-beta expression is significantly reduced in endometrial polyps compared to endometrium, and that this difference occurs in both tamoxifen and postmenopausal groups. The microarray study and subsequent pathway analysis revealed a subset of ‘polyp genes’ that were significantly either up- or down-regulated in endometrial polyps relative to the endometrium. MSH3 and WFDC2 were 2 such polyp genes that were highly up and down regulated respectively, and whose actions within the endometrium are likely to influence polyp formation through their actions on DNA mismatch repair, microsatellite instability, ER-beta signalling and involvement in other gynaecologically important pathological processes. Wnt and Notch signalling pathways were shown to be two of the most highly affected genetic pathways in endometrial polyps. The ability of levonorgestrel delivered via the LNG-IUS to induce stromal decidualisation and induce IGFBP-1 expression in tamoxifen treated women was confirmed, and just as this treatment also inhibits polyp formation it has been shown to reverse the expression patterns of 2 key polyp genes in-vitro.
6.2 Future Work

6.2.1 Endometrial origins of polyp pathogenesis

In order to identify why some patients are more susceptible to developing endometrial polyps, and indeed why polyps arise at certain sites within the endometrium, it would be useful to sample both polyps and endometrium from separate sites within the same uterus using similar experimental approaches.

6.2.2 Progestins and polyps

The cell culture model used here provides an opportunity for further study into the effect of progestins on polyp genes. Examining the effect of varying progestin doses in order to identify the lowest dose required to cause a change in gene expression would be useful in order to minimise the side effects of progestin treatment.

6.2.3 LNG-IUS and polyps in postmenopausal women without tamoxifen treatment

Clinical trials have demonstrated the efficacy of the LNG-IUS at preventing polyp formation in postmenopausal women taking tamoxifen, but it is not known whether it is equally effective at preventing polyps in non-tamoxifen treated women, or pre-menopausal women at high risk of polyp formation. A randomised controlled trial involving women at high risk of polyp formation (such as those who are obese) would be needed to answer this question.
Appendix 1: Ethics approval

University Hospitals of Leicester

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25 January 2008
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Dear Professor Konje

07517

ID: The application of the Levonorgestrel containing intrauterine contraceptive device in the prevention of endometrial changes induced by tamoxifen in women undergoing adjuvant therapy for

SSA Ref: 6498
Sponsor and Funder: Schering Health Care
Rec Ref:

Please note that Trust indemnity ceases 01/10/2009

Thank you for your annual report, dated 24/01/08

The report has been noted by the R&D Office and information has been updated on the R&D Office database.

In order for us to keep up to date can you please notify the Research Office of any changes in the personnel attached to the study, and ensure that any changes to the protocol are notified in the appropriate way.

We look forward to reading the final report of this study.

Below is a list of the Researchers Approved to work on this Application within UHL
Professor J.C. Konje
Dr Sarah Phillip

Yours sincerely

Sharon Turner
R&D Administrator
Cc Sarah Phillip
References


